Characterization Of Chromaffin Granule Membrane Glycoprotein IV

Fariha Mohammed Getlawi

Thesis submitted for the degree of Doctor of philosophy
University of Edinburgh
Scotland UK
March 1998

To whom I love so much
Alaa and Lujain

Declaration

This study was carried out under the guidance of Dr. David K. Apps and Dr. Jeff Haywood at the Department of Biochemistry, University of Edinburgh, Medical School between October 1992 and June1996.

This thesis has been composed by myself and the experimental work presented herein was carried out by myself while a member of a research group directed and supervised by Drs David Apps and Jeff Haywood. Some of the results presented have already been published.

Fariha Mohammed Getlawi

Department of Biochemistry,
University of Edinburgh Medical School,
Hugh Robson Building,
George Square,
Edinburgh, EH8 9XD
February 1998

Acknowledgments

First and foremost I would like to thank my supervisors, Dr. David K. Apps and Dr. Jeff Haywood for their guidance, help, invaluable advice and encouragement, as well as their help during the preparation of this thesis. I also would like to thank Dr. Jeff Haywood for all his help with screening monoclonal antibodies.

I would like to give my special thanks to Dr. H. Bülent Tugal whom I learned a great deal of basic techniques and to Leonora Ciufo for her help with molecular biological techniques.

I would like to give many thanks to Lorna Webster, Ian Thomson, Jan Mackintosh and Muna Sabri for their help with membrane preparations.

I would like to thank Dr. Herman Schägger at Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Germany for carrying out protein sequencing analysis for me, and Dr. Andrew Cronshaw at the Biochemistry Department, University of Edinburgh for his help in HPLC teqhniques.

Finally I would like to thank all members of the Faculty Animal Area, University of Edinburgh for their help with producing antisera.

Abstract

The membranes of chromaffin granules, the catecholamine storing vesicles of the adrenal medulla, contain several glycoproteins, which were initially identified simply as lectin-binding components and some of which have had functions ascribed to them subsequently. Thus glycoprotein I is dopamine β-monooxygenase, glycoproteins H, J, and K are intragranular proteinases, glycoproteins II and III have been sequenced but are still of unknown function, while glycoproteins VI and V are still uncharacterized glycoproteins of unknown function. A major component of the granule membrane is the H^+-translocating ATPase, the largest subunit of which (116kDa) is a glycosylated transmembrane protein and a second glycosylated subunit named Ac45 (now termed M45) was also recently reported.

Glycoprotein IV of bovine chromaffin granule membranes was purified by membrane fractionation with the non-ionic detergent Triton X-114, solubilization with n-octyl-β-glycoside and affinity chromatography on concanavalin A lectin-agarose, followed by electrophoresis from polyacrylamide gels. Purified glycoprotein IV was subjected to direct N-terminal amino acid sequencing, and purified glycoprotein IV was injected into rabbits in order to raise an antiserum used to characterize the protein further.

Blue Native polyacrylamide gel electrophoresis was used to confirm that glycoprotein IV is a component of the membrane sector of the H^+-ATPase, and the anti-glycoprotein IV serum recognized the same component as one directed against subunit M45 of this enzyme. Furthermore the N-terminal amino acid sequence of glycoprotein IV was identified within the M45 gene. Although this confirmed that glycoprotein IV and M45 are identical proteins, it also showed that they are derived from a larger precursor by removal of a 246-amino acid N-terminal sequence. In agreement with this, enzymatic deglycosylation of M45/glycoprotein IV indicated an apparent polypeptide molecular mass of 29kDa.

Study of the topology confirmed that glycoprotein IV although predicated to be a transmembrane protein, has no significant cytoplasmic domain since it is protected from proteolysis in intact granules but not in lysed granules.

Glycoprotein IV/M45 is found in the membranes from mouse pituitary AtT-20, from rat phaeochromocytoma (PC-12) and from human phaeochromocytoma but is absent in bovine kidney microsomal membranes and rat brain synptosomes.

Contents

Chapter 4: Partial amino acid sequence of glycoprotein IV

Chapter 5: Production and characterization of antibodies

Abbreviations

ACA:	6-amino caproic acid (6-aminohexanoic acid)
ATP:	adenosine-5'-triphosphate
ATPase:	adenosine-5'-triphosphatase (EC 3.6.1.3)
BN-PAGE:	blue native polyacrylamide gel electrophoresis
Bis-Tris:	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
BSA:	bovine serum albumin
cDNA:	complementary deoxribonucleic acid
CHAPS:	3(-3-cholamideopropyl)-dimethyl-ammonio-1-propane sulphate
Con A:	concanavalin A
$\Delta\mu H^+$:	proton electrochemical gradient ($\Delta\mu H^+ = \Delta\psi$ - (2.3 RT/ F) ΔpH
ΔpH:	transmembrane pH difference (pH_{out}-pH_{in})
$\Delta\psi$:	transmembrane potential
DBM:	dopamine-β-monooxygenase
DCCD:	N,N'-dicyclohexylcarbodiimide
DNTB:	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT:	dithiothreitol
ECL:	enhanced-chemiluminescence
EDTA:	ethylenediamineN,N' tetraacetic acid
Gal:	galactose
GalNAc:	N-acetylgalactosamine
Glc:	glucose
GlcNAc:	N-acetylglucosamine
Gp:	glycoprotein
H^+-ATPase:	proton translocating adenosine triphosphatase
HEPES:	N-(2-hydroxyethyl)piperizine-N'-2-ethanesulphonic acid
HRP:	horse radish peroxidase
HPLC:	high pressure liquid chromatography
IEF:	isoelectric focusing
IPTG:	isopropyl β-D-thiogalactopyranoside
kb:	kilo-base
kDa:	kilodalton
LCL:	lens culinaris lectin
Man:	mannose
MEGA 8:	ω-octanoyl-N-methylglycamide
MEGA 10:	ω-decanoyl-N-methylglycamide
Mr:	relative molecular mass

n-OG:	n-octyl-β-D-glucopyranoside
PAS:	periodic acid Schiff reagent
PC:	phosphatidyl choline
PI:	isoelectric point
PI-PlC:	phosphatidylinositol-specific phospholipase C (EC 3.1.4.10)
PMSF:	phenylmethylsulphonyl fluoride
PNGF:	peptide: N-glycosidase F (EC 3.5.1.96)
PNMT:	phenylethanolamine N-methyl transferase (EC 2.1.1.28)
rpm.:	revolutions per minute
SA:	sialic acid
SDS:	sodium dodecyl sulphate
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS:	tris-buffered saline
TCA:	trichloroacetic acid
TEMED:	N,N,N'N'-tetramethylethylenediamine
TLCK:	N-p-tosyl-L-lysine chloromethylketone
TPCK:	N-tosyl-L-phenylalanine chloromethylketone
Tris:	tris(hydroxymethyl)aminomethane
WGA:	wheat germ agglutinin

Chapter 1
Introduction

1.1 The adrenal medulla.

The adrenal glands are situated close to the top of the upper pole of the kidney and average 20 to 60 mm in size and weigh about 10g in bovine. The adrenal gland is divided both histologically and physiologically into two distinct portions, the adrenal cortex and the adrenal medulla, and there is accumulating evidence of hormonal interaction between the two. The cortex, the outer portion of gland, contains several cell types, which synthesise and secrete into the plasma several steroids of two main classes: the glucocorticoids and the mineralocorticoids. The medulla contains largely polygonal cells of about 16μm diameter and of two types: adrenalin-secreting and noradrenalin-secreting. Both types contain secretory granules that are characterised by a distinct staining reaction in which they are stained brown with chromium salts; they are therefore known as chromaffin cells. These cells have specialised functions for the biosynthesis, storage and secretion of catecholamines and some peptides and proteins such as enkephalins and chromogranins. Because of their similarity to other types of secretory vesicles and because relatively large quantities of these granules can be obtained in a highly purified state, chromaffin granules have been extensively studied as model secretory granules [Winkler and Carmichael, 1982].

1.2 The chromaffin granule.

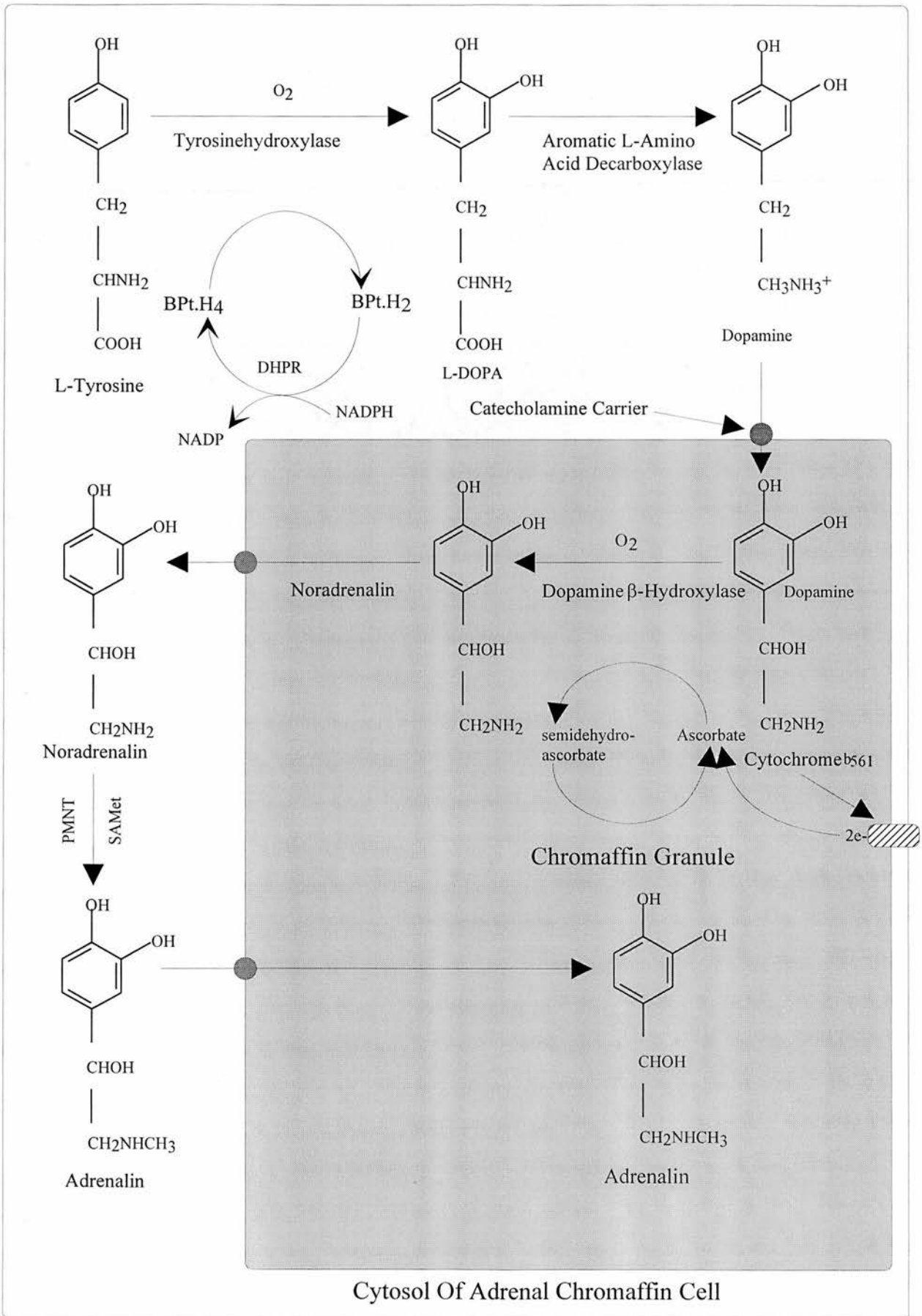
Chromaffin granules, the secretory vesicles of chromaffin cells, release their contents by the process of regulated exocytosis, in which the granules undergo Ca^{2+}-triggered fusion with the plasma membrane and release their contents into the blood- stream [Burgoyne, 1991]. A typical chromaffin cell contains up to 30,000 granules. These are roughly spherical and 100-500 nm in diameter (mean, 280nm in bovine adrenal glands) as determined by electron microscopy [Phillips, 1982; Coupland *et al.*, 1968]. Although often regarded as a homogenous population, the granules appear to vary in shape and size [Coupland, 1968; Kryvi *et al.*, 1979; Terland *et al.*, 1979].

This is because the adrenal medulla is composed of two different cell types (adrenalin- and noradrenalin-storing) in a ratio 3:1. These two types of cells are clearly distinguishable in electron micrographs, those secreting noradrenalin containing the denser-looking granules in comparison to those that secrete adrenalin [Coupland *et al.*, 1964; Coupland and Hopwood, 1966]. The chromaffin granule has a single membrane bounding an inner acidic (pH 5.5) soluble phase called the granule matrix [Johnson and Scarpa, 1976] .

1.3 The chromaffin granule matrix.

The matrix of the chromaffin granule is composed of a complex mixture of secretory products including low molecular mass constituents and some soluble peptides and proteins [Winkler, 1976]. All these products are summarised in Table 1.1. The small-molecule composition of the soluble fraction of chromaffin granules has been well characterised, most of the constituents being in high concentrations, for example catecholamines (~550 mM). The high concentration of catecholamines presents the problem of water entry by osmosis into the granules and it is thought that the nucleotides and proteins of the matrix interact with the catecholamines to lower their activity and prevent this process [Winkler and Carmichael, 1982]. The pathway of synthesis of catecholamines has long been established and is initiated in the cytosol with the hydroxylation of L-tyrosine into L-DOPA (Figure 1.1). This reaction which is catalyzed by the enzyme tyrosine hydroxylase (EC1.14.16.2) is the rate-limiting step of catecholamine synthesis, and is regulated by substrate and co-factor availability as well as multi-site phosphorylation of the enzyme. The product DOPA is then converted into dopamine by the cytosolic enzyme L-amino acid decarboxylase (EC 4.1.1.7.8). The synthesis of both noradrenalin and adrenalin requires the active uptake of cytosolic dopamine into the granules via an amine transporter and once in the granule, the enzyme dopamine β-monooxygenase (EC 1.14.17.1) catalyses its conversion into noradrenalin. This enzyme requires molecular oxygen and catalyses a redox reaction in which two electrons are transferred from ascorbic acid via the two copper ions of dopamine β-monooxygenase to dopamine.

Figure 1.1 Catecholamine biosynthesis in adrenal chromaffin cells



Adapted from Nicholks 1994

KEY : Bpt : biopterin,
SAMet : S-adenosy-L-methionine,
PMNT : ethanolamine-N-methyl transferase.

The ascorbic acid is regenerated from its oxidised form (semidehydro-ascorbate) by non-enzymatic dismutation followed by reduction by electrons supplied from the transmembrane protein cytochrome b561 [Skotland *et al.,* 1980; Diliberto and Allen, 1980]. In the cells with adrenalin-storing granules, noradrenalin leaves the granule and is converted into adrenalin by the cytosolic enzyme phenylethanolamine N-methyl transferase [PNMT EC 2.1.1.28]. The adrenalin is then taken up into the granule via the amine carrier and stored until its release from the cell.

Nucleotides (180mM) are another constituent of chromaffin granules. Most of the nucleotide is ATP which is present at about 150mM. Its actual function in the granules is not known, but it is thought to play an important role in reducing the osmotic stress caused by the high concentration of catecholamines, through ion pairing with Na^+ and molecular stacking with catecholamines [Kopell and Westhead, 1982]. ATP and other nucleotides are taken up into the granule by an atractyloside-sensitive nucleotide-translocase, driven by the membrane potential [Aberer *et al.,* 1978; Weber and Winkler, 1981]. Recently the presence of diadenosine polyphosphates, mainly Ap_4A, Ap_5A and Ap_6A, has been reported in chromaffin granules [Rodriguez del castillo *et al.,* 1988; Pintor *et al.,* 1992].

Ions (cations and anions), are another constituent of the chromaffin granule matrix; among the cations are protons (H^+) which are accumulated by membrane-bound ATPase I complex. The electrogenic accumulation of protons results in a lumenal pH of about 5.2 and generates a membrane potential of around -60 mV (inside positive), which is used in the uptake of small molecules and other ions.

Calcium ions are present in the granules at a total concentration of around 20mM. Only 10mM is free, the rest being sequestered by chromogranin A and as its ATP complex [Bulenda and Gratzl, 1985; Reiffen and Gratzl, 1986]. It is thought that accumulation of Ca^{2+} by the granule could be used as a mechanism for the long-term removal of Ca^{2+} from the cytoplasm. The uptake of the Ca^{2+} into the granules is mediated through the ruthenium red-sensitive Na^+/Ca^{2+} antiporter, which is linked to the proton gradient through of a Na^+ / H^+ antiporter [Haigh and Phillips, 1989].

However the distribution of Ca^{2+} and Na^{+} suggests that these anions do not equilibrate with protons. Magnesium ions are also present in the granules at a concentration of around 5mM, mostly complexed to ATP.

The anionic composition of the granules is less well defined: chloride ions are thought to cross the granule membrane passively driven by the membrane potential, and it has been suggested that a mechanism analogous to the bicarbonate/chloride exchanger of erythrocytes may be present in chromaffin granule membranes to prevent the lysis of granules as a result of Cl^- uptake, or that Cl^- uptake may be mediated through a Cl^-channel analogous to that present in the membrane of coated vesicles and the Golgi apparatus [Xie *et al.,* 1989; Barasch *et al.,* 1991].

Chromaffin granules are known to contain a large number of neuropeptides including enkephalin, neuropeptide Y, dynorphin (only found in noradrenalin granules), neurotensin and substance P [Schultzberg *et al.,* 1978; Goedert *et al.,* 1983; Bucsics *et al.,* 1981; Lemaire *et al.,* 1984; Dumont *et al.,* 1983]. The enkephalins, the first neuropeptides to be discovered in adrenal medulla, and neuropeptide Y are quantitatively the most important. Enkephalin-containing peptides are present predominantly as 23.3 and 18.2 kDa species in immature granules, and as 12.6 and 8.6 kDa species in mature granules [Fischer-Colbrie *et al.,* 1986], suggestive of a rapid processing of preproenkephalin followed by a slower processing to final [Met]- and [Leu]-enkaphalin. Later, Birch (1986) reported the presence of a 27-kDa species in chromaffin granules, which may represent the intact proenkephalin molecule. Proteolytic enzyme activities that have been implicated in the processing of enkephalins are discussed in section 1.3.1.3. Several C-terminally α-amidated neuropeptides have also been identified [Fisher-Colbrie *et al.,* 1986; Seizinger et al., 1985;] and the presence of the α-amidation enzyme has been inferred in chromaffin granules from their discovery. The activity of the α-amidation enzyme would be similar if not identical to that characterised in pituitary secretory granules [Tatemoto *et al.,* 1982; Seizinger *et al.,* 1985].

The α-amidating enzyme isolated from pituitary has been demonstrated to be a concanavalin A- binding glycoprotein and to have cofactor requirements similar to those of dopamine β-monooxygenase [Bradbury *et al.*, 1982; Eipper *et al.*, 1983]

An interesting recent discovery is the presence of amyloid β-peptide (Aβ) in the granules [Vassilacopoulou *et al.*, 1995]. Amyloid β-peptide (Aβ) is derived from the proteolytic processing of a family of proteins termed amyloid precursor proteins (APP). By immunological means both processed Aβ and unprocessed APP forms of the peptides were detected in granule lysate, but only the full length (APPs) is present in the membrane . Although solubilization of this precursor APP did not result in a detectable decrease in its size, APP was inhibited by several proteinase inhibitors as well as by preheating the membrane to 50-70 °C.

More recently it has been reported that bovine chromaffin cells also store and release growth factors like fibroblast growth factor-2 [Bieger *et al.*, 1995], and transforming growth β-factor like activity [Krieglstein *et al.*, 1995], but there has been no molecular characterization of these.

1.3.1 Matrix proteins of chromaffin granules.

In recent years, the combination of two-dimensional gel electrofocusing with immune and lectin blotting techniques has led to the separation and identification of an increasing number of protein components of the chromaffin granule membrane and soluble matrix. Approximately 40 soluble components have been resolved from the chromaffin granule interior [Apps *et al.*, 1985; Fischer-Colbrie *et al.*, 1985]. It is unlikely that each of these components represents a unique gene product, given the apparent processing of various proteins within the chromaffin granule interior. A large portion of the soluble proteins are of the class designated chromogranins (A, B and C) or secretogranins [Blaschko *et al.*, 1967; Fischer-Colbrie *et al.*, 1987]. This group of proteins, with other major soluble proteins (dopamine β-monooxygenase, carboxypeptidase H, glycoprotein III and proteoglycans) will be discussed individually later.

Among the proteins reported to be present in the granule matrix in small amounts is acetylcholinesterase and a membrane-bound form of this enzyme has also been reported [Gratzl *et al.,* 1981]. However, the location of this enzyme is controversial and its presence in the granules may be as a consequence of contaminating endoplasmic reticulum and plasma membrane where this enzyme is present in large amounts [Gratzl, 1984; Mizobe *et al.,* 1984; Burgun *et al.,* 1985]. Grigoryan et al 1981, have isolated another minor protein which represents only 0.25% of the total granule matrix protein. It is an extremely acidic (pI 3.5), 10 kDa copper-containing protein. The structure and the function of this protein are unknown. However, studies of its amino acid composition and antigenicity revealed that it is a member of the neurocuprein family of proteins that have been identified in mammalian brain, and the apo form of this protein has been reported to be a potent inhibitor of dopamine β-monooxygenase. A membrane-bound form of this protein has been reported, and its membrane anchor has been proposed to be an extended hydrophobic tail [Mikaelyan *et al.,* 1987].

A 45kDa enzyme that transfers the terminal phosphate group of ATP to ADP has been partly purified from the soluble proteins of chromaffin granules [Taugner and Wunderlich, 1981], but has not been further characterized.

Table 1.1

Components present in the matrix of bovine chromaffin granule[a]

Components	Approx no.of molecules per granule
1. Matrix small molecules	
Adrenalin	1.7×10^6
Noradrenalin	7.1×10^5
Dopamine	2.1×10^4
ATP	5.8×10^5
Other nucleotides	2.3×10^5
Ap_4A, Ap_5A and Ap_6A[b]	2.0×10^3
Ascorbic acid	1.0×10^5
Na^+	9.4×10^4
Ca^{2+}	4.1×10^4
Mg^{2+}	2.0×10^4
2. Matrix peptides	
Enkephalins	7.5×10^3
NeuropeptideY	5.0×10^2
Amyloid β- peptide (Aβ)[c]	-
Fibroblast growth factor(FGF-2)[d]	-
Transforming growth factor-β-like[e]	-
Neurotensin	0.8
Substance P	0.4
Dynorphin	9,000
	-
3. Matrix proteins	
Chromogranin A,B,C family	5000
Proenkephalin family	4000
Dopamine β-monoxygenase	140
Acetylcholinesterase	-
Proteases	-
4. Matrix glycosaminoglycans	
Chondroitin sulphate	250
Heparin sulphate	250

a. adapted from Winkler and Westhead (1980).
b,c,d and e. these are new compounds that have been recently added to the list.

1.3.1.1 Chromogranins.

A large proportion of the soluble proteins of chromaffin granules are highly acidic, and have been collectively called chromogranins (A, B, and C) [Blaschko *et al,* 1967]; chromogranins B and C were later designated secretogranins I and II. While they remain of unknown function, the presence of the highly acidic chromogranins in a wide variety of neural and endocrine tissues has led to the suggestion that they represent a new class of neuropeptide precursor [Winkler *et al.,* 1986; Fischer-Colbrie and Schober, 1987]. Chromogranin A (CGA) accounts for nearly 40% of the total soluble proteins. It has an apparent molecular weight of about 70 kDa as estimated by SDS/PAGE and a pI of 4.7-5.0. However the amino acid sequence of bovine CGA indicates a true molecular mass of 48 kDa for the unmodified form of this protein [Benedum *et al.,*1986; Iacangelo *et al,* 1986]. The difference between the apparent and theoretical molecular masses of chromogranin A possibly results from post-translational modifications (e.g. glycosylation, phosphorylation and sulphation), and the abundance of acidic amino acids (25%) causing a reduced migration in SDS/PAGE . CGA is a glycoprotein containing about 6% of carbohydrate consisting of tetrasaccharides or trisaccharides linked to serine or threonine by O-glycosidic bonds, and it has almost no defined tertiary structure [Kiang *et al.,* 1982; Smith and Winkler, 1967; Gerdes *et al.,* 1989]. It does not bind to mannose-specific lectins such as Concanavalin A following transfer to nitrocellulose [Apps *et al.,* 1985], but after removal of terminal sialic acid residues by neuraminidase it has been found to bind galactose-specific peanut agglutinin (PNA) (Table 1.3). CGA has also been found to be a phosphoprotein, containing five phosphorylated serine residues per protein molecule, and a little sulphate which is bound to O-glycans and not to tyrosine residues as is frequently the case. [Settleman *et al.,* 1985; Falkensammer *et al.,* 1985; Rosa *et al.,* 1985]. CGA is a substrate of carboxymethylase, an enzyme that modifies side-chain carboxy groups of proteins [Nguyen *et al.,* 1987; Diliberto *et al.,* 1976].

Chromogranin A has been claimed to be an integral membrane protein [Settleman *et al.,* 1985], but this seems unlikely in view of its behaviour on phase-partition in Triton X-114 and its almost complete removal from the membrane by washing with sodium carbonate [Pryde and Phillips, 1986], indicating that the chromogranin A associated with the membrane is a contaminant. Antisera raised against chromogranin A are found to react with a number of acidic low-molecular weight proteins ranging from 17-70 kDa, indicating that chromaffin granules contain endogenous proteinases which cleave chromogranin A to form these smaller proteins [Winkler, 1976; Hagn *et al.,*1986b]. These properties are also features of two other related proteins, chromogranin B (secretogranin I) and chromogranin C (secretogranin II). Simon (1989) has found that CGA and its degradation products are present in the same ratio in both immature and mature granules and also in the extracellular fraction after cell stimulation, suggesting early cleavage. Since part of the primary sequence of chromogranin A corresponds to the peptides pancreastatin and chromastatin, this suggested that chromogranin A may be the precursor of biologically active peptides [Tatemoto *et al.,* 1986; Iacangelo *et al.,* 1988; Hutton *et al.,* 1987a & 1987b]. The latter peptides inhibited secretion from pancreatic β-cells and have been shown to act in a similar fashion on cultured chromaffin cells.

Chromogranin B (CGB), was first identified in 1984 [Winkler *et al.,* 1984]. It is present in chromaffin cells in significantly smaller quantities than chromogranin A, representing approximately 2% of total soluble protein. A comparison between the primary structures of CGA and CGB has revealed that chromogranin A and B are more related to each other than either protein is to other sequenced members of the chromogranin family. CGA and CGB contain a highly similar disulphide-bonded loop structure near their N-termini and another homologous sequence at their C-terminus. Analysis of the mouse CGB gene [Pohl *et al.,* 1990] and the bovine CGA gene [Wu *et al.,* 1991] revealed that both the N-terminal and the C-terminal homologous domains are encoded by separate exons. The disulphide-bonded loop structure is not found in any of the other members of the chromogranin family (i.e. chromogranin C/ secretogranin II, 7B2 or secretogranin V) that have been sequenced.

Apart from their homologous N-terminal and C-terminal regions, there is no significant sequence similarity between CGA and CGB in the remaining 90% of the polypeptide chains. Chromogranin B is slightly less acidic than chromogranin A, and has an apparent mass of 100 kDa and pI=5.2. It has been found from molecular cloning to be a 76 kDa protein, and like CGA, to be subject to post-translational modification such as glycosylation, phosphorylation and sulphation. CGB is a glycoprotein containing 5.4% carbohydrate consisting of predominantly O-linked glycosides and some N-linked oligosaccharide chains. It can therefore, bind several lectins including Concanavalin A, and like chromogranin A, it binds peanut agglutinin [Apps *et al.,* 1985] after neuraminidase treatment. Chromogranin B has a sugar composition similar to that of chromogranin A but it contains significantly more fucose, mannose and sialic acid [Fischer-Colbrie and Frischenschlager, 1985]. It has also been shown to be a phosphoprotein, the phosphate being predominantly linked to serine residues, and to a small extent to threonine, but not to tyrosine residues [Rosa *et al.,* 1985]. Sulphate is also bound to CGB through O-glycans. CGB is a substrate of carboxymethylase [Nguyen *et al.,* 1987].

Chromogranin B was shown to have a widespread distribution in neuroendocrine tissues, [Rosa *et al.,* 1985; Lassman *et al.,* 1986]. In contrast to bovine adrenal medullary CGA, which is the predominant protein in secretory granules, CGB is the most abundant component in rat and human adrenal medulla [Hagn *et al.,* 1986a]. In addition CGB is processed to a greater extent than CGA, contrasting with the situation in other tissues where the precursor protein is the major component. [Fischer-Colbrie *et al.,* 1987]. Bovine chromaffin granule CGB was reported to be the precursor of biologically active peptides named secretolytin [Natori et al., 1994] and secretoneurin [Strub *et al.,* 1995].

A third family of acidic proteins present in the granule matrix is chromogranin C. These proteins react with an antiserum raised against secretogranin II, a protein described by Rosa and Zanini (1983) in the anterior pituitary. Chromogranins C are immunologically distinct from chromogranins A and B, and there is no observed immunological cross reactivity between these three distinct families of chromogranins [Winkler *et al.,* 1986].

Chromogranin C is a glycoprotein with an apparent molecular weight of 86-kDa and pI of 5.0, and represents only 1% of the total soluble protein.

The function of the chromogranins is elusive and although the members of the chromogranin family are now considered to be precursor molecules, little is known about their processing. Originally it was thought that the chromogranins were important for catecholamine storage, but the distribution of chromogranins in secretory granules and the absence of stable interactions between chromogranins and these compounds weakened this idea. Helle (1985a) suggested that the chromogranins may contribute to osmotic pressure regulation of chromaffin granules. Chromogranin A was found to bind Ca^{2+} suggesting that it may help to regulate the free Ca^{2+} concentration within chromaffin granules [Reiffen and Gratzl, 1986]. Two precursors of chromogranin A can be synthesised in reticulocyte lysate and wheat germ cell-free systems and these two precursors are converted into a single protein, most likely by the removal of different signal peptides. Only one precursor of chromogranin B is produced when mRNA from the adrenal medulla is translated in a wheat germ cell-free system. In the presence of microsomes the two chromogranin A precursors are converted into a single protein, apparently by the removal of different signal peptides [Kilpatrick *et al.,* 1983; Falkensammer *et al.,* 1985]. Endogenous chromogranin A and chromogranin B are larger and more acidic than the precursors translated in the presence of microsomes and O-glycosylation in the Golgi probably accounts for the increase in molecular mass. Mature chromogranin B focuses at pI 5.2 but the precursor has a pI of 5.7. The presence of sulphate residues in the mature form of chromogranin B may explain why it is more acidic than its precursor.

1.3.1.2 Dopamine β-monooxygenase (Glycoprotein I).

Dopamine β-monooxygenase (DBM) catalyses the conversion of dopamine to the neurotransmitter and hormone noradrenalin within catecholamine-secreting vesicles (chromaffin granules) of the adrenal medulla and the large dense-cored synaptic vesicles of the sympathetic nervous system. Originally it was isolated from bovine adrenal glands and was termed glycoprotein I.

Later it was identified as one of the two major membrane glycoproteins recognised as the enzyme dopamine β-monooxygenase (EC 1.14.17.1) [Levine *et al.*, 1960; Hörtingal *et al.*, 1972; Huber *et al.*, 1979]. In adrenal chromaffin granules dopamine β-monooxygenase is known to exist in both soluble and a membrane-bound forms, with an approximately equal distribution of enzyme activity between the two forms, although the soluble form accounts for only 3-6% of the total soluble proteins. The membrane-bound form represents approximately 20% of the total membrane proteins [Winkler *et al.*, 1986]. The native form of DBM is a tetrameric copper-containing glycoprotein of about 300-kDa [Wallace *et al.*, 1973] with the subunits arranged as pairs of disulphide-linked 75-kDa monomers species [Skotland and Ljones, 1977]. But more recent data show that the membrane-bound enzyme contains at least two subunits of higher molecular weight (77 kDa) than those of the soluble protein (73 kDa). Temperature-induced phase separation in Triton X-114 of chromaffin granule proteins is able to separate the two forms of DBM [Pryde and Phillips, 1986]. Both forms are N-glycosylated glycoproteins and Margolis (1984) analysed the sugar content of DBM and found each tetramer to contain an average of six oligosaccharide chains, two being of the high mannose type and four being complex biantennary chains with a molar ratio of 1:2 indicating microheterogeneity. It therefore it has affinity for a number of lectins including Concanavalin A(Con A), Lens culinaris lectin (LCL), and Wheat germ agglutinin (WGA), and these were used to purify the enzyme by affinity chromatography [Cahill and Morris., 1979; Gavine *et al.*, 1984; Apps *et al.*, 1985]. The carbohydrate portion of DBM has been analysed in detail and found to consist of residues of fucose, mannose, galactose-N-acetylgalactosamine, N-acetylglucosamine and sialic acid. It was also found that the membrane and soluble forms of DBM have identical carbohydrate compositions [Wallace et al., 1973; Gessler *et al.*, 1977; Fischer-Colbrie *et al.*, 1982]. In addition to heterogeneity in subunit structure, there is the potential dissociation of the tetrameric enzyme into dimeric (150 kDa) forms consisting of two disulphide-linked monomers, as well as possible higher-order oligomerisation [Park *et al.*, 1976; Rosenberg *et al.*, 1977].

The copper in DBM is relatively weakly bound. Early spectroscopic studies had shown that DBM is capable of binding up to eight coppers per tetramer (2 atoms of copper per subunit) [Ash *et al.,* 1984; Klinman *et al.,* 1984], and also proposed that the two electrons required for the hydroxylation of dopamine are donated by both copper atoms in each subunit. This would suggest that all the subunits are identical in function. The biosynthetic relationship between the membrane-bound and the soluble forms of DBM is controversial. DBM is synthesised on membrane-bound polysomes in bovine adrenal medulla and phaeochromocytoma cells (PC12) [Sabban *et al.,* 1983; Sabban and Goldstein, 1984] In phaeochromocytoma cells DBM is synthesised as a precursor of apparent molecular mass 67kDa which is rapidly glycosylated to a 77kDa form, which is then converted to a subunit of 73kDa within 15-90 minutes [Sabban *et al.,* 1983]. The membrane form of DBM appears to be composed of both types of subunit whereas the soluble form consists predominantly of the subunit of 73 kDa. Sabban (1983) proposed that soluble DBM in phaeochromocytoma may arise from post-translational processing of membrane DBM. This suggests the presence of a processing enzyme that functions at the acidic pH of mature granules. Bjerrum (1979) originally reported evidence for such an endogenous protease within chromaffin granules. Later, Helle (1985b) described the identification of a cathepsin-D-like enzyme capable of converting membranous DBM to a soluble form at pH 6.0. DBM has been cloned and sequenced from human phaeochromocytoma [Skotland *et al.,* 1977; Lamouroux *et al.,* 1987]. Despite the extensive evidence for two subunit forms little information is available regarding the structural basis for membrane attachment. The amino acid sequence for human phaeochromocytoma DBM shows no evidence of a hydrophobic domain other than the N-terminal signal sequence. Also studies by Stewart *et al.,* (1988) and Tayler *et al.,* (1989), both eliminated covalently-attached phosphatidylinositol as another possible mode of membrane anchoring for DBM, or the possibility of an uncleaved signal sequence as is the case with cytochrome P-450 [Sakaguchi *et al.,* 1987]. The mechanism of membrane attachment still remains unclear.

1.3.1.3 Proteinases.

Chromaffin granules contain several neuropeptides (Table 1.1). These molecules are derived from precursors by a post-translational mechanism of proteolytic processing which takes place within secretory granules. The processing occurs by a cascade of proteolytic steps involving several proteinases. Two endoproteinases that cleave bonds C-terminal to paired basic amino acids are related to subtilisin and to the kex2 proteinase of *Saccharomyces cerevisiae*, and are termed proenkephalin convertases: PC1 (also known as PC3) and PC2 [Kirchmair *et al.*, 1992]. PC2 corresponds to the previously-recognized chromaffin granule membrane glycoprotein H (molecular mass of 66 kDa and pI range of 5.2-5.8) as confirmed from sequencing data [Gavine *et al.*, 1984; Christie *et al.*, 1991]. PC2 is inhibited by the 27 kDa (but not the mature 21kDa) form of the neuroendocrine peptide 7B2 and its Ca^{2+} dependence suggests that it may correspond to the Ca^{2+}-activated protease identified earlier [Lindberg *et al.*, 1995; Laslop *et al.*, 1990]. A trypsin-like enzyme is another soluble adrenal endoproteinase [Hook *et al.*, 1984]. It can cleave intermediate-sized enkephalin-containing precursors. However, a portion of trypsin-like enzyme activity within chromaffin granule has been shown to be associated with the granule membrane [Shen *et al.*, 1989]. Another granule endoproteinase is a novel thiol proteinase [Krieger *et al.*, 1991], a glycoprotein of pI about 6.0 which cleaves at single or paired basic residues to generate methionine enkephalin. It has a pH optimum of 5.5, indicating that it is functional at the intragranular pH of 5.5-6.0. A metalloproteinase (molecular mass 45kDa) that cleaves at single basic residues, and a soluble aspartate proteinase (molecular mass 70kDa), also glycosylated, which cleaves between pairs of basic residues have been reported [Azaryan *et al.*, 1995]. Both bind Concanavalin A. All the above proteinases are minor granule components.

Carboxypeptidase H is an exoproteinase that removes C-terminal basic amino acids (arginine and lysine) from partially-processed pro-enkephalin and has no detectable activity towards other amino acids [Hook *et al.*, 1982; Fricker *et al.*, 1986].

Carboxypeptidase H has been well characterized with respect to its biochemical, molecular and regulatory properties and it appears to be involved in processing many neuropeptides. This enzyme (EC 3.4.17.10) was previously known as enkephalin convertase and carboxypeptidase E. The chromaffin granule-associated carboxypeptidase differs from other carboxypeptidases in that it is activated in several ways. It is stimulated by cobalt chloride and is inhibited by chelating agents similarly to carboxypeptidase B (CpB) and carboxypeptidase N (CpN). However CpB and CpN are both maximally active at a neutral pH [Fricker and Snyder 1982], whereas the chromaffin granule enzyme is maximally active at pH 5-6. Carboxypeptidase H occurs in two molecular forms, in approximately equal amounts which differ in their pI and molecular mass.

Both forms of carboxypeptidase are single chain glycoproteins that bind to Concanavalin-A and they are identical with the previously-identified membrane glycoproteins J (56kDa) and K (53kDa) as shown by sequencing data [Gavine *et al.,* 1984; Fricker *et al.,* 1986; Laslop *et al.,* 1986; Rodriguez *et al.,* 1989; Christie *et al.,* 1990]. Cloning and sequencing carboxypeptidase H from a bovine pituitary cDNA library has revealed that it is produced as a larger precursor. Removal of a basic C-terminal peptide would shorten the protein by 3280 Daltons, which is consistent with the observed difference in molecular weight between the two mature forms [Fricker *et al.,* 1986]. Both forms of carboxypeptidase H arise from the same precursor, which is encoded by a single gene, a member of a carboxypeptidase gene family that includes carboxypeptidase A and carboxypeptidase B. At the amino acid level, carboxypeptidase H has approximately 20% homology with bovine carboxypeptidase A and 17 % homology with bovine carboxypeptidase B [Fricker *et al.,* 1986]. Carboxypeptidase H has a broad tissue distribution, and it has been found to be present in many tissues that contain high or low levels of hormones or neurotransmitters. It is present in the pancreas, salivary glands, and endocrine cells of the gastrointestinal tract [von Zastrow *et al.,* 1986, Hook *et al.,* 1985], as well as in various regions of the brain. It is also present in moderate amounts in the lung, ileum and the colon, and at low levels in many other peripheral tissues including heart, pancreas, testis and spleen [Lynch *et al.,* 1986, 1987 & 1988].

1.3.1.4 Glycoprotein III.

Glycoprotein III is another chromaffin granule protein present in both soluble and membrane-bound forms. No differences were found between these two forms, both being acidic glycoproteins of pI 4.5-5.3. This wide range of pI may result in part from incomplete sialylation. The protein migrates in SDS-PAGE as a broad band of apparent molecular mass 74kDa under non-reducing conditions and 37kDa under reducing conditions [Fischer-Colbrie *et al.,* 1982 & 1984]. An antiserum raised against it has been used to show that the chromaffin granule membrane is returned to the Golgi following exocytosis and recycled into new secretory granules [Patzak and Winkler, 1986].

Glycoprotein III was also detected in other organs for example the anterior and posterior pituitary. It contains approximately 30% carbohydrate with major sugars being galactose, N-acetylglucosamine, sialic acid and mannose. For this reason it has a high affinity for wheat germ agglutinin (WGA), [Fischer-Colbrie *et al.,* 1982, Gavine *et al.,* 1984]. Glycoprotein III was purified from the soluble fraction of bovine chromaffin granules by chromatography using WGA Sepharose followed by reverse-phase high performance liquid chromatography, and its amino acid sequence was established [Palmer and Christie, 1990]. Amino acid analysis and partial N-terminal sequencing indicated that glycoprotein III was a disulphide-linked heterodimer of 37kDa subunits termed A and B chains. Both subunits contain carbohydrate: chain A has a polypeptide core of molecular mass 23,620 Daltons with three potential N-linked glycosylation sites, while chain B has a core polypeptide of 25,335 Daltons with five-potential N-linked glycosylation sites. When glycoprotein III from adrenal medulla was cloned [Palmer and Christie, 1990], a surprising finding was that its sequence was homologous with that of several other glycoproteins isolated from divergent sources: sulphated glycoprotein 2 from rat Sertoli cells, clusterin from ram testis fluid, SP-40,40 and apolipoprotein J from human serum. Since glycoprotein III has homology to glycoprotein 2 of rat Sertoli cells which can aggregate red blood cells, it is suggested that membrane binding by glycoprotein III may be dependent on the lumenal milieu.

Specific antisera were raised against the A and B chains of glycoprotein III, and immunoblotting using these antisera revealed that both chains with slightly differing molecular sizes are present in several endocrine tissues and in the brain, kidney, liver and serum. The function of glycoprotein III is obscure, although it has been suggested that it may be involved in cell death [Laslop *et al.*, 1993].

1.3.1.5 Proteoglycans.

The soluble proteoglycans of the chromaffin granule represent some 3-4% of the total soluble proteins (Table 1.1). Their glycosaminoglycan component mainly consists of dermatan sulphate and chondroitin 4- and 6-sulphate and heparan sulphate, which provide a total carbohydrate content of 25 and 45% [Blaschko *et al.*, 1976; Geissler *et al.*, 1977; Kiang *et al.*, 1982]. This heterogeneous sulphated component (86-100 kDa, pI 4.3-5.0) is susceptible to digestion with chondroitinase ABC. The protein core of the proteoglycans was initially thought to be immunologically similar to that of chromogranin A, despite differences observed in their peptide maps [Banerjee and Margolis, 1982; Kilpatrick *et al.*, 1983]. These differences were attributed to the different glycoside content of the two proteins. However the demonstration that the antibodies used in the comparison of the peptides were directed towards the glycosyl chain suggests that the protein core may indeed be dissimilar [Gowda *et al.*, 1990].

Chromogranin A was identified as existing in a form with attached proteoglycan by its sensitivity to chondroitinase treatment and also by [^{35}S] sulphate labelling [Falkensammer *et al.*, 1985; Rosa *et al.*, 1983]. This proteoglycan form of CGA represents only a minor component of the total proteoglycans in bovine chromaffin granules and only about 1-2% of CGA is present in this form. Proteoglycan CGA probably contains only a single chondroitin sulphate or dermatan sulphate chain of approximately 15kDa [Gowda *et al.*, 1990]. In parathyroid glands a proteoglycan form of CGA was only found in bovine, but not in porcine tissue. On the other hand in rat tissues the relative amounts of proteoglycan CGA varied from tissue to tissue [Gorr *et al.*, 1991].

1.4 The chromaffin granule membrane.

The basic structure of the membrane of chromaffin granules is a bimolecular lipid leaflet which behaves on freeze-fracturing just like any other membrane. The fractured membrane is split along its hydrophobic part yielding a convex fracture face and a concave fracture face on the cytoplasmic half of the bimolecular leaflet [Kryvi *et al.,* 1979]. The membrane composition of the chromaffin granule has to be compatible with its function, primarily the accumulation and storage of its contents, and consequently the membrane is highly impermeable to ions and small molecules [Johnson and Scarpa, 1976]. Chromaffin granule membranes have a high lipid to protein ratio: about 2μmole of phospholipid per mg of proteins [Winkler *et al.,* 1976].

1.4.1 Lipid composition of the chromaffin granule membrane.

The lipid composition is summarised in Table 1.2. Among the lipids, sphingomyelin and cholesterol are particularly abundant (e.g. 0.6μmol cholesterol/μmol phospholipid) [Winkler, 1976]. Chromaffin granule membranes also have an usually high content of lysophosphatidylcholine (approximately 20% of the total phospholipid) [Da Prada, 1972] which has been postulated to play an important role in the fusion between membranes [Howell and Lucy, 1969]. However only 10% of this phospholipid is in the outer leaflet of the lipid bilayer of the chromaffin granule membrane [Voyta *et al.,* 1978; de Oliveira-Filgueiras *et al.,* 1979]. In contrast, phosphatidylethanolamine, together with phosphatidylinositol, is enriched in the outer leaflet [Buckland *et al.,* 1978]; the latter is in the correct orientation for the generation of inositol phosphates through the action of phospholipase C. Phosphatidylinositol appears to be phosphorylated by a component of the chromaffin granule membrane [Phillips, 1973] and the product of this reaction, phosphatidylinositol 4,5-bisphosphate (PIP2) is implicated in the regulation of actin filamentation by proteins such as villin and severin during exocytosis [Matsudaira and Janmey, 1988]. Evidance that phosphatidylinositol 4-kinase is involved in exocytosis has been presented [Wiedemann *et al.,* 1996].

The other lipid component of the chromaffin granule membrane is the gangliosides which compose 2-3% of the total phospholipid. Three different species of GM_3 account for around 95% of the gangliosides [Dreyfus *et al.,* 1977; Serkine *et al.,* 1984]. As expected the oligosaccharide moieties of the gangliosides are exposed to the lumen of the granule [Westhead and Winkler, 1982].

1.4.2 Membrane proteins of the chromaffin granule.

There are at least as many proteins associated with the chromaffin granule membrane as proteins present in the soluble interior, as resolved by one-dimensional polyacrylamide gel electrophoresis and Coomassie blue staining. Abbs and Phillips, (1980) observed up to sixty bands but of these about twenty were present only in small amounts, and may well have been due to contamination. Some proteins which have different isoelectric points co-migrate on one-dimensional electrophoresis, and such proteins may be resolved by two-dimensional electrophoresis [Apps *et al.,*1980]. About half of these proteins are glycoproteins, which are poorly stained by Coomassie blue, and therefore may be missed if they are minor components, so in early studies the carbohydrate moieties of glycoproteins play an important role in the investigation of these proteins, for example Huber *et al.,* (1979) used periodic acid/Schiff stain on SDS polyacrylamide gels. This stain is relatively insensitive but is able to stain glycoproteins particularly those containing sialic acid. Using this method five membrane glycoproteins, including dopamine β-monooxygenase, were revealed.

Cahill and Morris (1979) used fluorescein-conjugated lectins to investigate glycoproteins, and after separation on SDS polyacrylamide gels, they were able to identify 12 glycoproteins. Abbs and Phillips (1980) used chemical and lectin labelling, and their results were in good agreement with those of Cahill and Morris (1979). The studies of Roda *et al.,* (1980) revealed a smaller number of concanavalin A binding proteins, many of them of low molecular weight.

The sensitivity and resolution of detection of glycoproteins are improved after transferring them to nitrocellulose membrane, and decorating them with radio-iodinated lectins [Clegg, 1981; Hawkes *et al.,* 1982].

Gavine *et al.,* (1984) used this method to investigate chromaffin granule membrane glycoproteins. After separating them by one- and two-dimensional electrophoresis and using several different radio-iodinated lectins, over 20 different glycosylated components were identified. Most of the membrane glycoproteins bind concanavalin A but the low affinity of glycoprotein III for Con A makes it difficult to detect on Con A overlays of chromaffin granule membrane. Glycoprotein II and III both have a high affinity for WGA however.

Later, the separation of membrane proteins into different fractions by phase separation in Triton X-114 or extraction with lithium di-iodosalicylate, followed by detection using radio-iodinated lectins, simplified and enhanced the analysis of these proteins. Using the non-ionic detergent Triton X-114 membrane proteins were separated into four fractions termed phospholipid-rich phase (P1), detergent-rich phase (P2), aqueous phase(S) and extrinsic membrane proteins [Pryde *et al.,* 1986], while extraction of chromaffin granule membrane glycoproteins with lithium di-iodosalicylate simply separated them into soluble and insoluble fractions [Christie *et al.,* 1990]. The major matrix and membrane glycoproteins together with their lectin-binding properties are listed in Table 1.3. The very low abundance of some proteins means that they can only be visualised by use of specific antibodies e.g SV2, p65 (synaptotagmin) and p38 (synaptophysin). In addition there are more membrane proteins which have not been identified on one- or two-dimensional gels but which are recognised by their activities in chromaffin granule membrane preparations (e.g the Na^+/H^+ antiporter) [Winkler *et al.,* 1980; Franson *et al.,* 1982; Treiman *et al.,* 1983]. The proteins of the chromaffin granule membrane have been categorised into three groups as summarised in Table 1.4. [Winkler *et al.,* 1986].

Synaptobrevin (p18) also termed vesicle-associated membrane protein (VAMP) is an intrinsic protein of synaptic vesicles with a molecular wight of 18kDa and pI of 6.6. It is present in chromaffin granules together with a smaller homologue known as cellubrevin [Baumert *et al.,* 1989; Foran and Lawerence 1995].

Synaptophysin is a glycoprotein with a pI ranging from 4.5-4.8 and molecular weight of 38kDa, but under non-reducing conditions it forms homo-oligomers and has an apparent molecluar weight 76kDa on SDS gels [Rehm *et al.,* 1986; Johnston

et al.,1989; Obendorf *et al.*, 1988]. The primary strucure of synaptophysin is known and like p65, it is highly conserved. It consists of 307 amino acids with membrane topology showing four hydrophobic transmembrane domains. Its N-terminus as well as its larger C-terminal domain are both exposed to the cytoplasm [Buckley *et al.*, 1987; Leube *et al.*, 1987; Südhof *et al.*, 1987]. Synaptophysin has been demonstrated to be a substrate for tyrosine kinase [Pang *et al.*, 1988].

In recent studies, more proteins have been added to the list of membrane proteins (Table 1.3, group c). These include a glycoprotein laminin, which is composed of one A-chain with an apparent molecular weight on SDS-PAGE of about 400-kDa, and one B1and B2 chain with an apparent molecular weight of about 215 and 205kDa respectively. Only the B-chain was identified in bovine and rat adrenal chromaffin granule membrane by immunoblotting and latter confirmed by an immunocytochemical study [Fujino *et al.*, 1994]. Since the presence of laminin A-chain in the granules was undetectable, the role of laminin in these secretory granules is still unknown. More recently two cysteine string proteins (Csps) termed Csp1 (36kDa) and Csp2 (70 kDa) were detected in the rat adrenal medulla by using immunoblot analysis against the C-terminal region of *Torpedo* Csps [Kohan *et al.*, 1995]. Later, these proteins were cloned from bovine adrenal medullary chromaffin cells. They were originally discovered in *Drosophila,* where they are apparently found localized excusively at synaptic terminals, and thought to be neuron-specific. Recent work has shown a more widespread distribution of these proteins, suggesting a more general role in synaptic exocytosis, resembling other proteins involved in exocytosis previously thought to be brain-specific [Chamberlain *et al.*, 1996].

In contrast to the soluble granule proteins, the structure and function of several membrane proteins have been well studied, in particular the proteins related to catecholamine biosynthesis; dopamine β-monooxygenase and cytochrome b561, which together constitute around 50% of the total membrane protein [Winkler *et al.*, 1980 & 1986]. Dopamine β-monooxygenase has been described earlier (section 1.3.1.2)

Among the other major proteins of the chromaffin granule membrane that are involved in biosynthesis of intraganular contents is carboxypeptidase H, previously known as carboxypeptidase E or enkephalin-convertase. One common feature of the biosynthetic enzymes dopamine β-monooxygenase and carboxypeptidase H is that they are present in both membrane-bound and soluble forms in approximately equal amounts. Another chromaffin granule protein, glycoprotein III, with unknown function, is also present in two forms. The reason for this dual distribution is not known. All of the above proteins have been cloned and sequenced (see sections 1.3.1.2, 1.3.1.3 and 1.1.3.4 for more details)

1.4.2.1 Cytochrome b561.

Cytochrome b561, which comprises about 20% of the total membrane protein, provides reducing equivalents for the function of dopamine β-monooxygenase (section 1.3.2) [Terland and Flatmark, 1980; Nujs *et al.*, 1983]. It is an integral membrane protein, with an apparent molecular mass of 28kDa on SDS gels. It is not glycosylated but its N-terminus is fatty-acylated and thus attached to the membrane [Kent and Fleming, 1990]. It is thought to be the only haem-containing protein in chromaffin granule membranes and it gives the membranes their pink colour. Spectroscopic and electrochemical studies of cytochrome b561 indicated that it contains a single haem redox centre within a midpoint potential of 140 mV [Apps *et al.*, 1984], but Degli Esposti *et al.*, (1989) predicted a di-haem structure on the basis of CD spectroscopy. The primary amino acid sequence of this transmembrane protein has been determined to be 273 amino acids [Perin *et al.*, 1988] and it is predicted to have six transmembrane helices, although from more recent data, a structure with only five transmembrane spans was proposed [Srivastava *et al.*, 1994]. The haem ligands appear to be conserved histidine residues in helices 2 and 4 of this new structure. Cytochrome b561 also occurs in both large dense-core and small dense core vesicles of sympathetic neurones, as well as in the secretory granules of the anterior and posterior pituitary [Asamer *et al.*,1971; Duong *et al.*, 1984; Pruss and Shepard, 1987].

1.4.2.2 Catecholamine transporter.

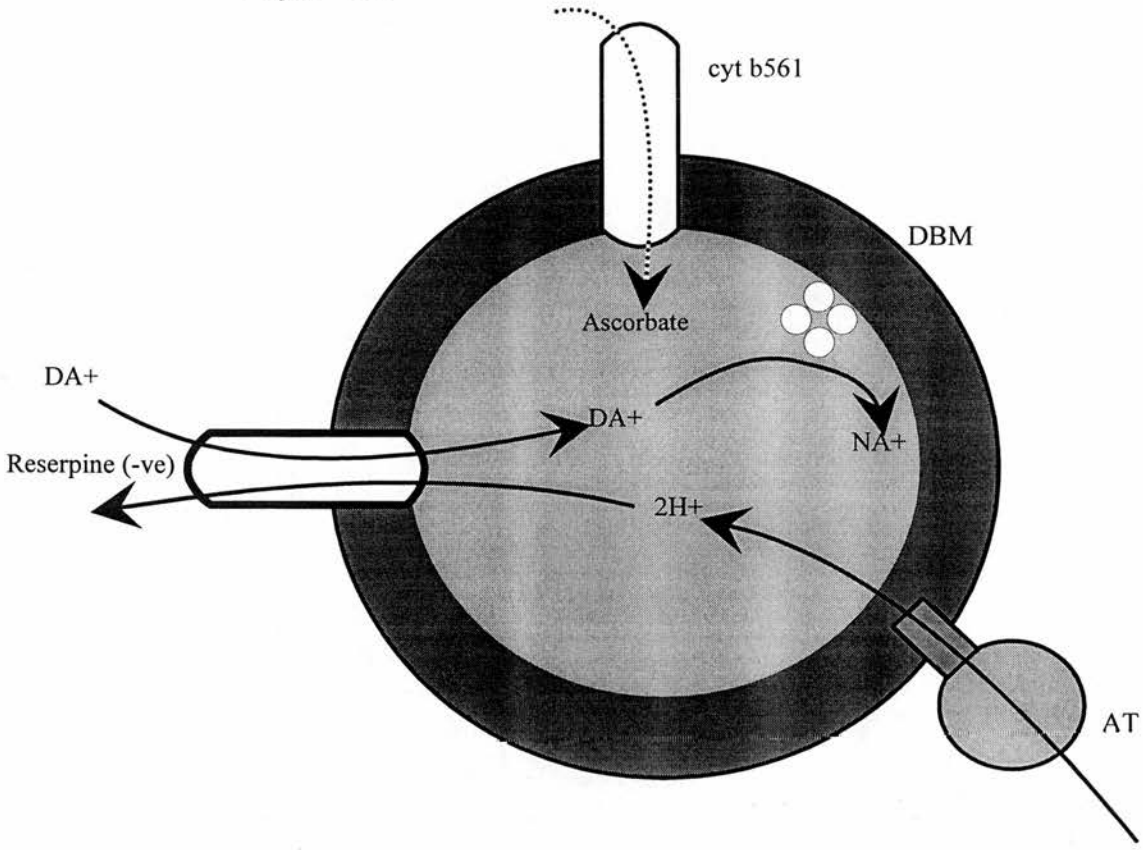
In contrast to cytochrome b561, the catecholamine transporter has not been well characterized. Uptake of catecholamines into chromaffin granules depends on the trans membrane potential ($\Delta\psi$) and pH difference (ΔpH) generated by a proton-translocating ATPase (Figure 1.2). Transport is observed to be greatest and most rapid when both (ΔpH) and ($\Delta\psi$) are present, with an empirical relationship between the driving force for amine transport ($\Delta\mu$) and total amine accumulation of

$$\Delta\mu = \Delta\psi - 2Z\,\Delta pH$$

where $Z = 2.3\,RT/F$ [Johnson *et al.*, 1979].

The stability of transported biogenic amines to leakage or release was studied by Schuldiner (1985), who found little or no loss over a period of an hour. The molecular mechanism whereby catecholamines can be retained by vesicles in the absence of ATP, yet undergo facile uptake and exchange, is unknown [Maron *et al.*, 1983]. Although the kinetics and thermodynamics of amine transport in chromaffin granules have been well studied, the nature of the transporter protein has made its purification and study very difficult. Attempts at isolating the carrier have primarily involved the solubilization of complexes of the protein bound to radiolabeled tight-binding inhibitors [Scherman *et al.*, 1983]. The transporter is inhibited by reserpine, tetrabenazine (TBZ), ketanserin and also by DCCD [Scherman *et al.*, 1983; Henry *et al.*, 1994]. To date, two forms known as VMAT1 and VMAT2 have been reported. These forms were cloned from rat adrenal and rat brain [Liu *et al.*, 1992; Erikson *et al.*, 1992], and both forms are also expressed in bovine adrenal medulla [Henry *et al.*, 1994]. VMAT2 is a more acidic glycoprotein (pI 3.5) than VMAT1 (pI 5.0). The two forms of the amine transporter differ in their kinetic properties, VMAT2 having a higher affinity than VMAT1 for all substrates tested, and in particular for histamine [Peter *et al.*, 1993]. Hydropathy plots of the derived aminoacid sequence of VMAT1 suggest that it has twelve transmembrane helices. A large extramembrane loop between helices 1and 2 contains three potential N-glycosylation sites and is presumably intravesicular.

Figure 1.2



Adapted from Nicholls 1994

The catecholamine (D^+A) is accumulated in exchange for $2H^+$ by a reserpine-sensitive carrier. Dopamine β Monooxygenase (DBM) attached to the lumenal face of the vesicle membrane hydroxylates DA to NA; this requires ascorbate which is reduced by electrons entering the vesicle through the transmembrane cytochrome b651

None of the other chromaffin granule membrane transporters have been characterized structurally. However SV2, a glycoprotein of 100kDa which was originally recognised as a synaptic vesicle antigen [Buckley and Kelly, 1985], has been identified in small synaptic vesicles of endocrine cells which are devoid of typical chromaffin granule marker proteins such as DBM and cytochrome b561. Recently, SV2 has been found to have an aminoacid sequence that places it in the 'major facilitator superfamily' of transporters [Feany *et al.*, 1992]. Two isoforms, SV2A and SV2B, are differentially expressed in brain [Bajjalieh *et al.*, 1994], and only SV2A is expressed in adrenal (PC12). As yet there is no direct evidence that SV2 is a transporter.

1.4.2.3 Adenosine triphosphatases (ATPases)

There are two different ATPases associated with chromaffin granule membrane, termed ATPase I complex (H^+-translocating ATPase) and ATPase II. ATPase I has been found to account for 70-75% of granule ATPase activity with a second vanadate-sensitive ATPase (ATPase II) activity reported to represent the remaining 20-25% [Dean *et al.*, 1986]

1.4.2.3.1 ATPase I complex (H^+-translocating ATPase)

The chromaffin granule H^+-ATPase is a member of the vacuolar (V-type) family of proton pumps, which is responsible for acidification of intracellular organelles such as lysosomes, endosomes, trans-Golgi vesicles and secretory vesicles. The chromaffin granule ATPase when first identified [Kirshner *et al.*, 1962] was thought to belong to the F_1F_o class of ATPases but recent studies of inhibitor sensitivity indicate that the proton pump of chromaffin granule (as well as other secretory granules) represents a third class of ion-translocating ATPase (vacuolar type ATPase). Viewed by electron microscopy the V-ATPase enzymes have a similar morphology to the F-type ATP synthase, i.e. a ball and stalk structure, which represents the two main parts of V-type ATPase: catalytic and membrane sector. This structure is seen on the surface of the lipid bilayer with the diameter of the ball being 10nm comparable to the 9nm F_1 particle [Bowman *et al.*, 1989; Moriyama *et*

al., 1991]. Taking into account this shape, it is suggested that the ball represents the catalytic-sector component of V-type ATPase, as was confirmed by the use of antibodies against the 72- and 57-kDa subunits of the chromaffin granule ATPase [Moriyama *et al.*, 1991].

All catalytic sector (V_1) subunits are accessible to treatment by impermeant reagents such as N-hydroxysuccinimidyl biotin, which suggests that they are exposed to the cytoplasm [Apps *et al.*, 1989]. The catalytic sector (V_1) contains five different polypeptides denoted A, B, C, D, E and possibly subunit F although this has not yet been definitely identified in chromaffin granules [Nelson *et al.*, 1992, 1994, 1995; Gräf *et al.*, 1994; Graham *et al.*, 1994]. The stoichiometry of these subunits, excluding F, was determined to be 3:3:1:1:1 respectively [Arai *et al.*, 1988; Supek *et al.*, 1994] .

Subunit A (68kDa) of V-ATPase contains the catalytic ATP- binding site of the enzyme [Moriyama *et al.*, 1987; Bowman *et al*, 1988; Zimniak *et al.*, 1988]. The aminoacid sequence of this subunit reveals a glycine-rich motif that is common to ATP-binding proteins [Walker *et al.*, 1982 (a), (b); Saraste *et al.*, 1990]. In V-ATPase this motif contains cysteine residues that confer on this class of ATPases their characteristic sensitivity to alkylating agents such as NEM. The sequence of this subunit shows a relationship to the protein sequence of the $F_1\beta$ subunit. It is not clear whether subunit B (57kDa) also contains an ATP-binding site [Moriyama and Nelson, 1987]. Sequence analysis reveals an extensive homology to the α subunit of F-ATPases [Bowman *et al.*, 1988; Manolson *et al.*, 1988]. However subunit B contains no glycine-rich sequence which would indicate the existence of a nucleotide-binding site. These and other observations suggest that subunit B may function in regulating the activity of V-ATPase, but may do so with or without direct involvement of bound nucleotides. The remaining subunits in the catalytic sector C (44kDa), D (28kDa), E (26kDa) and possibly F (14kDa) have no homology to F-ATPase subunits. Also there is no assigned function for these subunits of V-ATPase

The integral membrane component of the ATPase is designed V_o by analogy with F_o of F-type ATPase.

The function of this membrane sector (V_o) is to conduct protons through the membrane and to couple this vectorial action with the scalar process of ATP formation or hydrolysis. Chromaffin granule membranes contain V_o and V_1V_o in a ratio of about 11:1 [Webster and Apps, 1996]. Beltran and Nelson (1992) showed that V_o itself is not a passive conductor of protons unlike the F_o sector of F_o-ATPase, and that V_1 is not catalytically active when dissociated from V_o. While the membrane sector of *E.coli* F-ATPase consists of three different subunits (a, b and proteolipid), that of mammalian mitochondria may contain up to 10 different polypeptides [Schneider and Altendorf, 1987; Futai *et al.*, 1989; Senior *et al.*, 1990; Walker *et al.*, 1991]. In contrast, the membrane sector of archaebacterial V-ATPase may be composed only of the proteolipid subunit [Denda *et al.*, 1990]. The membrane sector of mammalian V-ATPase may be composed of at least five different subunits, the genes or cDNA encoding four of the subunits (M115, M45, M39, proteolipid) having been cloned [Nelson *et al.*, 1992]. All the above subunits were identified in V-ATPase from bovine and several other sources.

Among these subunits, two are glycosylated, the first glycosylated subunit identified being the largest V-ATPase subunit, M115. The sialic acid glycosylation of this subunit was detected by neuraminidase digestion and binding to peanut lectin [Adachi *et al.*, 1990]. The amino acid sequence of M116 suggests that it consists of two fundamental domains: a hydrophilic amino-terminal half and a hydrophobic carboxy-terminal half that contains at least six transmembrane helices and faces the luminal side of the organelle [Perin *et al.*, 1991].

The other glycosylated subunit is M45 which was discovered in V-ATPase from bovine chromaffin granules. Cloning of the cDNA encoding this polypeptide revealed seven potential glycosylation sites positioned on the luminal side of the membrane and a single transmembrane segment in the C-terminal part of the polypeptide. Immunological cross activity was revealed with the M45 of purified bovine kidney microsomes but not in the plasma membrane of epithelial cells which suggested that this polypeptide restricted is to internal organelles of mammalian cells with a potential function as an anchor for the enzyme. [Supek *et al.*, 1994].

Subunit M39 of V-ATPase, in contrast to M115 and M45, is a non-glycosylated polypeptide facing the cytoplasmic side of the membrane and contains no potential transmembrane helices. The lack of potential transmembrane helices suggests that this subunit is attached to V_o via another protein, and because M39 has no parallel in the membrane sector of the related mitochondrial F-ATPases, it was suggested that this polypeptide has no direct role in proton conductance across the membrane [Wang *et al.*, 1989]. Recently it has been found that M39 is identical to physophilin which was originally supposed to be located in the synaptic plasma membrane [Siebert *et al.*, 1994; Thomas *et al.*, 1990]. The proteolipid is the principle subunit of the membrane sectors of both F- and V-ATPase. The proteolipid of V-ATPase is a highly hydrophobic 16kDa protein that reacts with dicylohexylcarbodiimide (DCCD) and contains about 160 aminoacid residues [Sutton and Apps, 1981; Mandel *et al.*, 1988; Nelson and Nelson, 1989; Nelson *et al.*, 1992]. It probably exists as a hexameric proton-translocating channel as its sequence is homologous to other channel complex proteins such as the 15 kDa proteolipid of the mediatophore in *Torpedo* electric organs, and the 16kDa gap junction protein known as ductin [Mandel *et al.*, 1988; Leitch and Finbow, 1990]. The latest subunit to be attributed to the membrane sector of V-ATPase is M16 [Supekova *et al.*, 1996]. This is identical to subunit G, which was found to be part of V_1 in coated vesicle H^+-ATPase [Crider *et al.*, 1997]. It is distinct from the 16kDa proteolipid previously identified [Sutton and Apps., 1982; Mandel *et al.*, 1988], and exhibits homology to the b subunit of F-ATPase. M16 is smaller than subunit b and does not have an apparent transmembrane segment in its N-terminus but the reminder of subunit b is related to M16 not only by its amino acid sequence but also in its predicted structure of helix-turn-helix.

Addition of all V-ATPase subunits identified, in their apparent stoichiometry, would give a molecular mass for the ATPase of around 750 kDa, although the real value is probably greater than 900kDa which is consistent with that determined by gel filtration [Aria *et al.*, 1988; Gluck and Caldwell,1987; Moriyama and Nelson,

1989; Bowman *et al.,* 1989;] and by native gel electrophoresis [Webster and Apps, 1996] suggesting that additional subunits remain to be identified.

1.4.2.3.2 ATPase II (P-type ATPase).

ATPase II, a P-type ATPase, is a single polypeptide with an apparent molecular mass of 120 kDa on SDS gels. It is insensitive to DCCD or alkylating agents, weakly inhibited by trialkyl tin or quercetin, but strongly inhibited by vanadate. It has been purified but its amino acid sequence is not yet known [Apps *et al.,* 1983; Moriyama and Nelson, 1988]. A similar enzyme has been isolated from bovine brain clathrin-coated vesicles and the secretory granules of the pituitary. It is of unknown function, but it is speculated that it may be involved in aminophospholipid translocation within the granule membrane [Zachowski *et al.,* 1989 & 1993].

1.4.2.4 Synaptotagmin (p65).

The chromaffin granule membrane proteins involved in docking at, and fusion with, the plasma membrane are relatively minor components, in contrast to their abundance in synaptic vesicles. One of the first to be characterized was synaptotagmin which was discovered as a chromaffin granule protein using monoclonal antibodies prepared against rat brain synaptic vesicles. It is an integral membrane glycoprotein of molecular mass 65 kDa, and was termed p65 [Matthew *et al.,* 1981] but later named synaptotagmin. The discovery that synaptotagmin binds calmodulin, a protein which regulates the function of other proteins in response to the levels of intracellular Ca^{2+} concentration, has been an important step in implicating it as a component of the secretory machinary of regulated secretory cells [Fournier and Trifarõ, 1988; Trifarõ *et al.,* 1989]. The function of synaptotagmin in exocytosis is not yet defined. Biochemical and sequence studies show that it has a short glycosylated lumenal domain at the N-terminus, a single transmembrane span and a large cytoplasmic domain that contains binding sites for Ca^{2+}, phospholipids and inositol polyphosphates: for this reason it is thought to be involved in the sensing of cytoplasmic [Ca^{2+}] [Kelly *et al.,* 1995] . It also binds the adaptin AP2, and could therefore have a role in endocytotic retrieval of vesicle membranes

[Zhang *et al.*, 1994]. Synaptotagmin appears to be present in most, if not all neuroendocrine secretory vesicles. There are at least 9 isomers of synaptotagmin. Synaptotagmin I occurs in adrenal medulla, but it is not yet clear whether other isomers also exist there [Li and Ullrich, 1995].

1.4.2.5 Glycoprotein II.

Glycoprotein II is a heterogeneous membrane component. When separated by two-dimensional electrophoresis, it is resolved into two components: upper ($GpII_a$) and lower ($GpII_b$) with a pI of 4.2-4.7 and a molecular mass range of 80-100 kDa [Huber *et al.*, 1979; Pryde and Phillips, 1986]. Glycoprotein II is known to contain a large amount of carbohydrate, about 30% of the total molecular mass, with the major sugars being galactose, N-acetylglucosamine and sialic acid [Fischer-Colbrie *et al.*, 1982], and it was found to bind both Concanavalin A and wheat germ agglutinin [Gavine *et al.*, 1984]. Glycoprotein II has been identified in both endocrine and exocrine secretory vesicles. In more recent studies, glycoprotein II has been found in the membranes of kidney lysosomes [Weiler *et al.*, 1990], thus this glycoprotein is a common constituent of the two organelles arising from the trans-Golgi. Bovine chromaffin granule glycoprotein II was purified using lithium di-iodosalicylate extraction and WGA-Sepharose chromatography and was later cloned and sequenced [Hieber *et al.*, 1993]. The complete deduced amino acid sequence of $GpII_a$ was found to share a 72% identity with human LAMP-1, which belongs to a highly conserved group of lysosomal-associated membrane glycoproteins (LAMP proteins). The glycoprotein $GpII_a$ sequence encodes a 383 amino acid polypeptide with a calculated molecular mass of 40429 Da and contains a total of 19 potential asparagine-linked glycosylation sites. Topographical studies of this protein indicated that part of glycoprotein II is exposed on the outside of the chromaffin granules and can be degraded by exogenous proteinase [Abbs and Phillips, 1980], corresponding to the later prediction from the amino acid sequence [Hieber and Christie, 1993]. The C-terminal tail of glycoprotein II starts with paired basic amino acids which are potential sites for proteolytic processing. A specific

antiserum was raised against a synthetic peptide derived from the C-terminal tail of GpII (a cytoplasmic transmembrane region) and by immunological techniques it was confirmed that glycoprotein II is not proteolytically processed before reaching the chromaffin granules. The function of glycoprotein II in secretory granules remains unknown.

1.4.2.6 Glycoproteins IV and V.

These were identified by Huber *et al.,* (1979), together with dopamine β-monooxygenase, glycoprotein II and glycoprotein III by using PAS staining.

They are integral membrane glycoproteins, and both have an affinity for Concanavalin-A. They are both very heterogeneous glycoproteins: glycoprotein IV has an apparent molecular mass of 45kDa and pI of 5.2-4.2, and glycoprotein V has an apparent molecular mass 58kDa and pI of 4.8-3.6 [Gavine *et al.,* 1984; Pryde and Phillips, 1986]. Glycoprotein V appears to be present in pig and horse chromaffin granule membranes while glycoprotein IV appears to be absent in these species according to PAS stain [Huber *et al.,* 1979]. According to isotope labelling techniques, the carbohydrate chains of glycoprotein IV and glycoprotein V are localised on the inner face of granule membranes. Glycoprotein IV is the most characteristic glycoprotein of the phospholipid-rich pellet phase after the fractionation of membrane proteins with the non ionic detergent Triton X-114 [Pryde *et al.,* 1986], while glycoprotein V is mainly found in the detergent-rich phase, but both of them are extracted into the soluble fraction after treatment of chromaffin granule membranes protein with LIDS [Christie and Palmer,1990]. The structure and function of these glycoproteins are unknown.

1.5 The aim of the project.

Many proteins have been found in the chromaffin granule membranes and many of these are glycosylated, such as dopamine β-monooxygenase, glycoproteins II, III, IV, V, J, K, and H. The identification and characterization of chromaffin granule membrane glycoproteins is complicated by their relatively poor staining with Coomassie blue, and the predominance of proteins like DBM and other membrane

components. But the specificity of glycoproteins for lectin binding gives a convenient route for the purification of these membrane proteins. The development of methods for rapid purification such as membrane fractionation in the non-ionic detergent Triton X-114, or extraction with lithium di-iodosalicylate have facilitated the purification of some of these proteins and subseqently their structures were determined. But still the function of many remain unknown.

The overall aim of this project was to study the uncharacterized chromaffin granule glycoprotein IV. Since glycoprotein IV was the characteristic glycoprotein in the phospholipid-rich pellet after fractionation of chromaffin granule membranes with Triton X-114, as well binding strongly to concanavalin A, these methods were selected to purify it. Later, purified glycoprotein IV would be used to produce antibodies in order to investigate the structure of the protein for example determination of the core polypeptide of glycoprotein IV after deglycosylation, examining its topography in the membrane and its tissue localisation. The purified glycoprotein IV was also to be used to obtain its N-terminal sequence in order to isolate recombinant DNA and hence to obtain a complete amino acid sequence.

Table 1.2 Lipid composition of chromaffin granule membranes

Lipid	μmol/mg membrane protein
Cholesterol	1.44
Phosphatidylcholine	0.65
Lysophosphatidylcholine	0.41
Sphingomyelin	0.29
Phosphatidylethanolamine	0.84
Phosphatidylserine	0.06
Ganglioside GM3	0.05
Fatty acids	0.10

Adapted from Winkler, (1976) and Dreyfus *et al.*, (1977).

Table 1.3 Lectin-binding properties of the major matrix and membrane-bound glycoproteins of chromaffin granule

	Con A	ConA/ neur	PNA	PNA/ neur	PSA	PSA/ neur	WGA	WGA/ neur
1. Matrix glycoproteins								
Chromogranin A	-	-	-	+	-	-	-	-
Chromogranin B	+	+	-	-	-	-	-	-
Chromogranin C								
Dopamine β-monooxygenase	+	+	-	-	+	+	+	-
Glycoprotein III	-	-					+	+
Proteoglycan						-		
2. Membrane glycoproteins								
Glycoprotein I (DBM) as above								
Glycoprotein II	+	-					+	-
Glycoprotein IV	+	-					-	-
Glycoprotein V	+	-					-	-
Glycoprotein J	+	-					-	-
Glycoprotein K	+	-					-	-
Glycoprotein H	+	-					-	-
Synaptotagamin (p65)	-	-	-	+	-	+	-	-
ATPase subunit M115	-	-	-	+	-	+	-	-

Data from Apps *et al* ., 1985

Neur, neuraminidase treated; Con A, concanavalin A; PNA, peanut agglutinin; PSA, pisum sativum agglutinin; WGA, wheat germ agglutinin

(+) the glycoprotein can bind lectin, (-) the glycoprotein can not bind lectin, (blank) no information currently available.

Table 1.4

Membrane proteins of bovine chromaffin graunles[a]

Identifiable[b] on gel, and of known function.	**Identifiable only by their function.**	**Identifiable, but of unknown function.**
ATPase I complex Cytochrome b561 Catecholamine transporter Dopamine β-monooxygenase Glycoprotein K (PC2) Glycoprotein J (PC2) Glycoprotein H (Carboxpeptidase H) Synaptobrevin (p18, VAMP)	Ca^{+2}/ Na^{+}-antiporter Na^{+}/ H^{+}-antiporter Nucleotide transporter Phosphatidylinositol kinase	ATPase II Glycoprotein II Glycoprotein III Glycoprotein IV Glycoprotein V Synaptotagamin I (p65) Synaptophysin (p38) SV2 Laminin β-chain[c] Cysteine string proteins (Csp1 & Csp2)[d]

a Adapted from Winkler *et al.*, (1986).

b Identifiable by Coomassie-Blue staining.

c and d they are new proteins that have been recently added to the list of membrane proteins.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Chemical, Biochemical and Molecular Biological materials:

2.1.1.1 Chemicals

All common laboratory chemicals were of the highest available grades, purchased either from Sigma or BDH., unless specified.

2.1.1.2 Biochemicals

Acrylamide (Electran), bromophenol blue, N, N' methylene-bisacrylamide (Electran), Nonidet P-40, and polyacrylamide (molecular weight > 6000) were purchased from BDH, Pool, Dorset, U.K. Ampholytes (pH 3-10 and pH 9-11) were purchased from Bio- Rad Laboratories, Watford, Herts., U.K. Serva blue G was supplied from Serva, Heidelberg, Germany. Triton X-114 and ethanolamine were purchased from Fluka AG, Switzerland.

2.1.1.3 Enzymes and Proteins

Bovine serum albumin, Concanavalin-A agarose, Papain (EC 3.4.22.2), Proteinase K (EC 3.4.21.46), Pronase (EC 3.4.24.31), Endoproteinase Asp-N (EC 3.4.24.33), Endoproteinase V8 (EC 3.4.21.19), Trypsin (EC 3.4.21.4) and horse radish peroxidase (EC 1.11.1.7) were purchased from Sigma Chemical Co Ltd.

Peptide N-glycosidase F (PNGase F, EC 3.2.2.52), and Phospholipase C (EC 3.1.4.10) were purchased from Boehringer, Mannheim, Germany.

Maleimide-Activated Keyhole Limpet Haemocyanin was purchased from Pierce & Warriner U.K Limited.

2.1.1.4 Antibodies

Anti-rabbit IgG and anti-mouse IgG (whole molecule) coupled to horse radish peroxidase were purchased from Sigma Chemical Co. Ltd.

Samples of monoclonal antibodies raised against bovine chromaffin granule membrane dopamine β-monooxygenase, glycoprotein II and synaptotagamin (p65)

and samples of rabbit polyclonal antisera raised against bovine vacuolar ATPase subunits M116, and M39 and vacuolar ATPase subunits A (72kDa) and B (57kDa) from *Kalanchoe daigremontiana* were made available by Dr. David K. Apps and Dr. Jeff Haywood (Department of Biochemistry, University of Edinburgh, Medical School, Edinburgh U.K)

2.1.1.5 Tissue culture materials

RPM1-1640 was purchased from Flow, High Wycomb, Bucks., U. K.

Aminopterin and hypoxanthine-thymidine media supplement were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Myoclone foetal calf serum was purchased from Gibco BRL, Paisley, U.K. Tissue culture plates (Falcon) were purchased from Beveridge Ltd., Edinburgh, U.K. Polyethyene glycol 1500 (46%, Hepes-buffered and sterile) was purchased from Boehringer, Mannheim, Germany.

2.1.1.6 Cell lines

NS-0 myeloma cell line was a generous gift of Dr. Jeff Haywood (Department of Biochemistry, University of Edinburgh, Medical School, Edinburgh U.K)

2.1.1.7 Oligonucleotides

All the oligonucleotides (primers) were synthesised by Oswel DNA service Lab 5005, Medical & Biological Sciences Building, University of Southampton, Basset Crescent East, Southampton SO16 7PX.

2.1.1.8 Polymerase Chain Reaction (PCR)

Taq DNA polymerase, deoxynucleotide triphosphates (dNTPs), Taq buffer and pGEM-Tvectorwere supplied by Promega Ltd, Delta House, Enterprise Road, Chilworth Research Centre, Southampton SO16 7NS.

2.1.1.9 Enzymes for DNA manipulation

The restriction enzymes,Sal I, Sac II, Hinc 11and BamH1 were supplied by Northumbria Biologicals Ltd, South Nelson Industrial Estate, Cramlington, Northumberland.

2.1.1.10 Growth media

Agar no 1, Tryptone and yeast extract were supplied by Oxoid Ltd, Basingstoke, Hampshire.

Glucose, ampicillin, X-Gal and IPTG were supplied by Sigma Chemical Company, Poole Dorset.

2.1.1.11 Miscellaneous

Nitrocellulose (Schleicher & Schuell, 0.45μm pore size) was purchased from Anderman & Co. Kingston-upon-Thames, Surrey, U.K.

Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Sigma Chemical Co., Ltd.

Ultrafiltration apparatus with a PM-30 membranes were purchased from Amicon Inc., Beverly, MA 01915 USA.

Enhanced-chemiluminescence kit (ECL) was purchased from Amersham International, Amersham, Oxfordshire, U.K.

Fuji medical X-ray film was purchased from Fuji Photo Film Co., Ltd.

Samples of AtT-20 cell membrane fractions were generously given by Jan Mackintosh (Department of Biochemistry University of Edinburgh, Medical School, Edinburgh U.K)

E-coli strains NM522, bovine adrenal medulla cDNA library and rat brain synaptosomes were generously given by Leonora Ciufo (Department of Biochemistry University of Edinburgh, Medical School, Edinburgh U.K)

QIAEX II Kit for DNA extraction from agarose gel was supplied by QIAGEN Ltd, Unit 1 Tillingbourne Court, Dorking Business Park, Dorking, Surrey RH4 1HJ.

2.2 Methods

2.2.1 Preparation of pure chromaffin granule membranes

[A] Adrenal medullary homogenate

Fresh bovine adrenal glands (approximately 40-50 glands) were obtained from the local slaughter-house and were placed in ice within about 20 min of slaughter.

All preparations were carried out at room temperature with the glands and buffers being kept on ice. The adrenal medullae were dissected from the cortexes and placed in about 400 ml of 0.3M sucrose buffered with 10 mM Hepes-NaOH, 2mM EDTA (pH 7.2) at 0°C. The dissected glands were minced followed by homogenisation in buffered 0.3M sucrose.

[B] Crude chromaffin granules

The homogenate was centrifuged for 5 minutes at 4000 r.p.m (1500 g_{av}) in a Beckman JA-14 rotor at 4°C to remove intact cells, nuclei and debris. The resultant supernatant was centrifuged for a further 30 minutes at 14000 r.p.m (16000 g_{av}) in the same rotor at 4°C to pellet the granules. The pellet, which contains crude granules and mitochondria, was resuspended by homogenisation in the same buffered sucrose and centrifuged for 20 minutes at 15000 r.p.m (18000g_{av}) in a Beckman JA 20 rotor at 4°C. Mitochondria in the upper layer of the pellet were washed from the lower layer of crude chromaffin granules by gentle swirling. The granules were homogenized in 0.3M sucrose /Hepes-NaOH / EDTA (pH 7.2) and centrifuged through 1.6M sucrose, 10mM Hepes-NaOH, 2mM EDTA, (pH 7.2) for 60 minutes at 45000 r.p.m (161,000 g_{av}) in a Beckman 45 Ti rotor at 4°C. The pellet at this stage contains relatively pure granules [Smith and Winkler, 1967].

[C] Chromaffin granule membranes

The granules in the pellet were lysed by gentle homogenisation in 10 mM Hepes NaOH, 2mM EDTA, (pH 7.2). The membranes were pelleted by centrifugation at 161,000g_{av} (45000 r.p.m) in a Beckman 45-Ti rotor for 20 minutes at 4°C . The pellet of crude membranes was resuspended in 10mM Hepes-NaOH, 1mM dithiothreitol (DDT), 2mM EDTA, (pH 7.2), and then overlaid on 1.0 M sucrose, buffered with10mM Hepes-NaOH, 2mM EDTA (pH 7.2) and centrifuged for 30 minutes at 161,000g_{av} in the same rotor at 4°C [Apps and Schatz, 1979]. The pure chromaffin granule membranes were collected from the 0.3/1.0 M sucrose interface and washed to remove sucrose by resuspending them in 10 mM Hepes-NaOH, 2mM EDTA (pH 7.2), and pelleting by centrifugation for 30 minutes at 161,000g_{av} in the same rotor. Finally the membranes were resuspended in 12-15 ml of 10mM Hepes-NaOH, 1mM dithiothreitol (DTT), 2mM EDTA to give a final protein concentration of 5-9 mg/ml and were then stored at -20°C

2.2.2 Preparation of kidney microsomal membranes

Kidney microsomes were prepared by an adaption of the published procedure of Gluck and Caldwell, (1987). Bovine kidneys were obtained fresh from a local slaughter-house and transported on ice. All preparations were carried out at room temperature with the renal medullary tissues and buffers being kept on ice. All sucrose solutions contained 10mM Tris HCl, 1mM $NaHCO_3$, 1mM EDTA, 1mM DTT (pH 8.0). Bovine renal medullary tissue was minced, homogenised in buffered 0.25M sucrose and centrifuged for 10 minutes at 1,500 g_{av} (4000 r.p.m) in a Beckman JA-14 rotor . The resultant supernatant was centrifuged for a further 60 minutes at 41,000 r.p.m in a Beckman Ti-45 rotor at 4°C.

The pellet consisted of a fluffy white upper layer and a brown mitochondrial - lysosomal button. The upper layer, containing crude microsomes, was removed by gentle swirling and collected by centrifugation. The pellet was resuspended in buffered 0.25M sucrose and then overlaid onto step gradients composed of 4 ml each

of 0.7, 1.0, and 1.5 M buffered sucrose and centrifuged for 5 hours at 150.000g_{av} in a swing out rotor (Beckman SW41) .

The microsomal fraction was collected from the 0.7M/1.0M sucrose interface, diluted, centrifuged and finally resuspended in 0.25M buffered sucrose containing 2mM benzamidine, 5µg/ml pepstatin, and 5µg /ml leupeptin, to give a final protein concentration of 7-10 mg /ml and was then frozen at -20°C.

2.2.3 Treatment of Subcellular Organelle Membranes

2.2.3.1 Washing of membranes at pH 11

Membranes were resuspended at a protein concentration of 1mg/ml by gentle homogenisation in 0.1M Na_2CO_3 (pH 11) containing 1mM EDTA and incubated on ice for 30 minutes with occasional agitation [Higgins *et al.*, 1984, Howell and Palade 1982]. The membranes were then recovered from the solution by centrifugation at 412,000 g_{av} for 15 minutes at 2°C in a TL-100 rotor (Beckman Instruments) and resuspended in 20mM Hepes-NaOH (pH 7.2) at a protein concentration of 5mg/ml and centrifugation was carried out again under the same conditions. The supernatant was discarded and the pellet containing membrane proteins was resuspended in the same buffer at a protein concentration of about 4mg/ml.

2.2.3.2 Fractionation of membrane proteins with Triton X-114

Chromaffin granule membranes which had been frozen at -20°C in 10mM Hepes -NaOH, pH 7.2, 2mM EDTA, 1mM DTT and then thawed were pelleted by centrifugation, and resuspended to a protein concentration of 4mg/ml in 10mM Hepes pH 7.2, 150mM KCl, 1mM DTT, 1mM EDTA by gentle homogenisation in the presence of 2% (w/v) cold Triton X-114 at 0°C and incubated on ice for a further 5 minutes before being centrifuged at 100,000 rpm (412,000 g_{av}) for 15 minutes at 2°C in a TL-100.3 rotor (Beckman instruments). The insoluble precipitate (termed P1) was washed by resuspending to its original volume in the presence of 2% (w/v) cold Triton X-114 in the same buffer. After gentle homogenisation at 0°C the

mixture was kept on ice for a further 5 minutes before being centrifuged at 412,000 g for 15 minutes at 2°C. The supernatant was discarded and resulting P1 was washed twice with water.

The supernatant (S1) left after the removal of P1 was layered over a 1 ml cushion of 0.25M sucrose/ 10mM Hepes pH 7.2, 150mM KCl/0.06% (w/v) Triton X-114 in conical glass centrifuge tubes. The tubes were incubated at 30 °C for 5 minutes to separate the detergent phase (P2) followed by centrifugation at 2,500 g_{av} (swing-out bench centrifuge, top speed) for 5 minutes at room temperature. The supernatant "aqueous phase" was removed from above the cushion. The cushion was then carefully removed, the "detergent-rich" phase recovered and resuspended to its original volume in ice-cold Hepes buffer. The residual detergent in the aqueous phase was removed by dialysis against 100-volumes of 10mM Hepes-KCl containing 1% Amberlite XAD-2 resin, 0.2mM PMSF, 1mM benzamidine at 4°C for 5 days with two changes of buffer. The dialysed fraction was diluted with 3-volumes of 10mM Hepes-KCl buffer and centrifuged at 412,000g_{av}. The pellet "glycoprotein-rich fraction" was resuspended by homogenisation in 10mM Hepes-KCl pH7.2 with 0.1%(v/v) Triton X-100. All fractions above were subjected to one- and/ or two-dimensional electrophoresis and analyzed by Coomassie blue staining and/or immunoblotting with Concanavalin A.

2.2.3.3 Solubilizing phospholipid-rich pellet proteins with commercial detergents

The phospholipid-rich pellet termed P1, formed from the fractionation of membrane proteins with the Triton X-114, was washed twice with water as described above. The pellet was resuspended to its original volume by gentle homogenisation in Hepes buffer pH 7.2. The total volume was divided into 5 equal portions, each containing about 0.5 ml, and these were homogenized in the presence of 2% (w/v) of the following detergents: n-octyl-β-glycoside, ω-octanoyl-N-methyglycamide (MEGA 8), ω-decanoyl-N-methylglucamide (MEGA 10), and 3(-3-cholamideopropyl)-dimethyl-ammonio-)1-propane sulphate (CHAPS). The homogenates were then centrifuged at 412,000g_{av} (Beckman Instruments TL-100.3

rotor) for 10 minutes at 2°C to pellet unsolubilized material. The supernatant was removed and the insoluble material was resuspended in Hepes buffer pH7.2. Samples from the supernatant which contained solubilized proteins and the resuspended pellet were diluted with 4x SDS-sample buffer.

All samples were subjected to one-dimensional SDS-gel electrophoresis and analyzed by Coomassie blue staining and immunoblotting with Concanavalin-A.

2.2.3.4 Deglycosylation of membrane glycoproteins

Deglycosylation was carried out by using peptide N-glycosidase F (EC 3 .2.2.52) [Ausubel *et al.,* 1987]. The pellet (P1) obtained after fractionation of chromaffin granule membranes protein with Triton X-114 was washed twice with water as described earlier and resuspended to give a final concentration of 2mg /ml in 10 mM Hepes-NaOH (pH 7.2), 0.5% deoxycholate, 1% (v/v) Triton X-100, and 40 units/ml peptide N-glycosidase F. The digestion was carried out at 37°C overnight in the presence of 5mM benzamidine, 50µM PMSF, 5µg/ml leupeptin, 5µg/ml pepstatin, 50µM TLCK and 10mM EDTA (Tugal *et al.,* 1991). The digestion reaction was accompanied with a separate control sample (mock-digested) which was run without endoglycosidase F. The samples were lyophilised and resuspended in SDS-PAGE sample buffer and analysed by SDS-polyacrylamide gel electrophoresis followed by immuno-blotting. Samples to be analysed by two-dimensional electrophoresis were lyophilised and resuspended directly in electrofocussing sample buffer and then analysed by two-dimensional electrophoresis followed by immunoblotting.

2.2.3.5 Protease digestion of intact chromaffin granules and membranes

The crude granule fraction and chromaffin granule membranes were resuspended in 0.3M sucrose, 10mM Hepes-NaOH, 1mM DTT, 1mM EDTA, (pH 7.2) to give a final concentration of 1mg protein /ml, and were incubated with trypsin, papain, pronase, proteinase K or proteinase V8 at various concentrations at 20°C for up to 2 hours. The digestions were terminated by the addition of the

following protease inhibitors: 50μM TLCK, 5mM benzamidine, 5μg/ml leupeptin, 5μg/ml pepstatin, 20mM EDTA, 200μM PMSF, and additionally, 2mM iodoacetamide was added to samples treated with papain.

In the case of purified chromaffin granule membranes, the soluble peptides were separated from the membrane-bound peptides by centrifugation at 412,000 g_{av} for 20 minutes at 2°C. The resulting pellet was washed twice by resuspension and centrifugation in 10mM Hepes-NaOH pH 7.2 containing the same protease inhibitors. The supernatant fraction was recentrifuged as above to remove any residual membrane-bound proteins.

In the case of crude intact granules, the digestion was terminated as above and the granules were then purified by centrifugation through 1.8M sucrose/10mM Hepes-NaOH (pH 7.2) containing the protease inhibitors at 412,000g_{av} for 30 minutes in a TL-100.3 rotor (Beckman Instrument). The granule pellet was resuspended in 10mM Hepes-NaOH (pH 7.2) containing the same protease inhibitors and centrifuged again at 412,000g_{av} in the same rotor. The resultant membrane pellet and granule fractions were then denatured by the addition of hot 4x-acidic SDS-PAGE sample buffer (0.2M potassium biphthalate, 0.2mM EDTA, 20% (w/v) SDS, 40%(v/v) glycerol, 0.015% (w/v) bromophenol blue and 10mM DTT pH 4.0). The soluble fractions were diluted with 4x-SDS sample buffer pH 4.0 as above.

The samples were then analysed by SDS polyacrylamide gel electrophoresis followed by immuno-blotting.

2.2.3.6 Treatment of chromaffin granule membranes with phospholipase C

The membranes were recovered from washing of membrane with Na_2CO_3 (pH11) as described in section 2.3.1. These were suspended to give a final protein concentration of 2mg/ml. with the protease inhibitors (5mM benzamidine, 2mM EDTA, 50μM PMSF, 5μg/ml pepstatin, 10μM TLCK and10μM TPCK) .

The digestion with phosphatidylinositol phopholipase C was carried out at a concentration of 0.1unit/ml at 37°C overnight, accompanied with a mock digestion as control. The pellet containing membrane-bound peptides was separated from the supernatant containing soluble peptides by centrifugation at 412,000 $g_{av}$for 20

minutes at 2°C in a TL-100.3 rotor (Beckman Instruments). The pellet was resuspended in the same buffer and then samples from all fractions were denatured by the addition of SDS-PAGE sample buffer and their proteins separated by one dimensional gel electrophoresis, blotted to nitrocellulose and tested for reaction with Con A and the following monoclonal antibodies: anti-membrane dopamine β-monooxygenase, anti-glycoprotein II, and anti-synaptotagmin (p65)

2.2.4 Electrophoretic Methods

2.2.4.1 SDS-polyacrylamide gel electrophoresis

Proteins were analysed by electrophoresis in the presence of SDS basically following the method of Laemmli (1970), using the Hoefer Scientific Instruments "Tall Mighty Small" mini-gel apparatus. The gels usually consisted of a separating gel composed of 9% acrylamide (30:0.8 acrylamide-bisacrylamide), 375mM Tris HCl (pH 8.8), 0.1 % (w/v) SDS, and 0.5% (w/v) polyacrylamide to improve the mechanical strength of the gel. Gels were chemically polymerised with 0.05% (v/v) TEMED and 0.1%(w/v) ammonium persulphate. Separating gels were overlaid with stacking gels composed of 4.5% acrylamide in 125mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS and 0.5% polyacrylamide. Samples were solubilized by heating for 5 minutes at 65°C in a sample buffer composed of 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 0.2 mM EDTA and 0.001% (w/v) bromophenol blue in the presence of 10 mM DTT, and loaded into slots in the stacking gel using a Hamilton syringe.

Gels were normally run at a constant current of 15-20 mA for 2-2.5 hours at room temperature, using electrode buffer containing 50 mM Tris base, 380 mM glycine and 0.1% (w/v) SDS. After the gel had been run, the proteins were fixed and stained in the gels as follows:

[A] Coomassie blue staining

Coomassie blue staining was performed as described by Weber *et al* (1969). Proteins were fixed in the separating gels with 10%(v/v) acetic acid and 20% (v/v) methanol for at least 30 minutes . The gels were then stained with Serva blue R (0.25%(w/v) in 7.5% acetic acid and 50% methanol) for 5-10 minutes on a shaking platform. Gels were destained in 7% acetic acid and 10% methanol for 2-10 hours, polyurethane foam pieces being placed in the destaining solution to aid the destaining process. Fixing , staining and destaining steps were carried out at room temperature.

[B] Silver staining

Gels requiring sensitive detection of proteins were silver-stained using the method of Wray *et a.,l* (1981). Gels were fixed in 50% (v/v) methanol and 10% acetic acid overnight followed by three one-hour washes in 50% (v/v) methanol with 5-minute intermediate washes in distilled water. The gels were then stained for 20 minutes with freshly prepared silver stain solution (made by mixing 21ml of 0.36% NaOH with 1.4ml of 14.8 ammonia.) As this solution was rapidly stirred, 0.4g of silver nitrate dissolved in 2ml of distilled water was add dropwise and the resultant colourless solution was made up to 100ml. The gels were then rinsed in distilled water for 5 minutes before developer solution (0.01% (w/v) citric acid, 0.04% formaldehyde) was added. Stain development was stopped by pouring off the developer and rinsing the gel in fixing solution. Fixing, staining and washing steps were carried out at room temperature with the gel shaker gently on an orbital mixer.

2.2.4.2 Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out essentially as described by O' Farrell, (1975).

[A] First-dimension isoelectrofocusing gels (IEF)

Isoelectric focusing gel mixture was composed of 4.5% (w/v) acrylamide, 0.06% (w/v) bisacrylamide, 9.5M urea, 2% (v/v) Nonidet P-40.

The urea was dissolved by swirling the tube containing this mixture under warm running water and was degassed under vacuum before addition of 2 % (v/v) ampholine (pH range from 3-10 & 9-11 in the ratio 5:1(v/v)) and 0.4% (w/v) ammonium persulphate was added to chemically polymerise the gel. The isoelectric gels were cast to a height of approximately 12cm by displacement with water using capillary tubes of 130 x 2mm (internal diameter). The tubes containing polymerised gel were placed in the electrophoresis chamber which was filled with 0.2% (v/v) orthophosphoric acid (anode buffer) so the bottom of the tubes were immersed in the anode solution, while the top of the tubes were overlayed with the cathode buffer (0.06% (v/v) ethanolamine).

The samples of proteins were prepared by freeze-drying in 1.5 ml microcentrifuge tubes and dissolved in two-dimensional sample buffer containing: 9.5 M urea, 2% Nonidet P-40, 2% (v/v) ampholytes of pH (3-10), 10mM dithiothreitol and 0.001% (w/v) bromophenol blue. The samples of protein (up to 50 μg) were loaded from the cathodic (basic/ top) end of the gel.

The gels were focused for 1-2 hours at 100V and then the voltage was increased to 200V for a further 2 hours, and finally to around 300V for another 3 hours. The electrofocusing gels were removed from the tubes and incubated in soaking buffer, containing 3% (w/v) SDS, 10% (v/v) glycerol, 50 mM Tris-HCl (6.5), 5mM dithiothreitol, 0.001% bromophenol blue for about 20 minutes before loading onto the second dimensional gel.

[B] Second-dimension SDS polyacrylamide gel

The Laemmli (1970) SDS buffer system was used with a separating gel of 9% acrylamide. The electrofocusing gels were placed on the top of the SDS gel, then

hot molten agarose solution (1% w/v) dissolved in the soaking buffer was poured on the top of the electrofocusing gel to seal it to the separating gel. The gels were run as described earlier in section 2.4.1.

[C] Fixing and staining two-dimensional gels

2D gels requiring staining in Coomassie blue were incubated in two-dimensional gel fixer (25%(v/v) isopropanol, 10% (v/v) acetic acid) overnight to remove ampholytes from the gel. Gels were stained with the staining solution recommend by Bio-Rad, 25% (v/v) isopropanol, 10% (v/v) acetic acid, 0.04% (w/v) Coomassie blue R250, 0.5% crocein scarlet and 0.5% (w/v) cupric sulphate for 2hours. Gels were destained in 12% (v/v) isopropanol, 7% (v/v) acetic acid and 0.5% (w/v) cupric sulphate for several hours.

2.2.4.3 Blue Native gel electrophoresis

Protein complexes of the chromaffin granule membrane were analysed by blue native gel electrophoresis basically following the methods of Schägger and von Jagow, (1991) and Schägger *et al.,* (1994).

2.2.4.3.1 First-dimension Blue Native gel (BN-PAGE)

[A] Separating gel

Acrylamide stock for native gels consisted of: a 48:1.5% (w/v) mixture of acrylamide and bisacrylamide. Linear gradient gels were poured with a 2-channel peristaltic pump using the required percentage of acrylamide and bisacrylamide mixture in 5% glycerol, 50 mM Bis Tris-HCl (pH 7.0), 0.5 M aminocaproic acid (ACA). The gel was chemically polymerised with 0.01% ammonium persulphate and 0.05% TEMED.

[B] Stacking gel

The stacking gel containing 4% (w/v) acrylamide and bisacrylamide mixture was mixed in the same buffers as the separating gel.

[C] Sample preparation for blue native gel electrophoresis

Samples (membranes or P1), were dissolved by gentle homogenization in 0.75 M ACA, 0.05 M Bis Tris-HCl (pH 7.0) containing 2% (w/v) dodecyl maltoside, and the solubilized material was then centrifuged at 412,000 g_{av} in a Beckman TL-100.3 rotor, for 5 minutes at 2°C. The supernatant was collected and mixed with Serva Blue G to a final concentration of 0.4% before loading, and samples of protein up to 75µg protein per 1.5 cm well were loaded.

[D] Running conditions

The cathode buffer was 50 mM Tricine, 15mM Bis-Tris HCl (pH 7.0) and 0.02% Serva blue G. The anode buffer was 50 mM Bis Tris-HCl (pH 7.0)

After loading the samples the gel was run at 100V until the sample was within the separating gel then electrophoresis continued with voltage limited to 300V and current limited to 1.5 mA. Running time was 5-6 hours at 4°C. After the gel was run the protein bands were visualised by fixing and destaining in 10% acetic acid.

2.2.4.3.2 Second-dimension blue native gel (BN-PAGE)

The complexes isolated by first dimension BN-PAGE can be separated further into their subunits by second dimension.electrophoresis in Tricine-SDS PAGE.

24 x 18cm glass gel plates with 24 x 2.5 x 0.13cm spacers were used in Tricine-SDS second dimension PAGE. The lanes from the first dimension (native) gel were cut and placed on one of the second-dimension glass plates at the usual position of the stacking gel. The gel strips were soaked with about 250 µl of 1% (w/v) SDS, 10mM DTT, for 2 hours, turning the strips after 1 hour. After incubation any excess soaking buffer was removed with tissues as completely as possible, and two gels plates with spacers fixed together with clips, so that the strip was squeezed between the two plate about 1cm from the top and did not slide down when the gel cassette was brought to vertical position during the pouring of the gels. The gel plates were sealed with 1.5% (w/v) molten agarose. Three different gels poured one on the top of the other were used in the second dimension.

The separating gel (12% acrylamide in 1.0 M Tris (pH 8.45), 0.1 % SDS) was poured between two glasses leaving a 2.5 cm gap below the piece of native gel.

After polymerisation the separation gel was overlaid with a stacking gel of 4% acrylamide, in the same buffers as the separating gels.

The third gel was an overlay gel of 10% acrylamide in first-dimensional gel buffer containing 10% (v/v) glycerol and 0.02% (w/v) SDS, so the first dimension gel was embedded in the overlay gel.

The three gel layers were poured successively into the gel cassette taking care not to create bubbles. Each layer of gel was overlaid with distilled water and allowed to set before water was poured off, the plates dried with tissues and the next layer poured on top. After the overlay gel had polymerised the bottom spacers were removed, excess agarose was cleaned away, and the gel cassette was clipped to the slab gel stand. The upper reservoirs were filled with the cathode buffer composed of 0.1M Tris-HCl, 0.1 M Tricine (pH 8.25), 0.1% (w/v) SDS and the lower reservoirs were filled with the anode buffer composed of 0.1M Tris-HCl (pH 8.9).

The second dimension Tricine-SDS gels were run at a maximum of 200V, with the current limited to 30 mA, for about 8-9 hours at room temperature.

[A] Fixing, staining and destaining

Tricine SDS-PAGE gels were fixed for 1hour with the normal fix solution for SDS-polyacrylamide gels and stained with 0.025% (w/v) Serva blue G dissolved in 10% (v/v) acetic acid for 2-3 hours on a shaking platform at room temperature.

Gels were destained in 10% (v/v) acetic acid for 5-10 hours. Polyurethane foam pieces were placed in solution to aid the destaining process.

2.2.5 Binding of proteins onto nitrocellulose membranes

2.2.5.1 Electrophoretic transfer of protein to nitrocellulose

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to cellulose nitrate sheets was done according to Towbin *et al*, (1979). The SDS polyacrylamide gel containing the separated proteins and a sheet of nitrocellulose of similar size were sandwiched in tight contact and placed in an electro-blot tank containing transfer

buffer composed of 20mM Na_2HPO_4, 0.02% (w/v) SDS, 20% methanol with the nitrocellulose sheet oriented towards the anode. For thin gels (0.75 mm spacers) proteins were transferred with at current of 1A for 2 hours but in case of thick gels (1mm and 1.5 mm thick) proteins were transferred with current of 0.4 A overnight.

2.2.5.2 Dot-blots

Non-denatured proteins were applied onto a sheet of nitrocellulose previously incubated in distilled water for 5 minutes, using a BioRad Bio-Dot microfiltration apparatus by passive filtration to allow for quantitative binding of proteins. Up to 50 µg of protein were loaded onto each dot. After passive filtration excess solvent was removed by brief application of a vacuum.

2.2.6 Detection of Proteins Bound to Nitrocellulose with Antibodies and Lectins

In the case of electrophoretic transfer, after the protein had been transferred, the nitrocellulose sheet was washed briefly with three changes of distilled water and stained for 5 minutes with Ponceau S (0.4% (w/v) in 2% (w/v) TCA) to assess the efficiency of transfer. The nitrocellulose was destained initially with distilled water then with 20mM Tris HCl, pH7.4, 150mM NaCl (Tris buffered saline, TBS) /0.05% Tween 20 for 10 minutes. Non-specific protein binding sites on the nitrocellulose blot were blocked by incubation in 0.5% Tween 20 in TBS and 0.1 (w/v) % sodium azide for 60 minutes on an orbital mixer. In the case of dot blots, after binding of the sample, dot-blots were washed briefly with three changes of TBS and the non-specific sites were blocked as above.

2.2.6.1 Immunoblots

For detection of proteins with polyclonal antibodies, the sheet of nitrocellulose containing the bound samples was incubated with antisera at 1:200 to 1:1000 dilution in TBS containing 0.05 % (v/v) Tween 20, 1mg/ml (w/v) BSA, 5% normal horse serum and 0.01% (w/v) sodium azide, for 3-4 hours on a rocking platform at room temperature. For detection of proteins with monoclonal antibodies, the sheet of

nitrocellulose containing the bound samples was incubated with culture supernatant at 1:2 to 1:20 dilution in 1 mg/ml (w/v) BSA in TBS with 0.1% (w/v) sodium azide for up to 16 hours on rocking platform at room temperature.

After incubation, the blots were washed for 25 minutes with 5 changes of TBS containing 0.05% (v/v) Tween 20. The blots were then incubated in secondary HRP-conjugated antiserum at a dilution of 1:4000 in washing buffer with 1mg BSA / ml for 60 minutes and then washed for 25 minutes as above. The blots were incubated with enhanced chemiluminescence (ECL) reagent according to the manufacturer's instruction. The blots were wrapped in plastic sheeting and the antigen-antibody complexes were detected by exposing the blots to X- ray film for periods of 5 to 20 seconds in the dark room. For preliminary testing of antisera and culture supernatant the sheets of nitrocellulose containing the bound samples were cut into 15cm x 0.75cm strips and these were placed in a BioRad incubation tray and blocked as above. The strips were incubated with a minimum of 2ml of the culture supernatants or 2ml of different dilution antisera. Usually one strip was incubated with a antidopamine β-monooxygenase as a positive control and only in case the of polyclonal antisera one of the strips was incubated with 200 fold dilution of pre-immune bleeding. After incubation the strips were washing, incubated with second antibody and processed to detection as described in sections 2.2.6.1.

2.2.6.2 Lectin blots

Decoration of blots with Con A and horseradish peroxidase was essentially as described by Clegg *et al.*, (1982). The blocking buffer was discarded and replaced with freshly prepared Con-A solution (10μg/ml in the blocking solution). To enhance the Con-A binding, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $MgCl_2$ were added to the Con-A solution. After 1 hour incubation the blot was washed for 25 minutes with 5 changes of TBS/ 0.05% Tween 20 and then the blot was incubated for another 1 hour in horseradish peroxidase (50μg /ml in blocking solution without azide) . The blot was washed as above and the complexes were visualised using ECL reagent and autoradiography as described in section 2.2.6.1.

2.2.7 Other methods

2.2.7.1 Pre-Condensation of Triton X-114

Triton X-114 was condensed and purified as described by Bordier et al (1981). 16 mg of butylated hydroxytoluene dissolved in 100 µl ethanol and 20 g of Triton X-114 were added to 980 ml of 20 mM Tris HCl (pH 7.4), 150 mM NaCl (Tris-buffered saline, TBS) in a 1 litre separating funnel. The solutions were mixed together and incubated on ice at 4°C for about 8 hours or until the mixture turned clear. The mixture was then incubated at 30°C overnight to condense the Triton X -114. Next morning the detergent-rich phase was collected and the aqueous phase discarded and replaced with the same volume of fresh Tris buffered saline and the two solutions were mixed and incubated again on ice for about 8 hours, then at 30°C overnight.

This process of dissolution and condensation was repeated three times to yield around 100 ml of approximately 10% (w/v) Triton X-114.

2.2.7.2 Estimation of Triton X-114 concentration

The concentration of Triton X-114 was determined by the method of Garewal (1973), using Triton X-100 as the standard.

Samples of 2mg/ml Triton X-100 in 50% ethanol were added to glass conical tubes to give 0-400µg final. The volume was made up to 300µl with 50% ethanol before the addition of 0.4ml cobaltothiocyanate reagent (17.8% NH_4SCN, 2.8% $Co(NO_3)_2$ in distilled water. The samples were vortexed and left for 5 minutes at room temperature before the addition of 1.5 ml dichloromethane. The samples were vortexed for 2 minutes to extract the colour into the solvent phase and then the phases was separated by centrifugation at 3000 r.p.m in an MSE bench centrifuge.

The lower solvent layer was scanned in a Pye-Unicam recording spectrophotometer between 570 and 700 nm and the difference in absorbance between 622 and 687 nm plotted against the amount of Triton X-100 to calculate the amount of Triton X-114 (Figure2.1).

The baseline sample was that which did not contain any Triton. Assays were carried out in triplicate and an average taken for estimation of Triton X-114.

2.2.7.3 Protein concentration determination

Protein concentration estimation was performed according to the method of Bradford (1976). A stock solution of 1% bovine serum albumin in distilled water was used as the standard protein and the exact concentration of BSA was determined by measuring the absorbance at 280nm, using $A^{1\%} = 6.6$, and then was used to prepare a standard curve using 2-12µg of BSA. Samples for protein estimation (100µl) and BSA samples (100µl) were incubated 15 minutes with dye reagent (1ml) and the absorbance at 595nm was measured against blank of 100µl of distilled water in 1ml of the same dye reagent. Triplicate determinations were performed to give an accurate estimation. The graph of A_{595} against concentration of BSA is relatively linear between 2-10 µg of BSA/100µl (Figure 2.2).

2.2.7.4 Acetylcholinesterase assay

Acetylcholinesterase (EC 3.1.1.7) activity was assayed by a spectrophotometric method according to Ellman *et al.*, (1961) to test the effectiveness of the PLC reaction in all reaction mixtures described above

Fig 2.1 Typical standard curve For Triton X-114

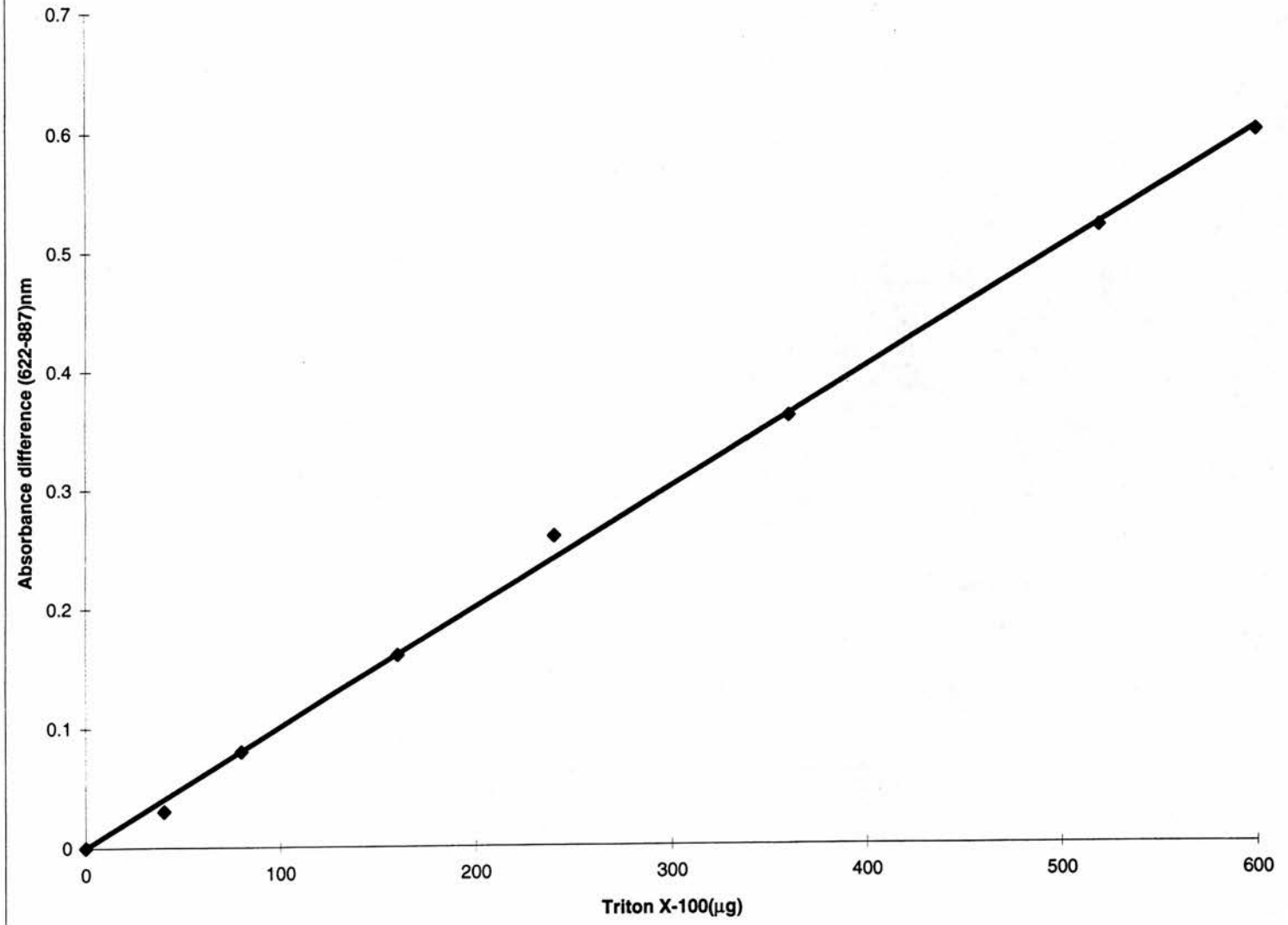
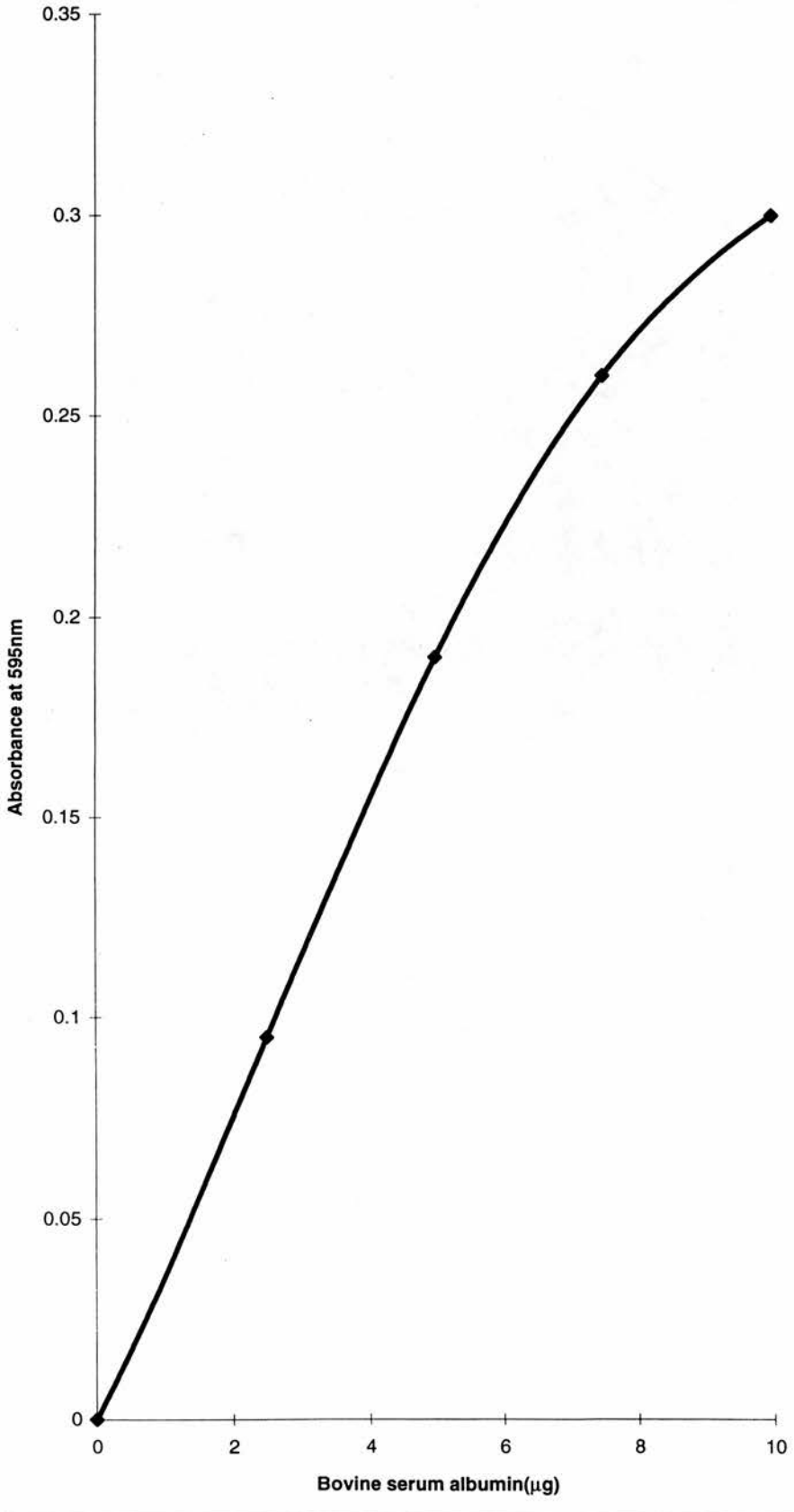


Fig 2.2 Typical standard curve for the estimation of protein concentration by Bradford method



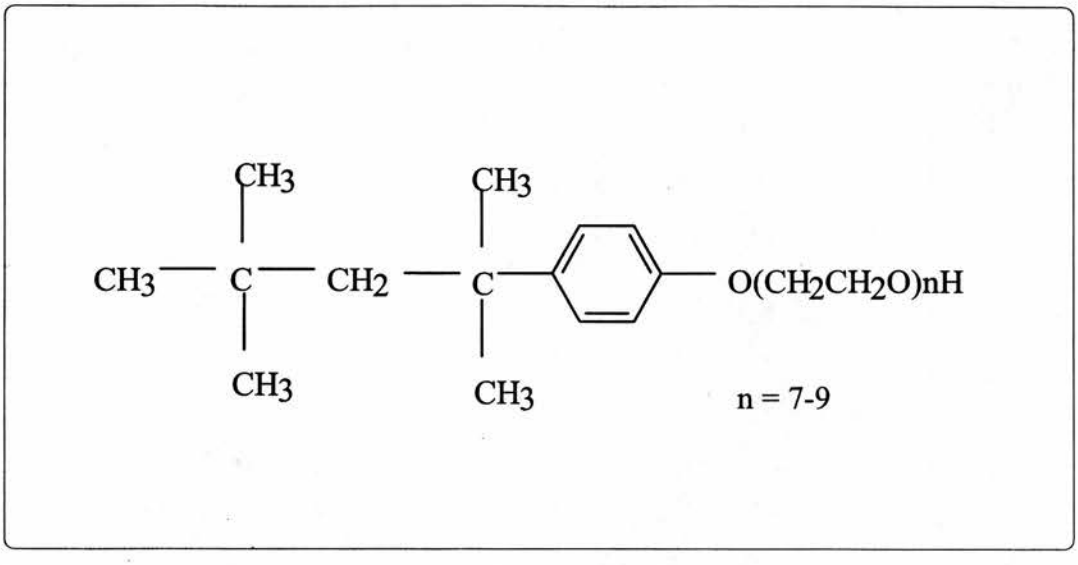
Chapter 3
Purification of glycoprotein IV from bovine chromaffin granule membranes

3.1 Introduction

Most of the information available regarding chromaffin granule membrane proteins is based on electrophoretic techniques. Over 40 membrane proteins have been identified [Abbs and Phillips, 1980] many of which are glycosylated. Five major glycoproteins termed I-V were originally identified by PAS- staining [Huber *et al.*, 1979]. Subsequently, improvements in analysis using two-dimensional gel electrophoresis with lectin binding and membrane fractionation using Triton X-114 have enabled the identification of at least 20 membrane glycoproteins including glycoproteins H, J and K [Gavine *et al.*, 1984; Pryde *et al.*, 1986]. While the composition of chromaffin granules has been increasingly well studied there is little detailed structural information available for membrane proteins, though some of the above glycoproteins have been purified from chromaffin granule membranes using sequential lectin affinity chromatography and later characterized by molecular cloning. Glycoprotein I was identified as dopamine β-monooxygenase. The deduced amino acid sequence of glycoprotein II revealed that it is identical with Lysosome-Associated Membrane Glycoproteins (LAMP-1 and LAMP-2) [Hieber *et al.*, 1993]. Cloning of glycoprotein III has shown that it represents the bovine counterpart of rat Sertoli cell sulphated glycoprotein 2 [Palmer *et al.*, 1990]. Glycoproteins J and K are different molecular forms of carboxypeptidase H [Christie *et al.*, 1990; Laslop *et al.*, 1986] and glycoprotein H is the bovine equivalent of the Kex2-related proteases [Christie *et al.*, 1991]. Glycoprotein IV is the subject of this thesis, and no molecular studies of glycoprotein V have yet been reported.

The fractionation of chromaffin granule membrane proteins with the non-ionic detergent Triton X-114 has been an important technique in providing material that could be used for further purification or for production of monoclonal antibodies. Detergents of the Triton series are amphipathic molecules composed of an aromatic, hydrophobic head and a repetitive hydrophilic polyoxyethylene tail which determines the physical properties of the molecule in solution (Figure 3.1). For example Triton X-100, which has an average repeat of nine polyoxyethylene groups, forms micelles composed of about 140 molecules at 0°C when at or above its critical micelle concentration (cmc).

Figure 3.1 Polyoxyethylene p-t octyl phenol: The Trition X-114 molecule



Increasing the temperature of such a solution has no visible effect until the characteristic "cloud-point" is reached, when the micelles aggregate and form a separate phase. For Triton X-100, this occurs at 64 °C, but the more hydrophobic detergent Triton X-114, which has an average around seven polyoxyethylene groups, has its cloud point at the more useful temperature of 20°C [Bordier, 1981; Pryde 1986]. In 1981 Bordier, using Triton X-114, showed that a mixture of proteins could be resolved into distinct groups depending on their hydrophobicities and he applied this principle to extract the membrane proteins from human erythrocytes. Biological membranes are composed of phospholipid bilayers containing integral membrane proteins anchored by hydrophobic domains, and when they are treated with Triton X-114 at 0°C, micelles containing detergent, phospholipid and membrane proteins are formed. At the cloud-point these mixed micelles aggregate and precipitate, forming a detergent-rich phase enriched in intrinsic membrane proteins, distinct from an aqueous phase containing extrinsic and soluble proteins. The ease with which Triton X-114 resolves membrane proteins while retaining their biological function has led to its application to other biological membrane preparations. In 1986, Pryde used this property to fractionate bovine chromaffin granule membranes with Triton X-114. Although rich in cholesterol and phospholipid, chromaffin granule memranes are apparently fully solubilized by treatment with Triton X-114 at 0°C, but after about 5 minutes on ice the solution becomes opalescent and a precipitate forms containing about 10% of the total membrane protein. It is enriched in cholesterol and phospholipid and can be separated by high speed centrifugation. Chromaffin granule membrane lipids appear to form large mixed micelles with the detergent so that aggregation and precipitation occur spontaneously.

Glycoproteins have sugar chains that can usually be recognised by specific lectins. These lectins are proteins frequently found in plant sources, although examples exist in many other species as well. These proteins are capable of reversible high affinity binding to specific carbohydrate sequences [Briles *et al.*, 1979]. Glycoproteins which have the specific sugar residue in an exposed position in the oligosaccharide unit will bind noncovalently to either soluble or immobilised lectins. The complexes formed can be dissociated with specific carbohydrate

ligands like α-methyl-D-mannoside and N-acetylglucosamine. This provides a method for separation and purification of glycoproteins and for study of the glycoprotein composition of macromolecular structures [Sharon *et al.,* 1993].

Table 3.1 is a list of common lectins and their sugar specificities used for glycoprotein purification. The most commonly used is Concanavalin A (Con A), a glycoprotein isolated from jack beans which was first reported to form a precipitate with glycogenous yeast mannan by Sumner and Howell in 1936. Concanavalin A is the first lectin whose three-dimensional structure has been elucidated and its physiochemical characteristics have been characterized in detail [Edelman *et al.,* 1972; Hardman *et al.,* 1972; Cunningham *et al.,* 1975]. At physiological pH con A has been shown to be a tetramer of identical subunits, while below pH 6 the tetramer dissociates into stable dimers or monomers [Gunther *et al.,* 1973; Griswold *et al.,* 1985]. Each monomer contains 273 amino acid residues (molecular weight 25,500Da) and has binding sites for one transition metal ion (normally Mn^{2+}), one Ca^{2+} and one saccharide, thus four saccharides can be bound by the tetramer [So and Goldstein, 1968; Yariv *et al.,* 1967]. Concanavalin A preferentially binds to α-D-mannopyranosyl and α-D-glycopyrosyl residues at the nonreducing termini of oligo- or polysaccharides [Goldstein *et al.,* 1965] or to certain non terminal mannosyl residues [Goldstein *et al.,* 1970], and its binding strongly indicates the presence of N-linked oligosaccharides chains [Goldstein *et al.,* 1973]. Low-resolution crystallographic studies have provided a geometric description of the shape and arrangement of the subunits [Hardman *et al.,* 1972].

Glycoprotein-linked carbohydrate chains have been found on the extracellular surface of the plasma membrane [Sharon and Lis, 1982]. To preserve the asymmetric arrangement of carbohydrates in the cell membrane, intracellular organelles that derive from or fuse with the membrane must have the carbohydrate of glycoproteins localized on the inner (extracytoplasmic) surface. Since chromaffin granules fuse with the membrane of the chromaffin cell it seems very unlikely that carbohydrates of glycoproteins are present on the cytoplasmic surface of the organelle membrane.

Eagles (1975) found that Con A binds to isolated chromaffin granule membranes but not to intact granules suggesting that carbohydrates are on the inner (matrix) surface of the membrane. Isolated chromaffin granule membranes were treated with galactose oxidase and then with sodium [^{3}H]-boro hydride and all the major glycoproteins were labelled. There was no significant labelling of glycoproteins when intact granules were treated in this way [Huber *et al.,* 1979; Abbs and Phillips, 1980].

Glycoprotein IV from chromaffin granule membranes is an uncharacterised glycoprotein of unknown function. It was identified on two-dimensional blots decorated with Concanavalin A as a protein with an apparent molecular mass of 45-50 kDa and pI range of 3.4-4.5. [Gavine *et al.,* 1984]. Also it has been found to be the most characteristic glycoprotein of the phospholipid-rich pellet phase after fractionation of membrane proteins with Triton X-114 [Pryde *et al.,* 1986]. In this current study fractionation of membrane proteins with Triton X-114 and lectin affinity chromatography were used in purifying glycoprotein IV from bovine chromaffin granule membranes.

Table 3.1

Lectins commonly used for glycoprotein purification

Lectin (a)	Specificity	Reference (b)
Concanavalin A (Con A)	Man and Glc	1, 2
Gorse lectin	Fucose	3
Helix pomatia lectin	GalNAc	4
Horse gram lectin	GalNAc	5
Lentil lectin	Similar to Con A	6
Ricinus communis agglutinin I	Non-reducing β–Gal	7, 8
Peanut agglutinin (PNA)	GAL (β–3)-GalNAc	
Pisum sativum lectin	Man, Glc	9
Soy bean agglutinin (SBA)	Terminal β–GlaNAc	10
Wheat germ agglutinin (WGA)	GalNAc (dimer or trimer)	11
	or sialic acid	12

[a]Abbreviations: man, mannose; Glc, glucose; GalNAc, N-acetylglucosamine; Gal, galactose and GalNAc, N-acetylgalactosamine

[b]Reference: (1) Goldstein and So, (1965); ,(2) Goldstein *et al.,* (1965); (3), Matsumoto and Osawa, (1970); (4) Hammarstrom *et al.,* (1969); (5) Etzler and Kabat, (1970); (6) Howard *et al.,* (1971; (7) Sharon and Lis, (1972); (8) Takahashi *et al.,* (1962); (9) Sharon and Lis, (1972); (10) Paulova *et al.,* (1971); (11) Lis *et al.,* (1970); (12) Burger and Goldberg, (1967).

3.2 Partial Purification of Gp IV by Sequential Affinity Chromatography on Concanavalin A-agarose.

3.2.1 Solubilizing GpIV from chromaffin granule membranes

The Triton-insoluble phospholipid-rich pellet, termed P1, formed from the fractionation of membrane proteins with Triton X-114 was washed twice with water (section 2.3a) and was resuspended in 20mM sodium phosphate pH 7.4 by gentle homogenisation in the presence of 2% (w/v) n-octyl-β-glycoside. The solubilized membrane proteins were then centrifuged at 412,000 g_{av} (Beckman instruments TL-100.3 rotor) for 10 minutes at 2°C to remove insoluble material which was discarded. The supernatant, containing solubilized proteins, was diluted 4-fold with 20mM sodium phosphate pH 7.4.

3.2.2 Preparation of Con A- agarose

This was based on the method described in Davey *et al.*, (1976). 2 ml of Con A-agarose was pre-washed with 20mM sodium phosphate pH 7.4, 0.5M NaCl, 1.7 M ethylene glycol, 0.05% (w/v) n-octyl-β-glycoside in a 50ml polyethylene tube.

The tube was placed on the rocker for 3 hours at 4°C, and the Con A-agarose then separated by centrifugation at 2000 r.p.m for 10 minutes in a MSE bench centrifuge. The washing buffer was discarded and replaced with the diluted solubilized protein in the presence of 0.1mM $CaCl_2$, 0.1mM $MgCl_2$, 0.1mM $MnCl_2$ and 0.1% (w/v) sodium azide and then the tubes placed again on the rocker for 3-4 hours at 4°C .

3.3.3 Concanavalin A column chromatography

This method was described in Wood *et al.*, (1985). The solubilized-Con A-protein-agarose mixture was poured into a 2 ml column (4cm x 0.5cm^2) and washed with 30ml of 20mM NaH_2PO_4 pH 7.4, 0.5M NaCl, 1.7 M ethylene glycol, 0.05%(w/v) n-octyl-β-glycoside for 3 hours at a flow rate of 10ml / h. Glycoprotein

IV was eluted from the Con A-agarose column using 10 % (w/v) α-methyl-D-mannoside in 20mM NaH_2PO_4 pH 7.4, 0.5 M NaCl, 1.7M ethyleneglycol, and 0.05% n-octyl-β-glycoside at a flow rate of 1ml / h. The proteins recovered from the Con A-agarose column were precipitated with 10 % (w/v) TCA and analysed by one dimensional SDS- polyacrylamide gel electrophoresis, staining with silver stain and Con A-blotting to estimate the degree of purity of glycoprotein IV that eluted from the column.

3.2.4 Electroelution of glycoprotein IV from one dimensional SDS polyacrylamide gel

Glycoprotein IV purified by Con A-agarose chromatography was further purified by electroelution from SDS polyacrylamide gels as described by [Harrington, 1990] as follows:

3.2.4.1 Preparing the sample for electroelution

The fractions eluted from the Con A-agarose column and analysed by Con A blotting were pooled usually in 15-20 ml. This volume of solution containing glycoprotein IV was reduced to 1-2 ml using an Amicon ultrafiltration apparatus with a PM-30 membrane, and then diluted 2-fold in SDS sample buffer.

SDS polyacrylamide gels containing 9% acrylamide were set up in cassettes with 1.5 mm spacers. A single slot was made right across the top of the stacking gel and the sample was loaded onto 2-3 gels, taking care to prevent overloading of the gels with protein, and electrophoresis was carried out as described before in section 2.4.1 at 25 mA for 3-4 hours. After the gels were run about 1cm from both sides of each gel were cut off and blotted onto nitrocellulose membranes and the main body of the gels were wrapped in cling film and stored, moistened with water, at 4°C.

The strip blots were probed with Con A and horseradish peroxidase to determine the position of glycoprotein IV on the gels as described in section 2.5.3.

3.2.4.2 Electroelution of glycoprotein IV

After the glycoprotein IV had been localized on the side strips, these were aligned with the untreated gel and the area of the gel containing the glycoprotein IV was excised using a sharp scalpel. The gel slices containing glycoprotein IV were further cut into small pieces (approximately 1-5 mm^2). These gel pieces were placed in the electroelution chamber which was then placed in the middle of the electroelution cassette orienting the chamber containing the gel pieces to the negative electrode. Electroelution buffer, containing (20mM Tris, 0.1M glycine, and 0.1% (w/v) SDS), was added to cover the gel pieces and to the reservoir of the chamber.

Proteins electroeluted from the gel slices at 100 V (approximately 15mA) overnight with the same buffer. The electroeluted glycoproteins were collected from the reservoir chamber. Fresh elution buffer (500 µl) was used to rinse the reservoir chamber and then was added to the sample. The sample volume was reduced to 1-2 ml by using an Amicon ultrafiltration apparatus with a PM- 30 membrane. A sample of electroeluted material was subjected to re-electrophoresis on SDS polyacrylamide gel, blotted to nitrocellulose and tested with Con A-lectin and horseradish peroxidase as described in section 2.2.6.2, to check the purity of protein. The protein concentration was estimated according to the method of Bradford (1976) using BSA as the standard.

3.3 Results and Discussion

3.3.1 Fractionation of membrane proteins with Triton x-114

Figure 3.2 is a Commassie blue stained two-dimensional gel showing the protein composition of chromaffin granule membranes: about half of these proteins are glycosylated and stain poorly with Commassie blue but are better visualised with lectin blotting [Gavine *et al.,* 1984]. Among the chromaffin granule membrane glycoproteins which bind the lectin Concanavalin A are the biosynthetic enzyme dopamine β-monooxygenase, glycoproteins J, H and K and also glycoproteins II, IV and V as shown in the two-dimensional Con A blot in Figure 3.3. Glycoprotein III binds a different lectin, wheat germ agglutinin. [Gavine *et al.,* 1984]. Upon treatment of membranes with the non-ionic detergent Triton X-114 [Pryde and Phillips, 1986], proteins were fractionated on the basis of their hydrophobicity into three distinct groups according to the scheme shown in Figure 3.4. The first of these fractions was removed by centrifugation and termed phospholipid-rich pellet (P1) which contains the subunits of the H^+-translocating ATPase and also glycoprotein IV as shown in Figure 3.5 lane 4 [Pérez-Castiñiera and Apps, 1990; Pryde and Phillips, 1986]. The enrichment of glycoprotein IV in this fraction was detected by two-dimensional Con A blot shows in Figure 3.6. The bulk of Triton X-114 remains in the supernatant, and warming to 30°C leads to separation of another group of hydrophobic proteins termed detergent-rich phase (P2) which contains several intrinsic proteins such as cytochrome b561, ATPase II, the membrane- associated form of dopamine β-monooxygenase, glycoprotein II and glycoprotein V as shown in Figure 3.5, lane 5 [Percy *et al.,* 1985; Pryde and Phillips, 1986]. But many intrinsic glycoproteins of the chromaffin granule membrane mixed with soluble chromaffin granule glycoproteins remain in the aqueous phase, and after the removal of the residual detergent by exhaustive dialysis, some integral membrane glycoproteins such as glycoprotein H, J and K and the minor membrane glycoprotein p65 (synaptotagmin) were precipitated and termed glycoprotein-rich pellet (p3) as shown in Figure 3.5 lane 6 [Pryde and Phillips, 1986; Tugal *et al.,* 1992].

The soluble secretory proteins such as chromogranin A, B and C and the soluble form of dopamine β-monooxygenase remain in the supernatant as shown in Figure 3.5, lane 7.

3.3.2. Solubilizing proteins from the phospholipid-rich pellet with detergents:

Several detergents with high critical micelle concentration (cmc) were tried in attempts to solubilize the P1 fraction. The solubilized material was separated from insoluble material by centrifugation and both fractions analysed by electrophoresis followed by Coomassie blue staining and Con-A blotting.

Even after treatment of the P1 fraction with these detergents, glycoprotein IV is still difficult to identify in the gel stained with Coomassie blue as illustrated in Figure 3.7a lanes 2-8. But a blot decorated with Con A-HRP complex shows that glycoprotein IV, dopamine β-monooxygenase and other proteins were solubilized from P1 by these detergents as shown in Figure 3.7b lanes 2-8. All the detergents tried were able to solubilize proteins from P1 but the amount of solubilized proteins was variable as shown in Figures 3.7a and 3.7.b. The amount of proteins solubilized with n-octyl-β-glycoside appeared to be greater than with other detergents as shown in Figures 3.3.2.1a and 3.3.2.1b lanes 2 (pellet fraction) and 3 (supernatant fractions); CHAPS, MEGA-8 and MEGA-10 solubilized similar amounts of protein.

3.3.3 Partial purification of membrane glycoprotein IV

Figure 3.6 is a two-dimensional Con A blot of P1 showing the partial isolation and enrichment of glycoprotein IV after fractionation of membrane proteins with Triton X-114. To further purify glycoprotein IV from P1, this pellet was solubilized using n-octyl-β-glycoside, diluted 4-fold in the solubilizing buffer and applied to a Con A-agarose column (as described in section 3.1.3). 0.1mM $CaCl_2$, 0.1mM $MgCl_2$, and 0.1mM $MnCl_2$ were added to the solution to enhance the binding of proteins to Con A. After the column had been equilibrated with buffer, the proteins were eluted with same buffer containing 10% (w/v) α-methyl-D-mannoside and 1.7M ethylene glycol. Fractions of 1 ml were collected and analysed with SDS-polyacrylamide gel electrophoresis followed by silver staining and Con A blotting.

Figure 3.8a shows the silver-stained gel of all fractions eluted from Con A-agarose column and shows that glycoprotein IV still was hardly visible even after this purification step. In Figure 3.8b the Con A-blotting of all fractions eluted from the Con A-agarose column shows that glycoprotein IV eluted from the Con A column was more pure than P1 proteins. Dopamine β-monooxygenase, which also binds Con A [Gavine *et al.*, 1984] was also eluted from the column (Figues 3.8a and 3.8b) because P1 was contaminated with soluble dopamine β-monooxygenase. Also the above Figures show that another protein of about 29kDa was eluted from the column; this is probably the Con A monomer (since probably not all subunits of the tetramer are covalently linked to the agarose). Because the main aim of this work was to purify glycoprotein IV in order to use it for producing monoclonal antibodies, and because eluted glycoprotein IV was contaminated with Con A which is a toxic material [Leist and Wendel, 1996] and should not be injected into laboratory animals, electroelution of glycoprotein IV from SDS-polyacrylamide gels was performed to remove this lectin.

3.3.4 Isolation of glycoprotein IV by electroelution from polyacrylamide gel slices:

Electroelution of proteins separated in various types of polyacrylamide gels can be employed from the analytical to the preparative scale. After separation, it is frequently necessary to extract or elute a specific protein from the gel for further study such as amino acid composition, sequence analysis or to produce antibodies. For optimal efficiency of elution it is desirable to have a simple technique that successfully extracts all protein from the gel and avoids any chemical modification of the protein.

Glycoprotein IV eluted from the Con A-column was further separated in SDS-polyacrylamide slab gels, and slices containing glycoprotein IV were cut from the gels and the protein electroeluted from the gel slices as described in section 3.1.4. The electroeluted glycoprotein IV was analysed by electrophoresis followed by Coomassie Blue staining or by transfer to nitrocellulose which was then decorated with Con A and horseradish peroxidase.

Figure 3.9a shows that electroeluted glycoprotein IV migrated as a single band and did not appear to be contaminated. Figure 3.9b also shows that this procedure provides considerable enrichment enabling the visualisation of this glycoprotein by Coomassie blue staining. This electroeluted glycoprotein IV was subjected to N-terminal sequencing (Chapter 4) and was also used for raising antisera (Chapter 5)

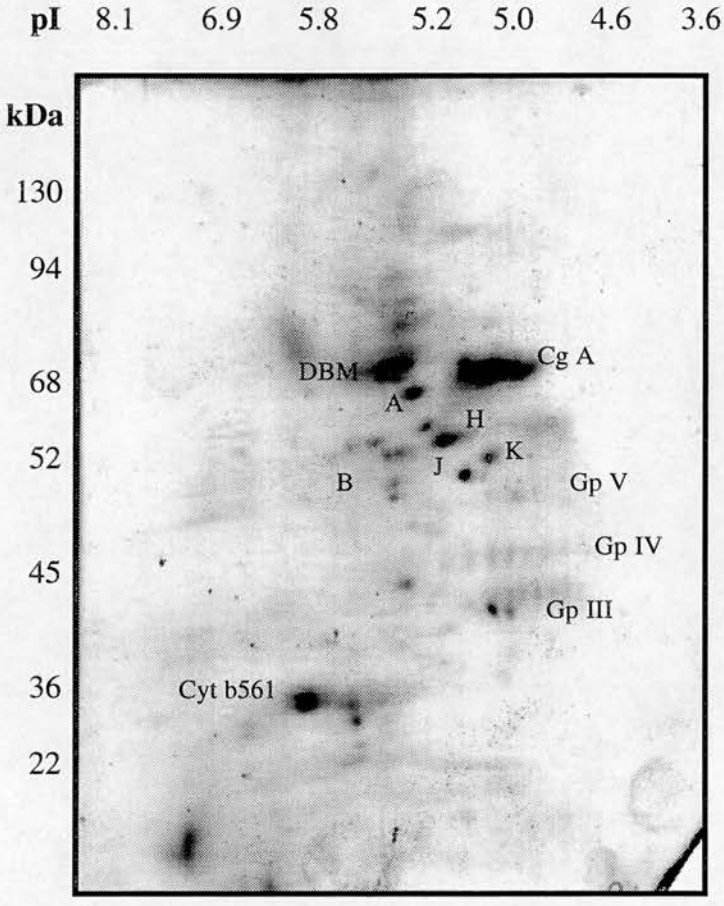


Figure 3.2 50μg of chromaffin granule membrane proteins were electrofocused in the first dimensional gel (1D) using ampholytes of pH range 3-10. The electrofocussing proteins were then separated in second dimensional SDS- PAGE (2D) followed by Commassie Blue staining.
DBM: dopamine β -monooxygenase,
Cg A: chromogranin A,
A and B: V-ATPase subunits,
H: proteinase CP2,
J and K: different molecular forms of carboxypeptidase H,
Cyt b561: cytochrome b561,
Gp III: glycoprotein III,
Gp IV: glycoprotein IV and
Gp V: glycoprotein V.

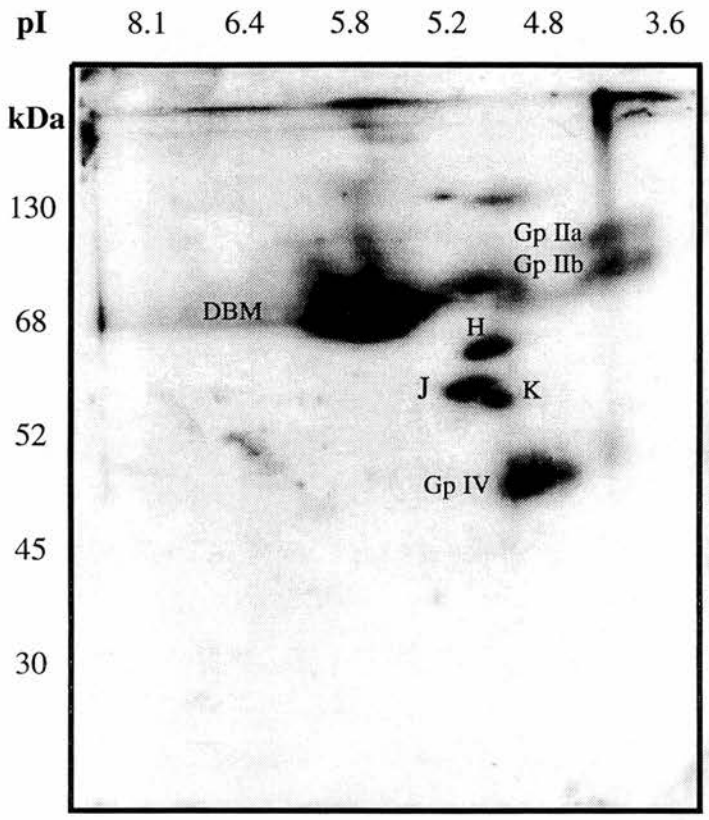
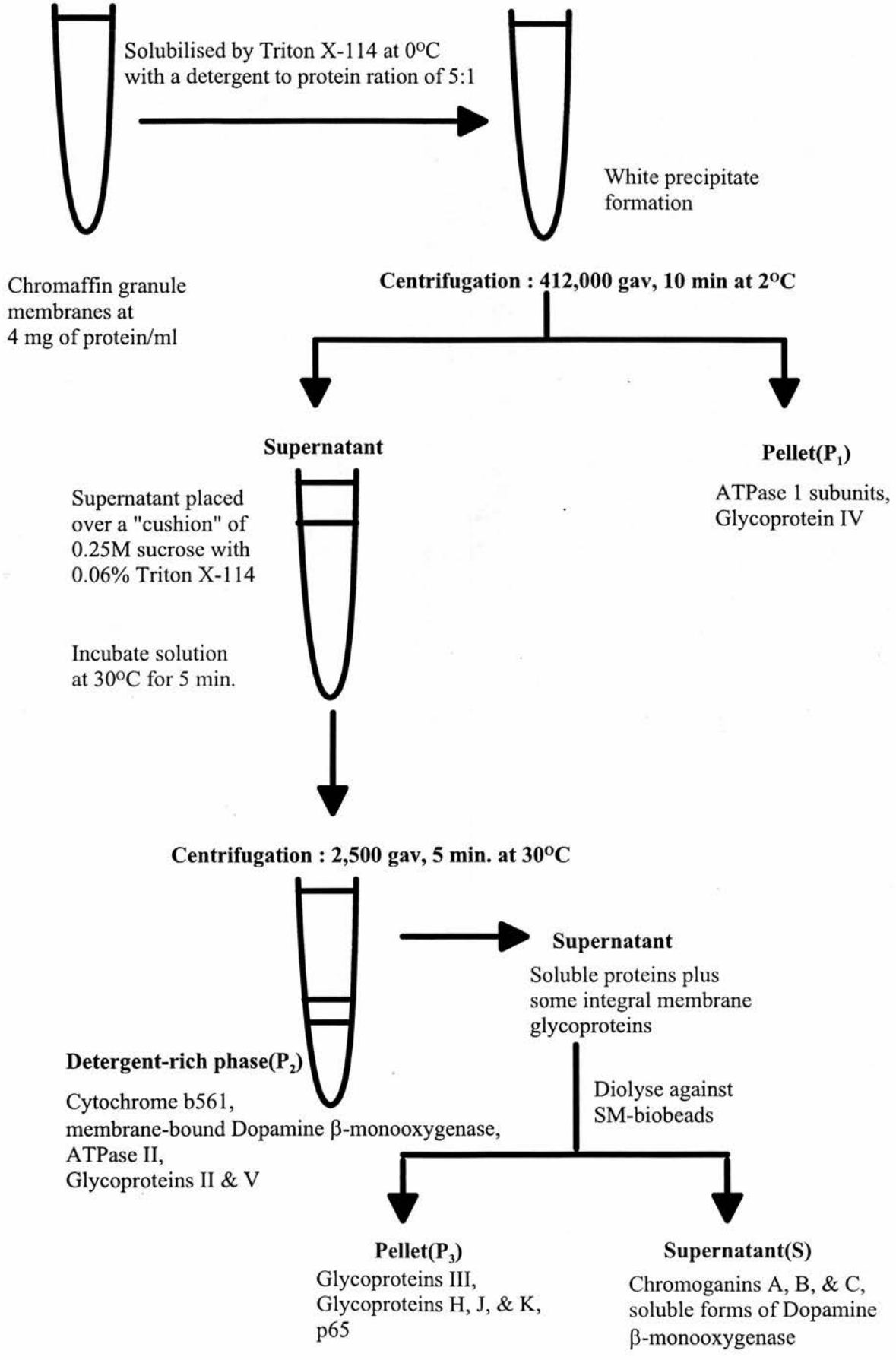


Figure 3.3 50μg of chromaffin granule membranes proteins were electrofocused in the first-dimension using ampholytes of pH range 3-10. The proteins were then separated in second-dimensional SDS-PAGE, blotted onto nitrocellulose and detected using Con A and HRP followed by ECL.
DBM : dopamine β-monooxygenase,
H: proteinase CP2,
J and K: different molecular forms of carboxypeptidase H,
Gp IIa and Gp IIb: different molecular forms of glycoproteins II,
Gp IV: glycoprotein IV.

Figure 3.4 Triton X-114 fractionation of adrenal chromaffin granule membrane proteins.



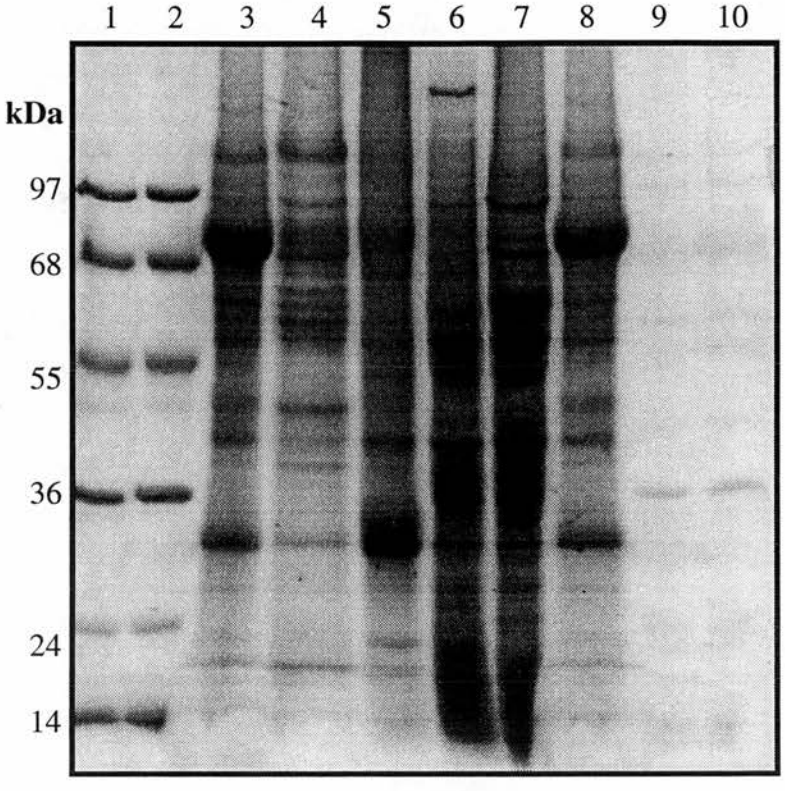


Figure 3.5 Proteins of Triton X-114 fractions derived from chromaffin granule membrane were separated by SDS-PAGE followed by Coomassie Blue staining.
Lane 1 and 2: molecular mass markers,
Lane 3 and 8: chromaffin granule membranes,
Lane 4: phospholipid-rich pellet (P1),
Lane 5: detergent-rich pellet (P2),
Lane 6: glycoprotein-rich pellet (P3),
Lane 7: aqueous supernatant,
Lane 9 and 10: molecular mass markers

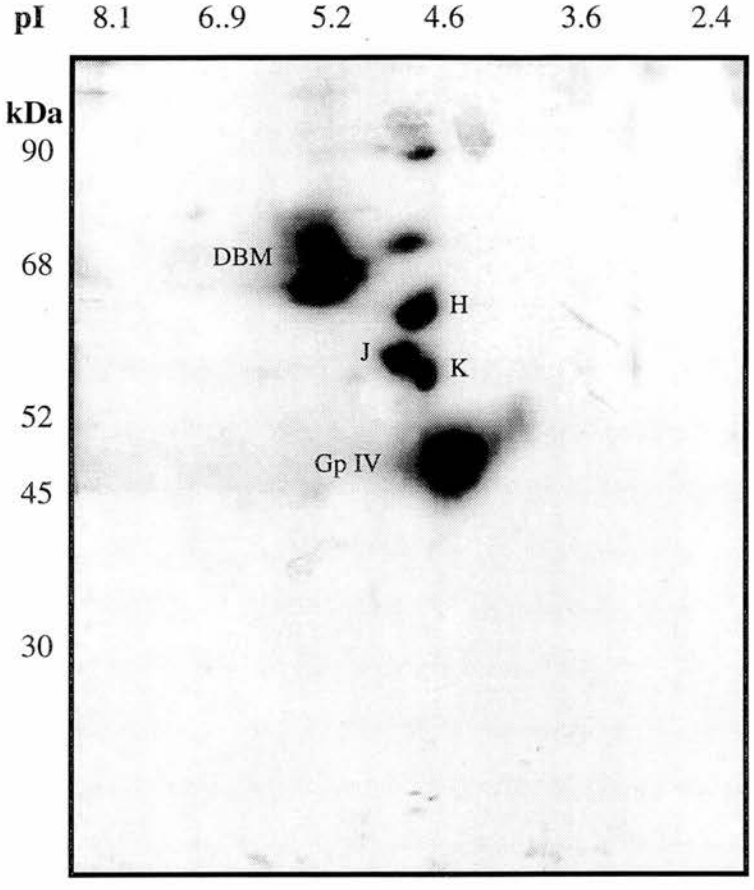


Figure 3.6 50μg of P1 fraction from chromaffin granule membrane proteins were electrofocused in the first-dimension using ampholytes of pH range 3-10. The proteins were then separated in SDS-PAGE, blotted onto nitrocellulose and detected using Con A and HRP, followed by ECL.
DBM : dopamine-β-monooxygenase,
H: proteinase CP2,
J and K : different molecular forms of carboxypeptidase H,
Gp IV: glycoproteins IV.

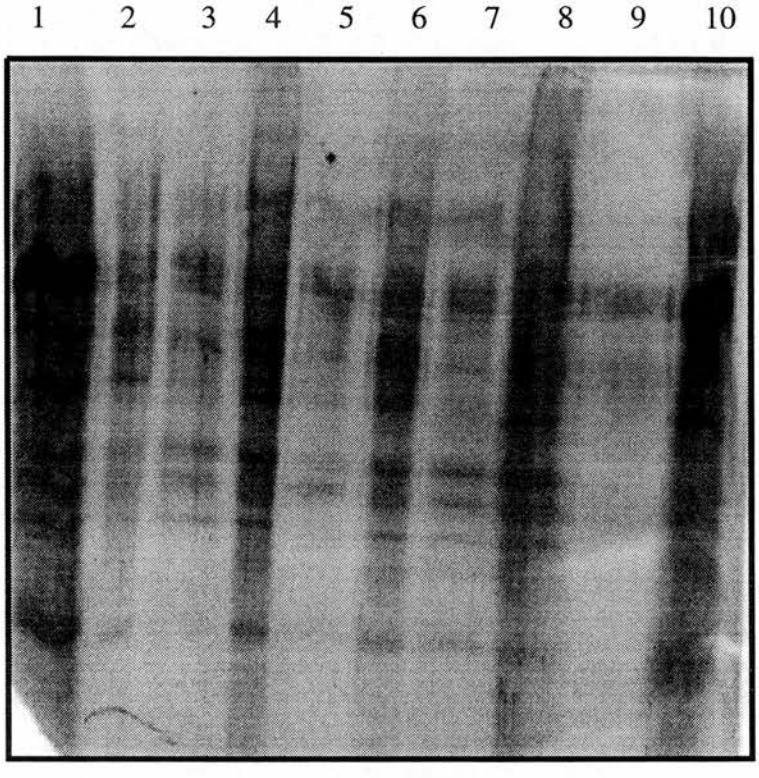


Figure 3.7a The phospholipid-rich pellet fraction (P1) derived from chromaffin granule membranes was washed with some commercial detergents. The solubilized material was then separated from insoluble material by centrifugation. Both fractions were analyzed by SDS-PAGE followed by Coomassie-Blue staining.
Lanes 1 and 10 : Chromaffin granule membranes;
Lane 2: Insoluble proteins left after washing the P1 with n-octyl- β-glucoside;
Lane 3: Solubilized proteins from washing the P1 with n-octyl-β-glucoside;
Lane 4: Insoluble proteins left after washing the P1 with MEGA 8;
Lane 5: Solubilized proteins from washing the P1 with MEGA 8;
Lane 6: Insoluble proteins left after washing the P1 with MEGA 10
Lane 7: Solubilized proteins from washing the P1 with MEGA 10;
Lane 8: Insoluble proteins left after washing the P1 with CHAPS;
Lane 9: Solubilized proteins from washing the P1 with CHAPS.

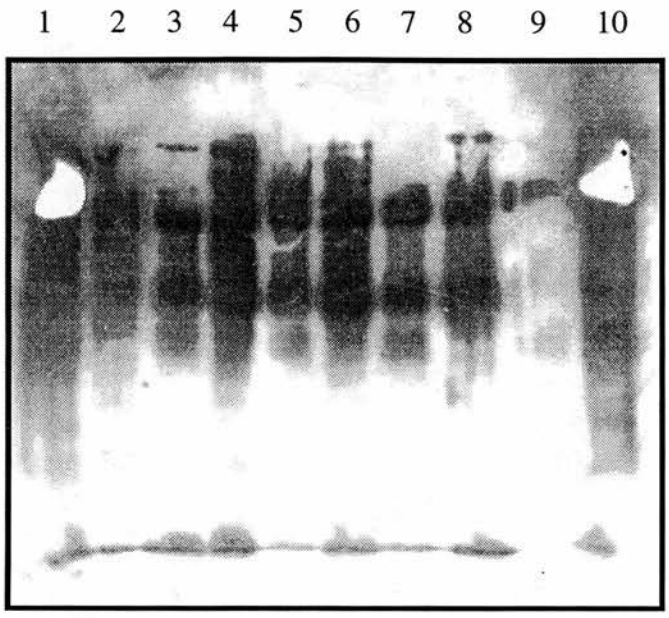


Figure 3.7b The phospholipid-rich pellet (P1) derived from chromaffin granule membranes was washed with some commercial detergents. The soluble material was separated from insoluble material by centrifugation. Both fractions were then separated in 9% SDS-PAGE and transferred onto nitrocellulose membrane. Immunoblotting was then performed using Con A and HRP and immunoreactivities were detected by using ECL.
Lane 1 and 10 : Chromaffin granule membranes,
Lane 2 : Insoluble proteins left after washing the P1 with n-octyl-β -glucoside,
Lane 3 : solubilized proteins from washing the P1 with n-octyl-β -glucoside,
Lane 4 : Insoluble proteins left after washing the P1 with MEGA8,
Lane 5 : Solubilized proteins from washing the P1 with MEGA8,
Lane 6 : Insoluble proteins left after washing the P1 with MEGA10,
Lane 7 : Solubilized proteins from washing the P1 with MEGA10,
Lane 8 : Insoluble proteins left after washing the P1 with CHAPS,
Lane 9 : Solubilized proteins from washing the P1 with CHAPS.

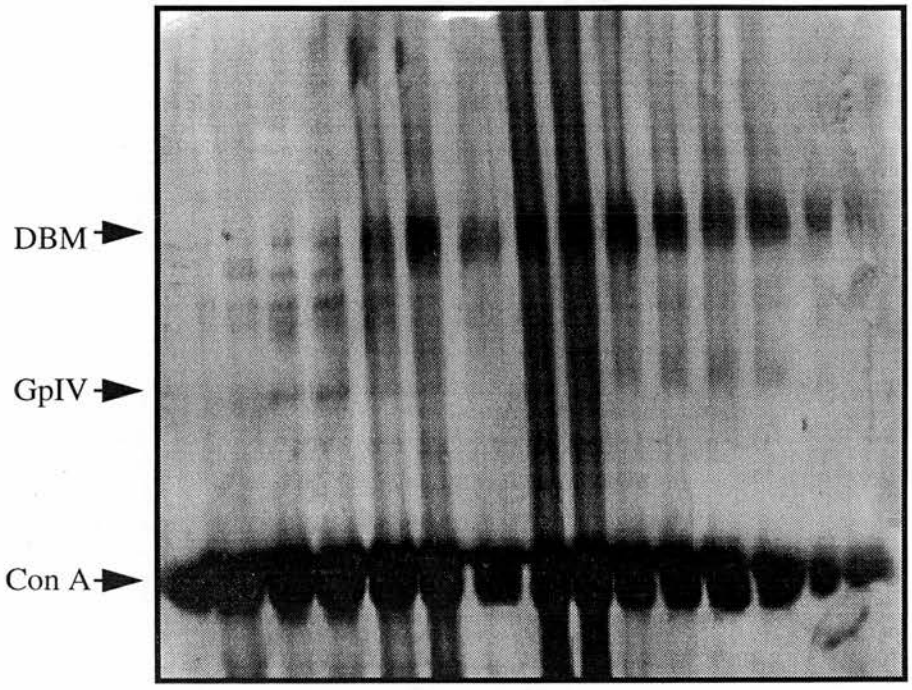


Figure 3.8a Elution of solubilized proteins from the Con A-agarose column. All the fractions were separated by 9% SDS PAGE followed by silver staining.

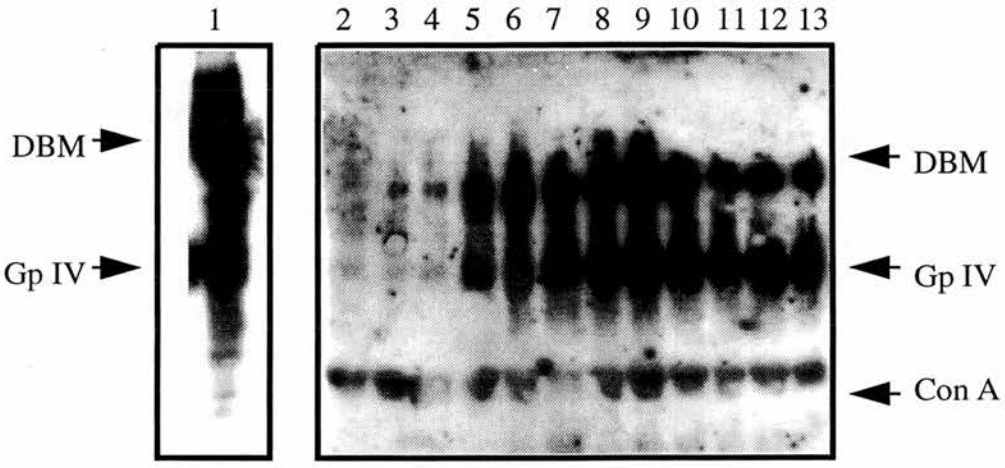


Figure 3.8b All the fractions eluted from Con A column chromatography were separated by 9% SDS-PAGE and immunoblotting was then performed by using Con A and HRP. Immunoreactivities were detected by using ECL.
Lane 1: Chromaffin granule membrane.
Lanes 2-13: fractions eluted from Con A-agarose column.

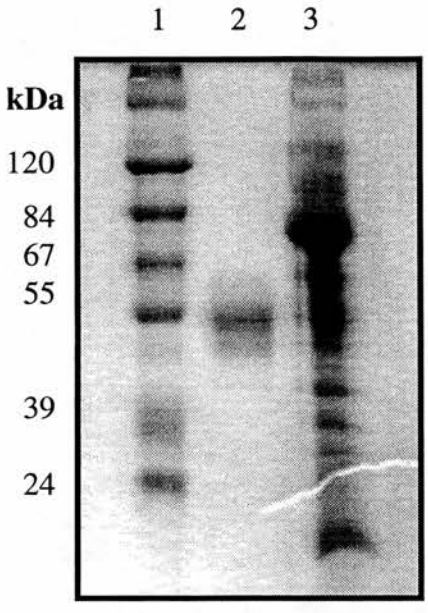


Figure 3.9a Electroeluted glycoprotein IV was analysed by 9% SDS-PAGE stained with Coomassie Blue.
Lane 1: Molecular mass markers,
Lane 2: Electroeluted glycoprotein IV,
Lane 3: Chromaffin granule membranes.

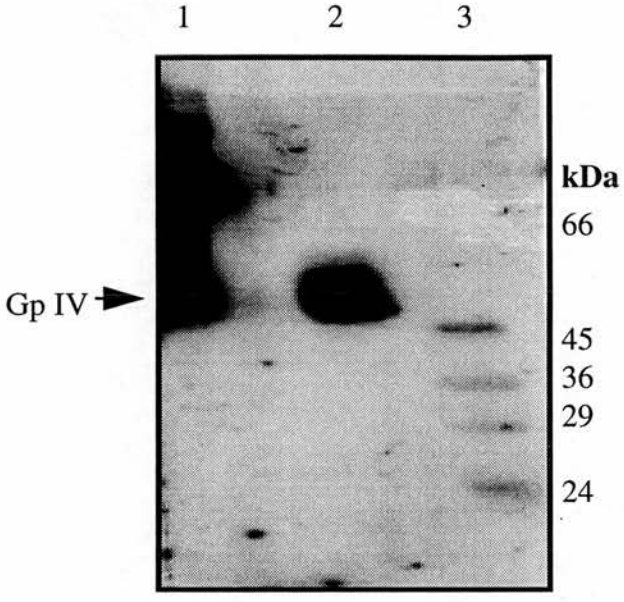


Figure 3.9b Electroeluted glycoprotein IV was separated by 9% SDS-PAGE and immunoblotting was then performed using Con A and HRP. Immunoreactivities were detected by using ECL.
Lane 1: P1 fraction from chromaffin granule membranes,
Lane 2: Electroeluted glycoprotein IV,
Lane 3: Molecular mass markers.

Chapter 4
Partial amino acid sequence of glycoprotein IV

4.1 Introduction

In recent years many biological research projects have been a co-operative approach between protein chemistry and molecular biology. The major practical integration of the two fields is the use of partial aminoacid sequence of proteins for the design of oligonucleotide probes to clone the corresponding gene, and analysis of the protein to obtain information on post-translational modification, processing, domain structures, surface topographies and folding [Hunkapiller *et al.,* 1984]. One limiting factor of this combined method is the sensitivity of protein sequencing technology. The most difficult part of any protein sequencing project is to obtain a sample that is sufficiently free of both protein and non-protein contaminants. One-dimensional gel electrophoresis is used because of its resolving power and its ability to maintain the solubility of almost any protein. Many proteins of interest are identified only by migration in analytical gels or by immunoreactivity in immunoblots. In the last few years, direct sequencing of proteins separated on polyacrylamide gels and electroblotted onto a suitable solid support has been introduced [Aebersold *et al.,* 1986; Matsudaira *et al.,* 1987], following the demonstration that polyvinylidene difluoride (PVDF) membrane can be used in immunoblotting [Pluskal *et al.,* 1986].

Polyvinylidene difluoride membranes are mechanically strong solid phase supports that bind proteins hydrophobically. They have been used in immunoblotting, and because they are inert to most organic solvents (e.g. acetonitrile, trifluoroacetic acid, ethyl acetate) they can be used in automated gas-phase sequenators. Matsudaira (1987) coupled SDS-PAGE and electrophoretic transfer to PVDF membranes which made amino-terminal sequence analysis possible for proteins not amenable to purification by other means, following which many protein sequence determinations have been done directly on samples immobilised on PVDF membranes.

The combination of two-dimensional gel electrophoresis with direct sequencing has also been reported [Bauw *et al.*, 1987; Eckerskorn *et al.*, 1988]. Acceptance of these techniques was slow due to the inability of many investigators to obtain adequate sequencing signals.

Evaluation of several parameters has indicated that pre-electrophoresis of gels with a scavenger such as glutathione or mercaptoacetic acid to eliminate reactive by-products of acrylamide polymerization, and lowering the separation pH to reduce the reactivity of the N-terminal amine, can dramatically improve the signals [Moos *et al.*, 1988, Dunbar *et al.*, 1994]. Tris-Tricine gels have became popular, especially for low molecular-weight proteins and peptides [Schägger and von Jagow 1987]. These gels address the concerns of pH-dependent N-terminal blockage and give good results in sequencing experiments.

Approximately 50% of eukaryotic cellular proteins are thought to have naturally occurring N-terminal blockage due to posttranslational modification, and in addition many reagents commonly used in protein sequence purification procedures can also modify N-terminal amino groups. These modifications make N-terminal sequence determination of the intact protein impossible and the ability to sequence these proteins is an important improvement in protein analysis. In the absence of an adequate remedy, internal sequence analysis is the only alternative. This requires chemical cleavage using cyanogen bromide or enzymatic digestion procedures and separation of the resulting peptides. Different strategies to obtain internal sequence from proteins separated on 1D or 2D gels, have been reported in the literature. Proteins were either electroblotted onto PVDF membrane and digested *in situ* , and the resulting peptides were separated by HPLC followed by automated sequencing [Aebersold *et al.*, 1987] or proteins can be digested *in situ* in the polyacrylamide gel matrix following staining and destaining and the resulting peptides are either separated by HPLC and sequenced (each peptide should analysed individually in an automated microsequencer) or separated on another 1D gel followed by immunoblotting on onto PVDF and direct sequencing of the bands. For many applications described in the literature, trypsin was the enzyme of choice for the digestion of proteins. However, chymotrypsin, subtilisin, and the endoproteinases Lys-C, Asp-N and Glu-C can work equally well [Houmard *et al.*, 1972; Brown and Wold 1973; Darpeau, 1980]. For all of the above application a standard protein, such as β-lactoglobulin or cytochrome c, should be analysed as a control.

4. 2 Determination of the N-terminal sequence of glycoprotein IV electroblotted onto polyvinylidene difluoride membranes

Glycoprotein IV eluted from a Con A-agarose affinity column was freeze-dried and redissolved in a minimum volume of distilled water. A SDS polyacrylamide gel containing 9% acrylamide was poured with a single slot right across the top of the stacking gel. The sample, containing about 250μg protein, was mixed with 4x-sample buffer and loaded into the single sample well. Electrophoresis was carried out as described before in section 2.4. After electrophoresis the gel was soaked in transfer buffer for 5 minutes to reduce the amount of Tris and glycine. During this time a PVDF membrane was rinsed with 100% methanol and stored in transfer buffer for 2-3 minutes. The proteins were then electrophoretically transferred to it as described before. The PVDF membrane containing bound protein was washed in deionized water for 5 minutes and blocked with 1x TBS/0.05% Tween 20 for 1 hour. About 1cm from each side of the PVDF membrane was cut off and the main body of the blot was kept in blocking buffer at 4°C. The side strips were decorated with Con A-HRP complex as described before in section 2.6.3 to locate the band of glycoprotein IV.

After the bands had been localized on the side strips, these were aligned with the untreated blot to determine the position of glycoprotein IV on the main body of the blot. The glycoprotein IV band was then cut out with a clean razor, rinsed in deionized water for 5-10 minutes, air dried and subjected to Edman sequencing. This sequencing was performed by Dr Ruth Hogue-Angeletti, University of New York.

4.3 Enzymatic digestion in solution

10 ml of glycoprotein IV eluted from the Con A-agarose column was reduced to about 1-2ml using an Amicon ultrafiltration apparatus with a PM-30 membrane. The sample (250 μl) containing about 300μg protein was divided into 5 portions (50μl each). The samples were incubated with Glu-C (V8) proteinase at various concentrations (0.0, 0.04, 0.2, 1 or 5μg total) at 37°C for 30 minutes.

Following digestion the samples were diluted with 4x sample buffer and immediately analysed by electrophoresis on a12% SDS polyacrylamide gel followed by blotting onto a nitro-cellulose membrane. The peptides generated from digestion of glycoprotein IV were localized with Con A and HRP. After analysis of this Westarn blot, the reaction mixture containing 1μg of V8 proteinase appeared to ahow the optimum digestion conditions and so a preparative 12 % SDS gel with a single-slot comb was set up 500μl of protein digested with 1μg of V8 proteinase.

Electrophoresis and blotting onto PVDF membrane were performed and the position of the band of interest was determined as described in section 4.1. After the peptides had been localized on side strips, these were aligned with the untreated blot and the strip of membrane aligend with them was cut from the blot. The PVDF membrane strip was washed with deionized water, air dried and sequenced immediately.

4.4 Fragmentation of intact glycoprotein IV bound to PVDF membrane

Protein was electrophoretically transferred to PVDF membranes, and the position of glycoprotein IV was determined on the side strips exactly as described as above, and the glycoprotein IV band excised from the main body of the blot. The PVDF membrane strip was washed 2-3 times with water, cut into approximately 1x1-mm squares which were placed in an Eppendorf tube and subjected to enzymatic digestion or cleavage with cyanogen bromide.

4.4.1 Enzymatic digestion using endoproteinase Asp-N

The proteolytic digestion of membrane-bound proteins was carried out using endoproteinase Asp-N [Fernandez *et al.*, 1992]. 200μl of 1% RTX-100 (hydrogenated Triton X-100)/10% acetonitrile/100mM Tris-HCl, pH 8.0 (digestion buffer), was added to the strips, followed by 20 μl of the proteolytic enzyme. All digestions were carried out at 37°C for 16 hours, accompanied by a control digestion composed of 50 μl digestion buffer, 2μl of proleolytic enzyme and a few PVDF strips. Following digestion, samples were sonicated for 5 minutes and then centrifuged at 1700 r.p.m. for 5 minutes.

The supernatant was transferred to another Eppendorf tube. The centrifuged strips were washed again with about 100µl of digestion buffer, sonicated and centrifuged again as above. Consecutive washes with 100 µl of digestion buffer (1x) and 0.1% TFA (trifluoroacetic acid) were performed with sonication and centrifugation as described above. All supernatants were pooled to give a total volume of about 500 µl. Both digested and control samples were reduced to 100µl using a Speed-Vac rotary drier prior to reversed-phase HPLC analysis to separate peptides before subjecting them to sequencing. The eluted peptide-containing fractions were collected manually into Eppendorf tubes based on their absorption at 220nm. The large peptide volume was reduced using a Speed-Vac rotary drier. The peptide-containing fractions were frozen immediately until sequencing

4.4.2 Chemical cleavage using cyanogen bromide

400µl of 10 mg/ml cyanogen bromide solution (10 mg /ml CNBr in 70% (w/v) formic acid) were added to the strips of PVDF membrane attached protein (section 4.4). The tube was covered with aluminum foil and kept in the dark at room temperature for about 16 hours accompanied by a control digestion composed of 50µl of cyanogen bromide and some PVDF membrane. After digestion the samples were centrifuged at 1700 r.p.m. for 5 minutes, and the supernatants were transferred to Eppendorf tubes. The supernatants were dried by evaporating the formic acid under nitrogen. The dry samples were wetted with water and dried as in the previous step. The digestion mixtures were completely dried in a Speed-Vac, solubilized in 6M guanidine-HCl, 0.1M Tris, pH8.5, 0.1M DTT and injected into a reverse-phase column immediately. The eluted fractions were collected manually into Eppendorf tubes based on their absorption at 220nm. The peptide-containing fractions were frozen immediately until sequencing

4.5 Preparation of pre-run SDS-polyacrylamide electrophoresis gels

To prevent the N-terminus of glycoprotein IV from becoming blocked, polyacrylamide gels for this sequencing work were pre-run with a free radical scavenger prior to electrophoresis of the proteins, according to Dunbar *et al,* (1994). Both the separating and stacking gels were loaded in a 0.75 mm gel cassette using 0.375M Tris-HCl buffer pH 8.8 containing 0.1% (w/v) SDS (separating buffer) the Laemmli stacking gel buffer being replaced by separating gel buffer. Prerunning was carried out using the Laemmli stacking gel buffer (0.125M Tris-HCl pH 6.5, 0.1%(w/v) SDS), 5mM reduced gluthathione was used as a free radical scavenger in the upper reservoir (cathode) and 0.375 M Tris-HCl buffer pH 8.8, 0.1%(w/v) SDS (separating buffer) in the lower reservoir (anode), for 2h at 10 milliamp. The progress of the prerun was monitored by placing 50µl of sample buffer in a single sample well. The prerun was terminated when the tracking dye in the sample well had reached the bottom of the gel. The sample was then loaded onto the prerun gel, and the electrophoresis was carried out at 20 milliamp using Laemmli electrode buffer in both reservoirs. Glycoprotein IV was detected by staining with Coomassie Blue R for 1 hour and destaining until the background was clear. After the localization of glycoprotein IV on the gel, the protein band was cut out and prepared for sequencing.

4.5.1 Preparation of the SDS polyacrylamide gel for protein sequencing

The piece of gel containing glycoprotein IV was crushed and 400 µl of 100 mM NH_4HCO_3 was added. After addition of 0.1% SDS the sample was incubated for 24 hours before 0.6 ml of water was added, and then a 100 mm^2 piece of PVDF which had been wetted with methanol was added too. This membrane was then washed with water, dried and cut into small pieces (about 30-40 mm^2) before being subjected to N-terminal protein sequencing by Edman degradation in a 473A gas-liquid protein sequentor (Applied Biosystems).

Identical pieces of membrane were also treated with 50µl of 80% HCOOH for 16 hours at 4°C in order to specifically hydrolyse aspartate-proline bonds (D-P bonds). Upon vacuum-removal of HCOOH the proteins rebind to the membrane which can be used for sequencing. Cytochrome b from bovine heart, which is present in N-formylated form (von Jagow *et al.*, 1978) and contains a single D-P bonds was used as a control (Anderson *et al.*, 1982).

4. 6 Results and discussion

One of the aims of this work was to obtain the N-terminal amino acid sequence of membrane glycoprotein IV. Glycoprotein IV was purified from bovine chromaffin granule membranes by fractionation of membrane proteins with Triton X-114 and Con-A agarose column chromatography as described before in Chapter 3. This purification procedure provided a protein pure enough to be subjected to the Edman sequencing procedure. After SDS-PAGE, glycoprotein IV was electroblotted onto polyvinylidene difluoride membrane and Figure 4.1 shows the position of glycoprotein IV on both side strips of the blot. The piece of PVDF containing glycoprotein IV was excised from the main body of the blot and subjected to10 cycles of sequence analysis in a gas-phase sequenator. The N-terminal sequence of Con A lectin (ADTIVAVELDTYP) [Cunningham *et al.*, 1975] was obtained from this work, suggesting that the N-terminus of glycoprotein IV was blocked during the purification procedure and that it was contaminated with this lectin. During protein sequencing it is not uncommon to find that a protein is blocked at its amino terminus. In an attempt to remove the block, glycoprotein IV was subjected to proteolytic digestion using endopeptidase Glu-C (V8) to generate an internal peptide that could be sequenced. Glycoprotein IV eluted from the Con A column was digested with endopeptidase Glu-C (V8) and the resulting peptides were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis followed by blotting onto polyvinylidene membrane. The peptides were visualised by decoration of the blot with Con A and HRP.

Figure 4.2 shows the Con A blot of peptides produced by digestion of glycoprotein IV with V8 proteinase. The peptide of about 35kDa produced by this digestion (indicated by arrows), was of interest for sequencing because it was produced consistently and in larger quantities than the other digestion products making it easier to analyse by N-terminal sequencing. In addition, it was necessary to choose a product with a greater molecular weight than Con A (29kDa) as this would ensure that the product was derived from GpIV rather than Con A.

PVDF membrane with bound peptide was prepared as described above and subjected to the sequencing procedure. Once again no sequence was obtained from this peptide, suggesting either that the peptide still carried the N-terminal blocking group of glycoprotein IV or that it had been re-blocked after cleavage or blotting. Usually this fact can be discovered only after the sample has been committed to sequencing . After obtaining this result different procedures were considered, in order to obtain sequence information for glycoprotein IV. If more peptides could be generated, these could be separated by reversed-phase HPLC, and analyzed in the automated microsequencer. Chemical fragmentation of glycoprotein IV using cyanogen bromide as well as enzymatic digestion with endoproteinase Asp-N were performed on the immobilized glycoprotein IV on PVDF membrane, after the glycoprotein IV had been subjected to a further purification step. The protein eluted from Con A column was further purified by electroelution from SDS-polyacrylamide gel and this more highly purified form of the protein was subjected to electrophoresis and immunoblotting onto PVDF membrane and then digested as described before. The eluted peptides produced from the digestion of protein on PVDF were directly isolated by reversed-phase HPLC as shown on Figure 3.4a. Also the control sample run alongside of digestion reaction was analysed with HPLC in order to avoid any background peaks resulting from autodigestion of the protease or from PVDF membrane as shown in Figure 4.3b.

After a comparison of the HPLC profiles (Figures 4.3a and 3.4b) resulting from the two proteolytic digestions, fractions corresponding to peaks number 14 and 15 were chosen for sequencing. Since these peaks were absent from the profile of the control sample of these fractions must containing peptides produced from GpIV by digestion with endoproteinase Asp-N. Once again the fractions containing these peptides failed to give any sequencing information. Generally a peptide peak with an absorbance of 0.01-0.02 should be easily sequenceable but in this case the procedure failed. After the intact glycoprotein IV was fragmented with cyanogen bromide as described above, the eluted peptides were directly separated by reverse-phase HPLC and sequences immediately.

CNBr cleaves specifically after methionine (Met) residues and because Met is a relatively infrequent amino acid (ca 2%) fragments tend to be larger and fewer than proteolytic fragments. Figure 4.4a shows the HPLC profile of glycoprotein IV chemically cleaved with cyanogen bromide which contains fewer peaks (peptides) than the proteolytic HPLC profile (Figure 4.3a) as expected. Also the control sample run alongside of this cleavage reaction was analysed with HPLC in order to avoid any background peaks Figure 4.4b.

After a comparison of the HPLC profiles (Figures 4.4a and 4.4b) resulting from cleavage of GpIV with CNBr, fractions corresponding to peaks number 2 and 5 were chosen for sequencing. Because these peaks were absent from the profile of the HPLC control sample they must contain peptides produced from GpIV by cleavage with CNBr. These fractions were subjected to sequencing. While this procedure was successfully used to deblock and sequence the amino-terminal of the proteolipid subunit of proton translocating ATPase of bovine chromaffin granules membrane [Mandel *et al.*, 1988], application of this procedure to the sequencing of glycoprotein IV was unsuccessful. The objective of the above methods was the generation and isolation of fragments for internal amino acid sequencing and since no sequence was obtained, this may attributed to various reasons, possibly due to some adsorption of the peptide on the HPLC column which would reduce the effective concentration of the peptide submitted to amino acid sequencing or the peptides may lost during dring down the volume of peptides. In the above both experiments, generally the overall recovery of peptides amounted to 65% of the protein initially present on the PVDF and this amount represents 50-80% of the amount of the initial sample on the gel. The amount of protein that is needed for sequencing is typically 30-60% of the amount of the sample that is loaded onto the gel. However after the above two trials for sequencing of GpIV by obtaining internal peptides, it was decided to avoid using the electroblotting system from gel to PVDF membrane as in many cases blockage is attributed to impurities in the gel which can only be eliminated by electrophoresing the gel prior to loading the sample and by including antioxidants such as thioglycolate.

Finally the sample of glycoprotein IV purified by electroelution from SDS-polyacrylamide gels was subjected to electrophoresis on a prerun one-dimensional SDS-gel to avoid any reactive by-products of acrylamide polymerization. Following staining with Coomassie Blue and destaining, a protein band was excised from the gel and then prepared for direct N-terminal sequencing as described before. After Edman degradation was performed on a 473 gas-liquid protein sequencer (Applied Biosystems), the aminoacid sequence VPPTMNVP was obtained. This eight amino acid sequence for the N-terminal of glycoprotein IV was found to be an internal sequence within the open reading frame of Ac45 at the position 247-254 as shown in Figure 4.5 (see also Figure 5.11). All the sequencing results obtained from the above experiments are summarised in Table 4.1.

The amino acid sequence of Ac45, claimed to be a novel subunit of vacuolar H^+-ATPase of chromaffin granule membranes, was published by Supek *et al.*, 1994. This protein was first observed as a diffuse band between subunits B and C at a position corresponding to the mass of 45kDa when purified V-ATPase from chromaffin granules membranes was analyzed on polyacrylamide SDS gels. After proteolytic digestion a short internal aminoacid sequence was obtained, and the cDNA was then cloned. The DNA-deduced sequence of Ac45 encodes an open reading frame for a protein of 486 amino acids with a molecular mass of 52kDa. Supek *et al.,* (1994) also suggested that this sequence included a potential N-terminal signal sequence of 35 amino acids, removal of which would leave a mature protein with a molecular mass of about 48kDa. The hydropathy plot indicates the presence of a potential transmembrane helix at the C-terminal membrane, and the sequence also contains seven potential N-glycosylation sites.

Ac45 was postulated to be a subunit of the H^+-ATPase, on the basis of its co-sedimentation through a glycerol density gradient with other H^+-ATPase subunits and was suggested to be part of the membrane sector (V_o) of the enzyme. This polypeptide is present in similar amounts to other subunits, and there is at least one copy of Ac45 per V-ATPase complex [Supek *et al.*, 1994].

Glycoprotein IV was confirmed to be an H^+-ATPase subunit using Blue-Native polyacrylamide gel electrophoresis followed by SDS-tricine gel electrophoresis and immunoblotting using anti-glycoprotein IV serum even before obtaining amino acid terminal sequence, as will be described in Chapter 6, the characterization of glycoprotein IV. The N-terminal amino acid sequence of glycoprotein IV (VPPTMNVP) was confirmed by different approach: Ac45 was prepared by blue native polyacrylamide gel electrophoresis to isolate the V_o sector of ATPase and subsequent SDS-gel electrophoresis to separate the subunits, which were then sequenced. This work was performed by Hermann Schägger [Getlawi *et al* ., 1996] and confirmed that glycoprotein IV and Ac45 are the same related protein.

Within the structure of Ac45 there are seven potential glycosylation sites, one positioned very close to the transmembrane helix at the C-terminal of the protein. It was suggested [Supek *et al.*, 1994] that the protein is synthesized with an N-terminal signal sequence which would direct the protein into the ER and after cleavage and glycosylation, the protein is attached to the membrane by the C-terminal helix leaving the glycosylation sites on the lumenal site of the membrane.

When the cDNA encoding Ac45 was transcribed and translated *in vitro*, a product of about 50kDa was obtained. If dog pancreas microsomes were included in the incubation, the product was even larger, but reduced to its original mobility when treated with endoglycosidase F. The glycosylated protein had been translocated into endoplasmic reticulum vesicles since it was protected from proteolytic digestion. [Supek *et al.*, 1994]. These results are consistent with cotranslational translocation, but rather suggest that the signal sequence had not been removed. It is not clear how these authers identified the potential signal sequence as there is no apparent reason for proposing a cleavage site between A35 and A36 (Figure 4.5) furthermore the proposed signals sequence contains 3 arginines (R11, R15, R22) which would make it much more hydrophilic than is usual in a signal sequence. Deglycosylation of Ac45 [Supek *et al.*, 1994] or GpIV from purified V-ATPase produced a smaller polypeptide than would be expected from the full Ac 45 sequence (as will be discussed later under deglycosylation of glycoprotein IV), but which would be

consistent with cleavage between amino acids 246 and 247. This suggests a specialised post-translational processing which may determine the assembly of the enzyme in various organelles.

The results obtained from N-terminal sequencing of glycoprotein IV and deglycosylation of glycoprotein IV suggest that Ac45 (now termed M45) is further processed by cleavage between two valines (residues 246 and 247 Figure 4.5) to a mature protein of only 222 amino acids. This cleavage may occur in the Golgi, or even the granule. Presumably the apparent molecular mass of 45-50 kDa of this fragment is due to glycosylation (see Chapter 6). Proteolytic degradation during purification can be excluded since the bands were transferred directly from the SDS gel to PVDF membranes, and then sequenced. Also the acidolytic cleavage with 80% formic acid should have cleaved a single D-P bond present in Ac45 at the position of 77-78 amino acids and in the fragment a newly generated N-terminal sequence PALELGPR (residue 77-84) should then be observed. No such sequence was obtained. In a control experiment with cytochrome b from bovine heart, similarly prepared, cleavage of a D-P bond was observed, but on treatment of Ac45 in this way no cleavage was observed which thus confirmed the absence of this bond (D77 and P78) from mature Ac45/GpIV.

Table 4.1

Purification step	Treatment	Sample for sequencing	Result
1. Con A column chromatography	-	PVDF-bound	N-terminal sequence of con A
2. Con A column chromatography	digestion of protein with V8 proteinase in solution	PVDF-bound	No sequence
3. Con A column chromatography + electroelution from SDS gels	Digestion of Gp IV with endoproteinase Asp-N on protein bound to PVDF	Peptides produced from proteolytic digestion were separated by HPLC	No sequence
4. Con A column chromatography + electroelution from SDS gels	Chemical cleavage of protein bound to PVDF with CNBr	Peptides produced from chemical cleavage were separated by HPLC	No sequence
5. Con A column chromatography + electroelution from SDS gels	pre-run gel	Piece of gel stained with Coomassie Blue	N-terminal of Gp IV was obtained

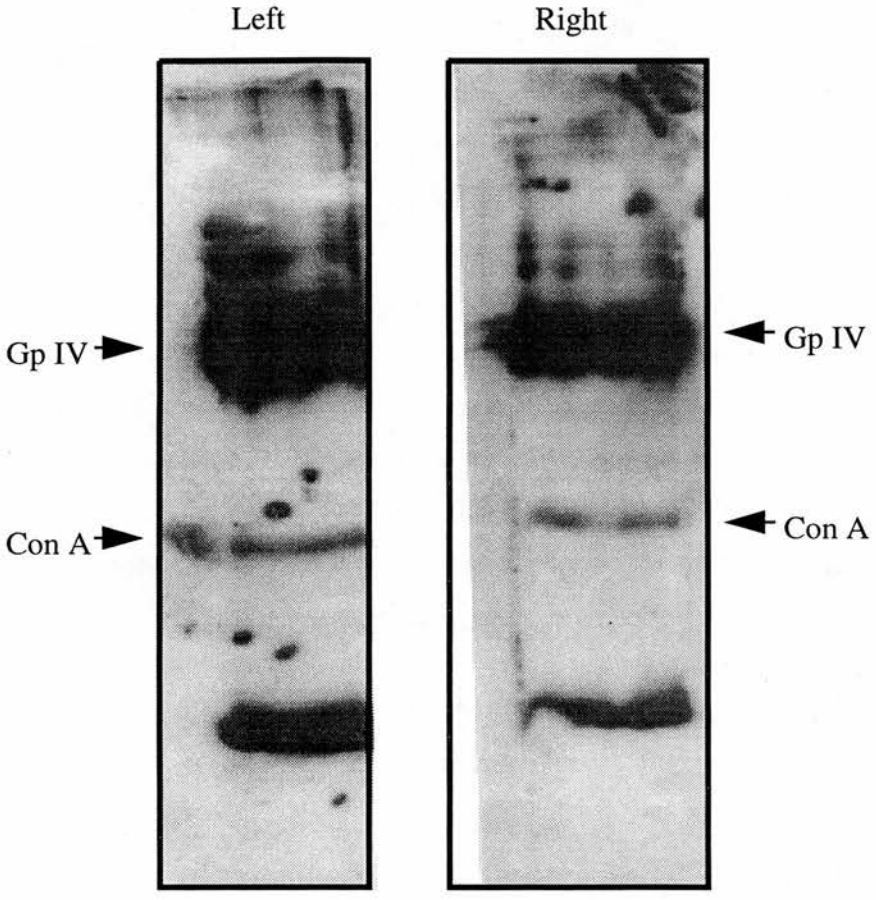


Figure 4.1 Glycoprotein IV was purified by Con A-agarose column chromatography ,then a sample was subjected to blotting into a PVDF membrane. Both sides of the blot were detected with Con A-HRP complex followed by ECL

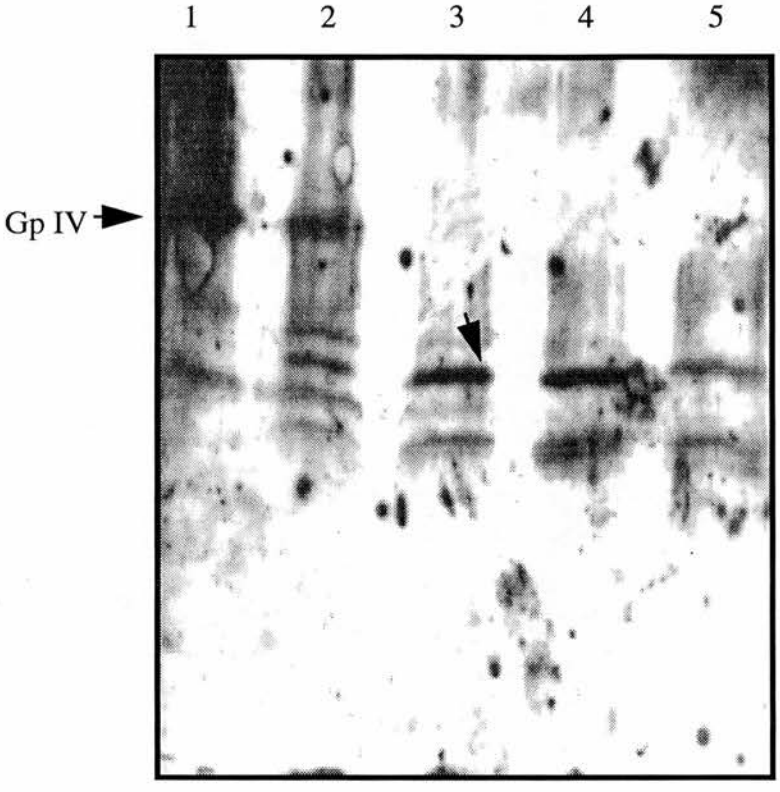


Figure 4.2 Gp IV purified by Con A-agarose column chromatography were incubated with 0-5µg of Glu-C (V8) endoproteinase as described in Section 4.3. The peptides generated from enzymatic digestion were analyzed by immunoblotting using Con A and HRP.
Lane 1: 0.00µg
Lane 2: 0.04µg
Lane 3: 0.20µg
Lane 4: 1.00µg
Lane 5: 5.00µg
The peptide chosen for sequencing is indicated by an arrow (lane 3).

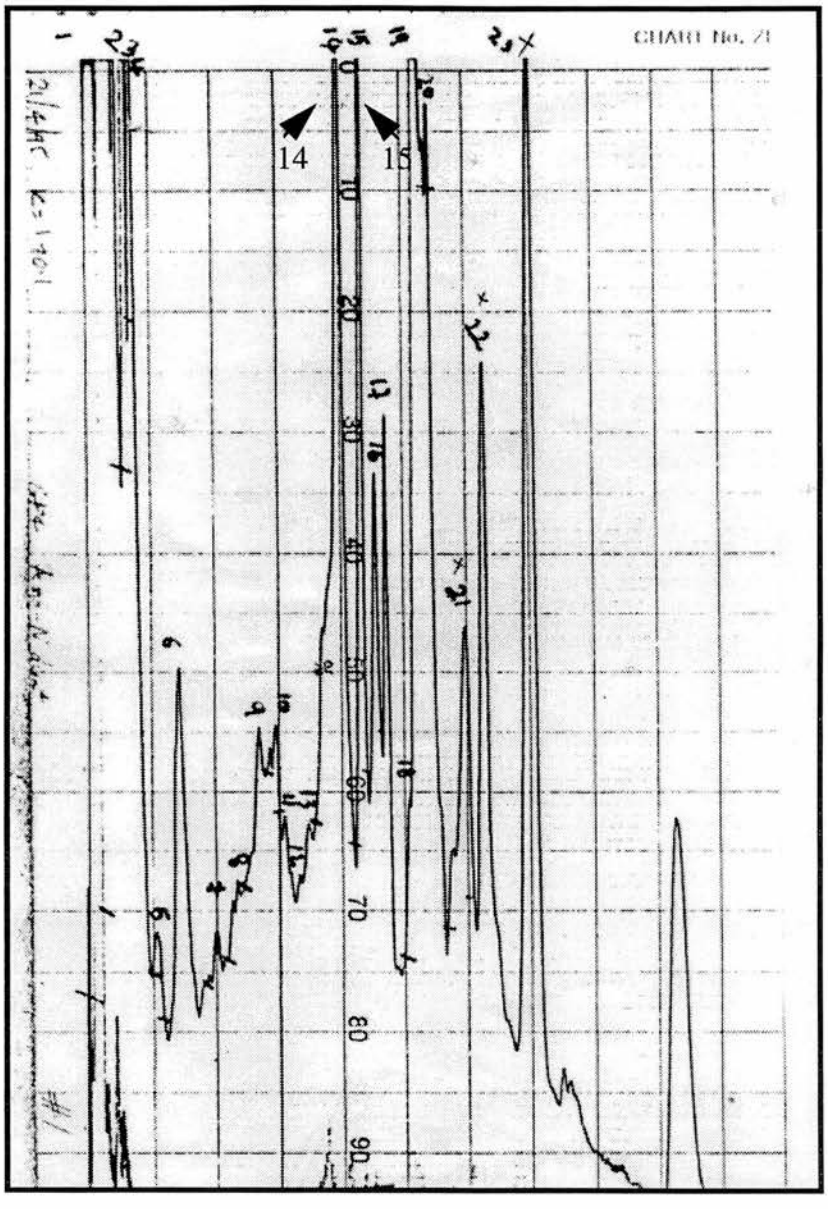


Figure 4.3 a Glycoprotein IV was digested with endoproteinase Asp-N and the generated peptides were separated by HPLC.
The peptides (14 and,15) chosen for sequencing are indicated by arrows.

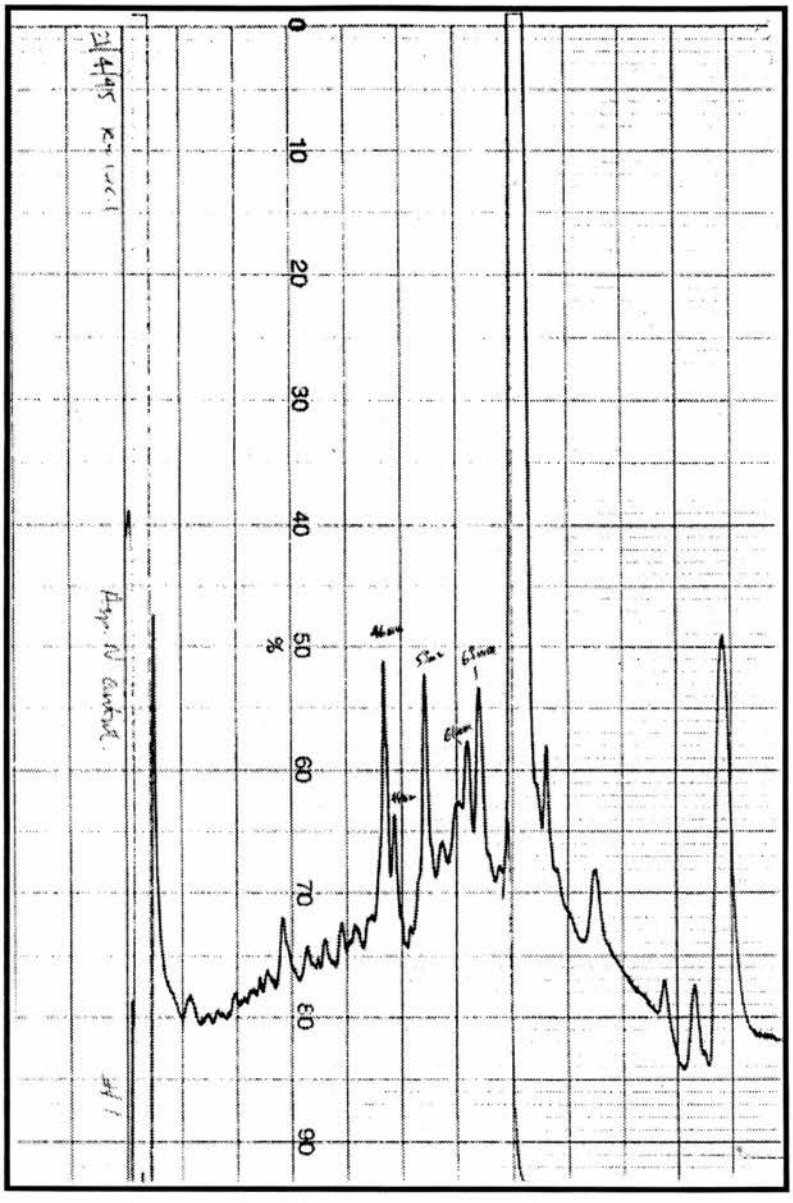


Figure 4.3b The control sample for the digestion of glycoprotein IV by endoproteinase Asp-N was also subjected to separation by HPLC.

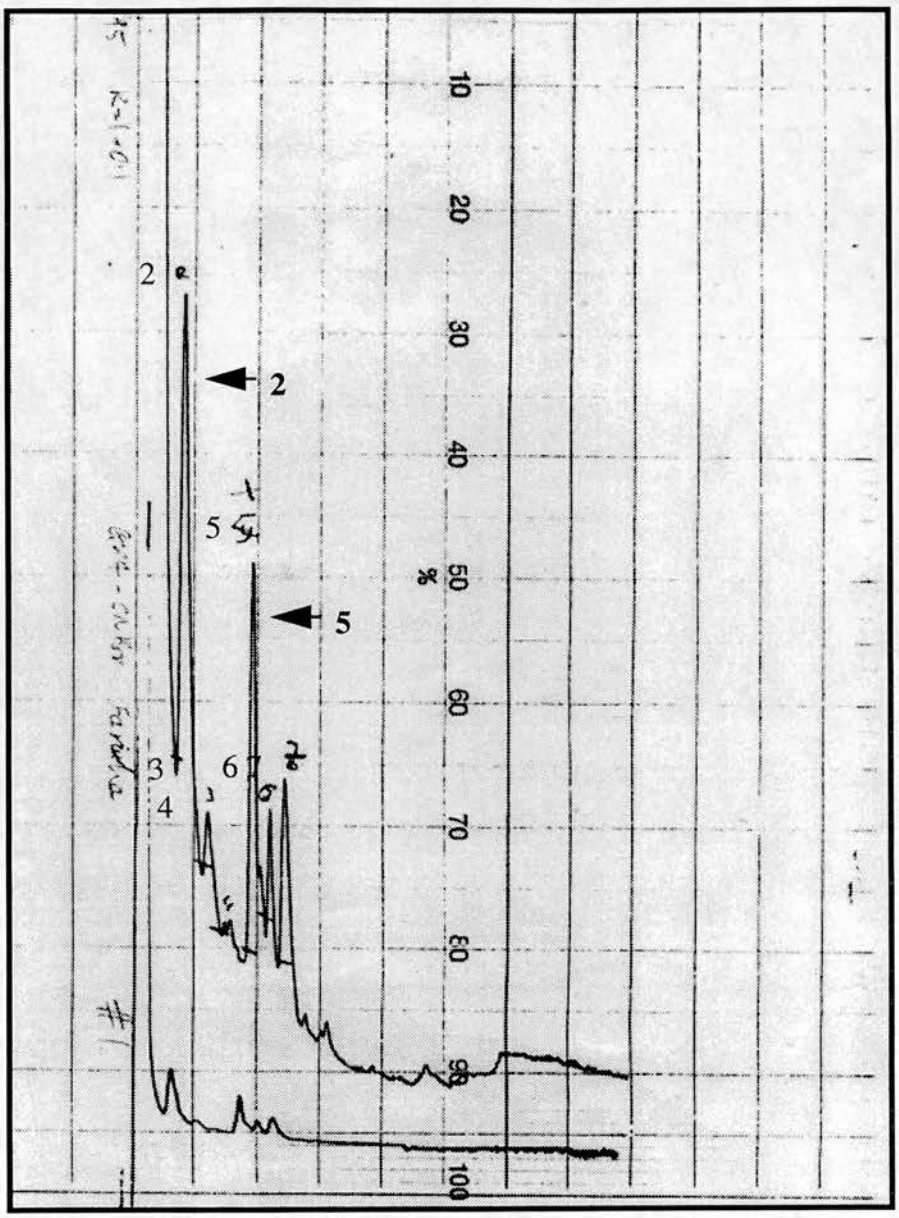


Figure 4.4a Glycoprotein IV was cleaved with CNBr and the generated peptides were separated by HPLC.
The peptides (2 and 5) chosen for sequencing are indicated by arrows.

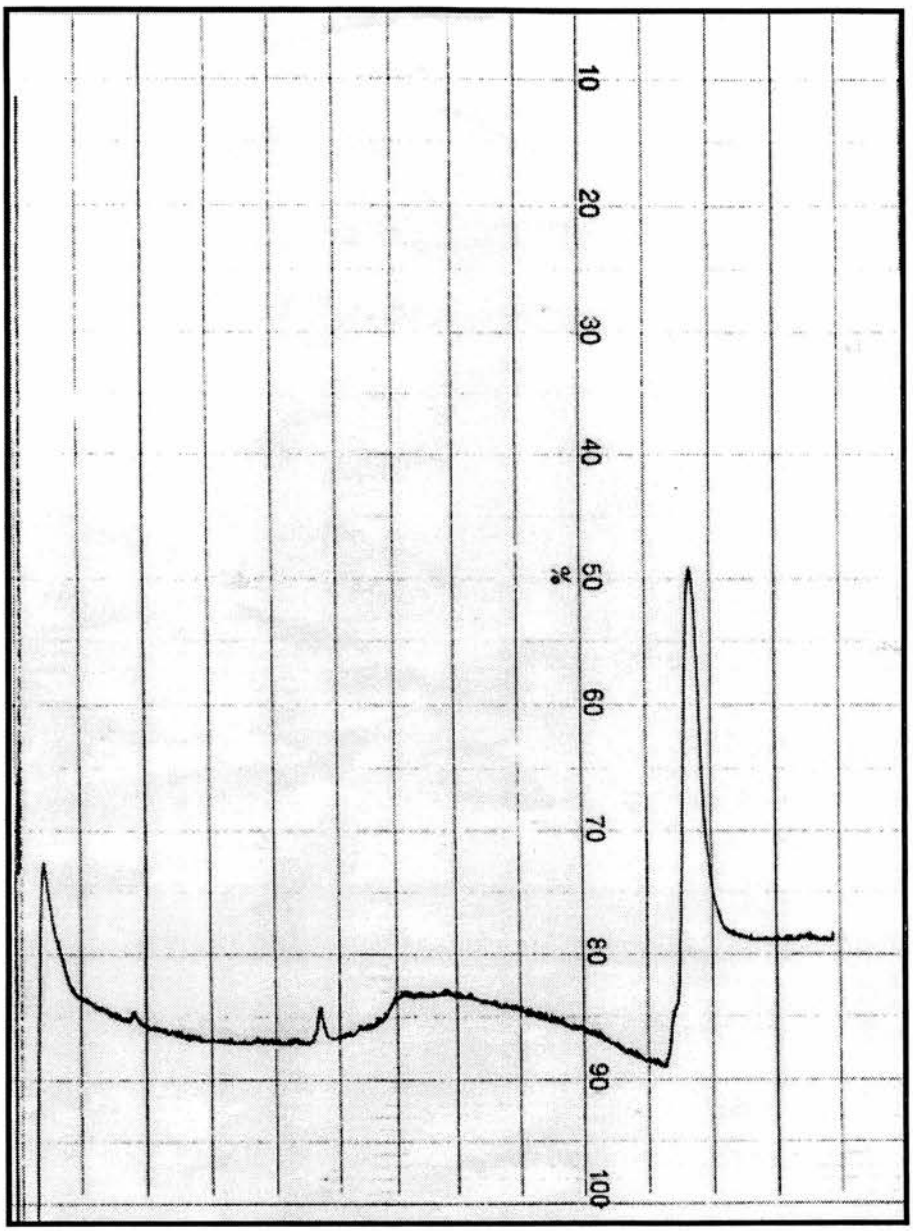
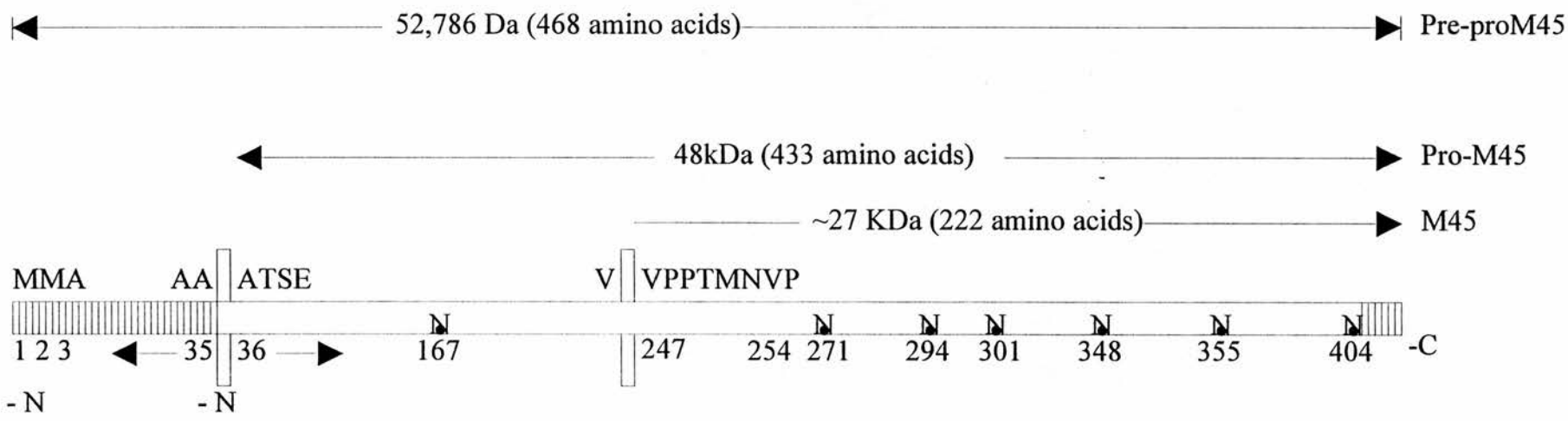


Figure 4.4b. The control sample for the cleavage of glycoprotein IV was subjected to separation by HPLC.

Figure 4.5 Diagram showing the relationshipbetween GpIV and its proposed precursor



N1, N35 Shadows area show the N-terminal signal sequence of pre-pro M45.
N Shows the position of glycosylation sites
-C Shadows area shows the C terminal.

Chapter 5
Production and characterization of antibodies

5.1 Introduction

Antibodies are products of the immune system of higher eukaryotes, their role being to tag foreign molecules and initiate their specific degradation by the phagocytic cells. Their specific affinity for almost any type of biological molecule has made them indispensable tools in biological research because they provide probes for the molecule of interest and allow its detection against a complex background of other molecules. The usual methods for eliciting antibodies involve immunization with purified antigen preparations. Antigens used are most commonly proteins or peptides and small organic molecules (haptens) can also be used after conjugating them to appropriate protein carriers. Polyclonal antibodies are the total population of antibodies present in the serum of the immunized animal. This complex population contains different antibody subclasses including IgG, IgM, IgE, IgA and IgD. Each antibody represents the secretory product from a single stimulated lymphocyte and its clonal progeny. Polyclonal antibodies are particularly valuable for immunoprecipation and immunoblotting. The introduction of monoclonal antibody technology by Köhler and Milstein in 1975 greatly accelerated the successful application of antobodies in the investigation of biological systems. The major advantages and disadvantages of polyclonal and monoclonal antibodies are outlined in Table 5.1.

The membranes of chromaffin granules contain several major glycoproteins, including the glycoproteins termed I-V and glycoproteins H, J and K. Glycoprotein IV is an uncharacterised protein of unknown function, and a specific antibodies were required for studying this protein. Con A is a convenient ligand for isolating glycoprotein IV by affinity chromatography but it is not specific because it binds to many other glycoproteins in chromaffin granule membranes (Gavine *et al.,* 1984) , and of different tissues. Because pure proteins are required for the production of antibodies, glycoprotein IV was purified by fractionation of chromaffin granule membrane proteins with Triton X-114 followed by chromatography on Con A - agarose then electroeluted from SDS-polyacrylamide gels as described in chapter 3.

Proteins from different purification steps were used to immunize mice and/or rabbits in order to produce monoclonal and polyclonal antibodies directed against glycoprotein IV.

The cloning and sequence determination of genes have greatly increased our knowledge of the structure of proteins and suggested mechanisms by which some are synthesized, processed and transported. However there is a gap between the ease with which a gene can be cloned and sequenced and the unequivocal assignment to a protein product. A solution to this problem was offered by the demonstration that one could produce antibodies to a few chemically-synthesized peptides predicted from newly-solved nucleotide sequences and then use these antibodies to define the product of the gene in question. The most important feature of antibodies made in this way is that they are directed against a small region of the protein, determined in advanced by the investigator. Synthetic peptides have been used also to prepare antibodies specific for previously uncharaterized proteins [Sutcliffe *et al.*, 1980; Walter *et al.*, 1986; Lerner, 1984]. Peptides are normally synthesized using solid-phase techniques [Merrifield *et al.*, 1963]. The synthetic peptides are incomplete immunogens but can be made more immunogenic by coupling them to a suitable carrier protein to form a conjugate proteins which can then be used to immunize animals. In these cases, the peptides serve as epitopes for binding to the antibodies on the B-cell surface, and the carriers provide the class II-T-cell receptor binding sites. In general, peptides are coupled to soluble carriers, the most commonly used carriers being bovine serum albumin (BSA; 67kDa), keyhole limpet haemocyanin (KLH; 4.5×10^5 to 1.3×10^7 Da) and ovalbumin (OVA; 45kDa). KLH is useful because of its large molecular mass, strong immunogenicity and many available lysines. KLH is a copper-containing protein belonging to a group of non-haem proteins called hemocyanins, which are found in arthropods and mollucs. KLH is isolated from the mollusc *Megathura crenulata*, and unlike other gastropod hemocyanins, this protein does not dissociate on removing divalent cations although divalent cations aid the formation of larger aggregates. The protein exists as 5 different aggregate states in Tris buffer, pH 7.4.

It will reversibly dissociate into subunits with moderate changes in pH and at pH 8.9 it is completely dissociated. Removal of oxygen will also dissociate this protein. Increased antibody binding can be expected when it is dissociated to its subunits because of improved availability of antigenic sites [Senozan *et al.*, 1981].

The coupling mechanism will vary with different peptides. Peptide-carrier conjugates seldom fail to elicit a response because of tolerance. Consequently, the peptide can usually be seen as epitopes, and high-titered antisera are obtained. Characteristically, these antibodies bind well to denatured proteins, but may or may not recognize the native proteins. The fraction of antipeptide antibodies that will bind to the native protein will vary from antigen to antigen. The two most important advantages of anti-peptide antibodies are that they can be prepared immediately after determining the aminoacid sequence of a protein and that particular regions of a protein can be targeted specifically for application in molecular biology. Similarly, the production of site-specific antibodies has immediate implications for functional or clinical studies.

A novel accessory subunit for vacuolar H^+-ATPase from bovine chromaffin granules membrane named Ac45 has been described [Supek *et al.*, 1994]. The DNA- deduced amino acid sequence of Ac45 subunit includes an open reading-frame for a protein of 468 amino acids with a calculated molecular mass of 52kDa. In the present work, chromaffin granule membrane glycoprotein IV was purified and the first 8 residues of its N-terminal sequence were determined . This sequence appeared as an internal sequence (amino acids 247-254, as shown in Figure 4.5) within the published Ac45 sequence. To determine whether Ac45 and glycoprotein IV are identical or are related proteins an anti-peptide antibody was raised. A conjugate was made from a selected peptide from the published sequence of Ac45 and coupled to maleimide-activated keyhole limpet haemocyanin. The conjugate was purified and then was used to produce polyclonal antiserum in rabbits.

Table 5.1

Advantages and disadvantages of polyclonal antibodies

Polyclonal antibodies	
Major Advantages	1-Multiple subclasses and high-affinity antibodies are present in antibody population 2-Multiple specificities of antibodies are likely to recognize sequential as well as conformational antigenic determinants 3-Antibodies should recognize multiple determinants specific for a protein 4-Amenable to experiments involving immunoprecipitation and immunoblotting
Major disadvantages	1-Immunogen must be highly purified to obtain desired specificity 2-Individual domains of complex are difficult to study because multiple antigenic determinants are recognized by the polyclonal antisera 3-Quantities of antibodies are limited to the immunized animal 4-Different bleedings have to be characterized individually due to changes in antibody affinity, specificity, and subclass.

Table 5.1 (continued)

Advantages and disadvantages of monoclonal antibodies.

Monoclonal antibodies	
Major Advantages	1- Single homogeneous antibody to a defined antigenic determinant 2- specific antibody can be used to study functional domain 3- large quantities of antibody can be obtained since immortal cell lines can theoretically be developed 4- Antibodies with low affinity can be selected during screening procedure
Major disadvantages	1-Procedure is expensive and time consuming 2- Well-equipped tissue culture facilities are needed 3-Epitope recognized by antibody may be shared among many different antigens not related to antigen of interest 4-Hybridoma cell lines are frequently unstable due to chromosome loss or may be lost because of tissue culture contamination.

5.2 Production of a monoclonal antibodies

5.2.1 Immunisation protocol

Samples containing 100 μg antigen (P1 fraction or glycoprotein IV purified by Con-A column chromatography) in Freund's complete adjuvant were injected subcutaneously into mice (6-10 week old). Four weeks after the first immunisation, a similar dose of the antigen was injected intraperitoneally and this was repeated up to twice more at four-weekly intervals. Three days before the fusion, the mice were immunised once more with 100μg of the antigen intravenously.

5.2.2 Media and conditions for culturing myelomas and hybridomas

Myeloma and hybridoma cell lines were cultured in RPM1-1640 media with 25 mM Hepes-NaOH (pH 7.2) supplemented with 0.1 mM hypoxanthine, 16 μM thymidine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM $NaHCO_3$, 0.1 mM β-mercaptoethanol and 15% (v/v) myoclone foetal calf serum (RPM1/HT/FCS). This medium was further supplemented with 0.5 μM aminopterin for the culture and selection of hybridomas for at least 1 week after the fusion. Both myeloma and hybridoma cell lines were cultured at 37 °C in a sterile incubator containing 5% (v/v) CO_2 at100% humidity.

5.2.3 Fusion and cell culture protocols

Cell fusion and cloning were carried out by Dr. Jeff Haywood (Biochemistry Department). The spleen of an immunised mouse was removed under sterile conditions and disrupted into a cell suspension in serum-free RPM1/HT media equilibrated to 37 °C according to Bestin *et al* (1982). Cells were washed twice by centrifugation at 500 g_{av} for 5 minutes at room temperature and finally resuspended in 15 ml of the same medium. Myeloma cells (NS-O) were cultured in RPM1/HT media containing 15% foetal calf serum and passaged into two 75 cm^2 flasks approximately 40 hours before the fusion. NS-O cells were harvested in log growth and washed as above and resuspended in 5 ml serum-free RPM1/HT medium

equilibrated to 37°C. The lymphocyte and the NS-O concentrations were determined using a haemocytometer. The NS-O culture supernatant was retained and mixed with a suspension of thymus (feeder T-cells) cells obtained from a 6-8 week old non-immune female mouse. The NS-O and the spleen cells were mixed in a ratio of 1:5 and the mixture centrifuged at 500 g_{av} for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in 1ml sterile 46 % polyethylene glycol (Hepes buffered PEG-1500, equilibrated to 37°C) over 30 seconds whilst gently agitating. The PEG was diluted using serum- free RPM1/HT medium 90 seconds after the start of the fusion, initially at a rate of 0.5 ml over 1 minute, followed by 2 ml over 2 minutes and finally 10 ml over 2 minutes. The fusion mixture was centrifuged at 500 g_{av} for 5 minutes at room temperature and the cells were resuspended in 60 ml of the T-cells in RPM1/HT media containing 15 % foetal calf serum and 0.5 μM aminopterin at 37°C.

The fusion mixture was dispensed into six 96-well culture plates. The cells were fed with RPM1/HT media containing 15% foetal calf serum and 0.5 μM aminopterin at 3 days after the fusion and cultured for a further 4-6 days before the hybridoma cell density was sufficient to remove the culture supernatants for immunoassays. The cells in the wells showing positive immunoreactivity were transferred into 24-well plates at about 50 to 80% confluency and the culture supernatants were assayed again. The immunoreactive cells were then transferred into 25 cm^2 flasks. The cells were allowed to grow to confluency. The supernatants of the confluent cells were harvested and stored at 4°C in the presence of 0.1% (w/v) sodium azide.

5.2.4 Cloning of hybridoma cell lines

Hybridoma cell lines producing antibodies of the required specificity were cloned by limiting dilution. To aid cell growth in the wells with low numbers of cells, 100 μl of mouse thymus (feeder) cells in RPM1/HT media containing 15% foetal calf serum and 1μM aminopterin were placed into the wells of sterile 96-wells tissue culture plates.

Usually three plates were prepared for each cell line. Into column "1" wells of each cloning plate, and100 µl of hybridoma cells at a density of 10000/ml were dispensed, mixed and 100 µl of the mixture was transferred into the wells in column "2". Such 1- in 2- dilutions were made across the plate. After three days in culture, 50 µl of fresh RPM1/HT medium containing 15% foetal calf serum was placed into each well without disturbing the cell colonies. The number of colonies in each well was determined after a further two days in culture days, wells with up to 3 colonies were chosen to be screened for antibody production. Cells originating from a single colony and producing the desired antibodies were expanded to flasks and were harvested at log growth to be frozen down for long-term storage in liquid nitrogen. The remainder of these cells were taken through another round of cloning using the above procedure.

5.2.5 Storage of hybridoma and myeloma supernatants

Hybridoma cell line supernatants were kept with the addition of 0.1% (w/v) sodium azide at either 4°C for immediate use or frozen at -20°C.

5.3 Production of antisera

5.3.1 Production of anti-glycoprotein IV serum

A sample of electroeluted glycoprotein IV (500 µl) containing about 150µg of protein was emulsified with an equal amount of Freund's complete adjuvant, and the mixture was sonicated for 3-5 bursts at 10 seconds. The mixture was cooled between each sonication, and then was taken into a syringe with a 25- gauge needle. The mixture was injected at multiple sites subcutaneously into a New Zealand white female rabbit on day 3 after preimmune bleeding of the rabbit.

One month later a booster injection of electroeluted glycoprotein IV emulsified with the same volume of Freund's incomplete adjuvant and sonicated was injected intramuscularly and booster injections were repeated every month.

A blood sample was obtained 10 days after the first booster injection from the marginal ear vein and was allowed to clot at room temperature for 3 hours, and then

the edges of clot blood were loosened with a thin plastic rod and the blood stored at 4°C over night (16 hours). The clotted blood was centrifuged at 20,000 r.p.m in a Beckman JA-20 20 rotor for 30 min at 4°C to remove blood cells. The antiserum was decanted, filtered through nitrocellulose (0.45 μm pore size) and stored at -70°C in 0.05% (w/v) sodium azide.

5.3.2 Production of antiserum against a defined peptide sequence

As an alternative strategy to obtaining an anti-glycoprotein IV serum, a peptide synthesized according to part of the Ac45 sequence was used as antigen, after coupling to KLH.

5.3.2.1 Coupling peptide to carrier protein (KLH)

The procedure for coupling was described in Harlow and Lane (1988). Maleimide-activated keyhole limpet hemocyanin (KLH) was reconstituted with deionized water to give a final concentration of 10 mg/ml of activated KLH according to the manufacturer's instructions. The peptide was dissolved in 0.083M phosphate buffer pH 7.0 to give a final concentration 3 mg/ml. 2 mg (200μl) of activated carrier was added to 0.6 mg (200μl) of peptide solution and mixed immediately. The mixture was incubated and allowed to react for 2 hours at room temperature.

5.3.2.2 Quantitation of conjugation

The degree of conjugation was estimated according to Tsao *et al.*, (1991). Ellman's reagent (1mg /ml reagent in 0.083 M phosphate buffer) was prepared as described in section 2.3.7. 980 μl phosphate buffer was placed in a cuvette, and 10μl of Ellman's reagent was added to phosphate buffer. The mixture was mixed well before 10 μl of peptide solution was added to the mixture. The whole mixture was incubated for 15 minutes at room temperature and the absorbance measured at 412nm.

The same procedure was repeated 30, 60, 90 and 120 minutes after the start of the conjugation reaction. The absorbance was measured at 412nm against blank of a mixture of phosphate buffer and Ellman's reagent.

5.3.2.3 Purification of conjugate

Uncoupled material was removed by use of a gel filtration column. A 10ml column (10 x 1.1 cm^2) containing Biogel P-6DG was equilibrated with phosphate/NaCl buffer (pH 7.2) before the sample was loaded onto it. The conjugate sample (total volume was 350µl) was passed through the column and the pure conjugate was eluted from the column with the same buffer. Fractions of 0.5ml were collected from the column. The absorbance of all fractions was measured at 280nm, and the fractions that contained conjugate were mixed together and frozen at -20°C until use.

5.3.2.4 Raising anti-peptide serum

A sample containing about 150-200µg of purified conjugate (500µl) was mixed with 500µl Freund's complete adjuvant and sonicated for 3-5 bursts at 10 seconds. The immunization protocol was carried out as described in section 5.3.

5.4 Screening procedures for antibodies.

5.4.1 Dot-blotting assay

Nitrocellulose sheets were set up in 96-well format as described in section 2.2.5.2 After dotting the antigen, the nitrocellulose sheet was removed from the dot-blotting apparatus, and placed in a separate blocker box. Non-specific sites were blocked with 0.5% (v/v) Tween-20 in TBS for 1 hour. The nitrocellulose membrane was replaced in the dot-blotting apparatus with the position of the dots corresponding to the wells. The apparatus was sealed to the atmosphere and the wells were incubated with the culture supernatants overnight and processed as described for immunoblotting (section 2.5.3).

5.4.2 Westarn blotting

Samples for protein analysis were separated by SDS-PAGE and transferred onto nitrocellulose membranes as described in section 2.2.5.1and 2.2.6.1, except that the nitrocellulose membrane was cut into 7.5mm strips and placed in a BioRad incubation tray, and incubated with a minimum of 2 ml of culture supernatants that show positive reactivity with dot-blot assay. For detecting polyclonal antiseum, 2ml of the antisera diluted to 200 and 2000 fold in TBS containing 0.05%(v/v) Tween 20, 5% (v/v) normal horse serum, 5mg/ml bovine serum albumin and 0.05% (w/v) sodium azide were added to the strips.

5.4.3 Two-dimensional immunoblots

Culture supernatants and the antisera showing positive reactivity after detection with Westarn blotting were further characterized by two-dimensional immunoblotting as described in section 2.2.6.1.

5.5 Results and discussion

5.5.1 Monoclonal antibodies against phospholipid-rich pellet

One of the objectives of this work was to obtain monoclonal antibodies against the uncharacterised chromaffin granule membrane glycoprotein IV. The phospholipid -rich pellet generated by fractionation of chromaffin granule membrane proteins with Triton X-114 provided an enrichment of this protein. Membranes were first washed with 100mM sodium carbonate (pH11), to ensure removal of the highly immunogenic intragranular component Chromogranin A (as this protein is completely removed by this washing step) [Howell *et al.,* 1982; Higgins *et al.,* 1984; Pryde and Phillips, 1986]. The washed membranes were fractionated with Triton X-114 and the phospholipid- rich pellet (P1) was isolated and used to immunise mice. Hybridomas were prepared and were screened against phospholipid-rich pellet proteins by dot-blotting, bound mouse antibodies being detected by enhanced chemiluminescence.

Figure 5.1 shows the primary screening results of the hybridomas obtained from fusion after phospholipid-rich pellet immunisation. The hybridomas secreting chromaffin granule -specific antibodies were expanded and their supernatants were tested against phospholipid-rich pellet proteins by immunoblotting. Figure 5.2 shows the immunoreactivities of these supernatants: only cell lines 7A,4B,8B, 5C and 7D were reactive: 7A and 4B reacted with a ~75kDa protein, but the remaining cell lines (8B, 5C & 7D) did not show specific reactivity. To further characterize supernatants of cell lines 7A & 4B two-dimensional immunoblots were carried out. Figure 5.3 and Figure 5.4 shows the two-dimensional immunoblot of cell line 7B and 4B. These antibodies detected an antigen of 75kDa with a pI of range 5.8-6.7, so it was concluded that the antibodies secreted by cell lines 7A & 4B were directed against the catecholamine biosynthetic enzyme dopamine β-monooxygenase. These antibodies were used as positive controls for further work. Although the aim of this work was to obtain a monoclonal antibody against glycoprotein IV, which was the main glycoprotein of the phospholipid-rich pellet, immunisation with this pellet was

unsuccessful, since it failed to produce a hybridoma secreting an antibody against glycoprotein IV.

5.5.2 Monoclonal antibodies against proteins eluted from the Con A-agarose column

Since the immunisation of mice with the phospholipid-rich pellet (P1) was unsuccessful, glycoprotein IV was further purified from this pellet. Chromaffin granule membrane proteins were fractionationed with Triton X-114 and the phospholipid-rich pellet was isolated and proteins were solubilized with 2% n-octyl β-glycoside. The solubilized proteins were subjected to Con A-agarose column chromatography as described in chapter 3. The proteins eluted from Con A were used to immunise mice and hybridomas were screened against P1 proteins by dot-blotting, bound mouse antibody being detected by enhanced chemiluminescence.

Figure 5.5 shows the primary screen results of hybridomas from this fusion, the hybridomas secreting chromaffin granule membrane-specific antibodies were expanded and their supernatants were tested against membrane proteins by immunoblotting. Figure 5.5, lane 7 shows that the antibody secreted by hybridoma number 6B recognized an unknown high molecular mass protein (~200-kDa). As shown from Figure 5.5, lane 7, this supernatant was cross-reactive with one of the P1 proteins at the top of the blot made from 9% gel so another blot was prepared from a 6% gel and tested. This antibody, compared to the monoclonal recognizing dopamine β-monooxygenase, is shown in Figure 5.7. but no further characterisation of this antibody was carried out.

5.5.3 Antiserum against pure glycoprotein IV

The polyclonal rabbit antiserum raised against electroeluted glycoprotein IV was tested against chromaffin granule membrane proteins and Triton X-114 phospholipid-rich pellet (P1) by one- and two-dimensional Western blotting as described above. Figure 5.8 (a) shows that antiserum raised against glycoprotein IV, used at 200 fold dilution, recognised a band at a position of 45-50 kDa similar to the position of glycoprotein IV on SDS-gels. Figure 5.8 (b) shows the antiserum used at 2000 fold dilution also strongly recognised the same band with a much clearer background. Figure 5.8 (c) shows that the pre-immune serum did not react with any membrane component of chromaffin granules. To further characterise the antiserum, two-dimensional immunoblots were carried out using chromaffin granule membrane and P1 (Triton X-114 phospholipid-rich pellet) proteins. Samples of chromaffin granule membrane and P1 proteins were focused in the first dimensional as described before, using the same ampholyte mixture (pH 3-10 & 9-11) that was used to test for glycoprotein IV in two-dimensional Con-A blots. Proteins focused in the first dimensional were subjected to SDS-second dimensional electrophoresis, transferred to nitrocellulose, and tested with an antiserum dilution of 1: 2000 as described in section 2.6. Figure 5.9 shows a two-dimensional immunoblot of chromaffin granule membrane proteins decorated with anti-glycoprotein IV serum. The antiserum recognised a band at 45-50 kDa with a pI range of 4.3-5.4. Figure 5.10 shows a two-dimensional immunoblot of P1 proteins with anti-glycoprotein IV serum, which shows that it recognised the same protein in the P1 fraction.

There is some non-specific labelling of other proteins by the antisera. Two bands at (65-70-kDa) were visible on two-dimensional blots. The appearance of these bands has been reported by Ochs *et al.,* (1983) and they probably correspond to keratin subunits (Mr 65-68-kDa). Contamination was reduced as the concentration of DTT in sample buffer was decreased, presumably because disulphide bonds in the keratin molecules were not broken and more keratin therefore, remained at the top of the gel.

Despite non-specific binding to keratin subunits in these blots, the antiserum was suitable for investigating chromaffin granule membrane glycoprotein IV.

5.5.4 Antiserum against peptide

The peptide (underlined in Figure 5.11) was selected from the Ac45 sequence as having the highest antigenic index. The selection of peptide sequence was made according to peptide structure computer programme which makes a prediction of different parameters as shown in Figure 5.12. For surface probability (calculated according to a formula of Emini *et al.*, (1985) the internal peptide sequence that give the highest peak underlined in Figure 5.12 was chosen to raise antibody. The hydrophilicity of Ac45 was calculated according to the algorithm of Hopp and Woods, (1985) and the chosen peptide found to show high hydrophilicity, as shown in Figure 5.12. The peptide was synthesized and purified at the Department of Chemistry, University of Edinburgh. A cysteine residue was added to the NH_2 terminus to allow coupling of peptide to a carrier protein (KLH), using the m-Maleimidobenzoyl-N-hydroxysuccinimide ester mechanism. The peptide was coupled to the carrier protein, keyhole limpet hemocyanin, (KLH) through the thiol of the N-terminal cysteine in the peptide. The degree of coupling was estimated using Ellman's reagent which estimates the total number of peptide sulphydryls present before and after conjugation as shown in Figure 5.13. The conjugate was purified by Biogel P-6DG column chromatography. Rabbits were immunised with the conjugated protein, and the anti-peptide serum was tested by one-dimensional immunoblotting against phospholipid-rich pellet (P1) proteins.

Figure 5.14(a) shows that the antiserum raised against the conjugate, used at 2000 fold dilution, recognised a band at a position of 45-50 kDa similar to the position of glycoprotein IV shown in Figure 5.14(b), while Figure 5.14(c) shows that the pre-immune serum did not react with any membrane component of chromaffin granules.

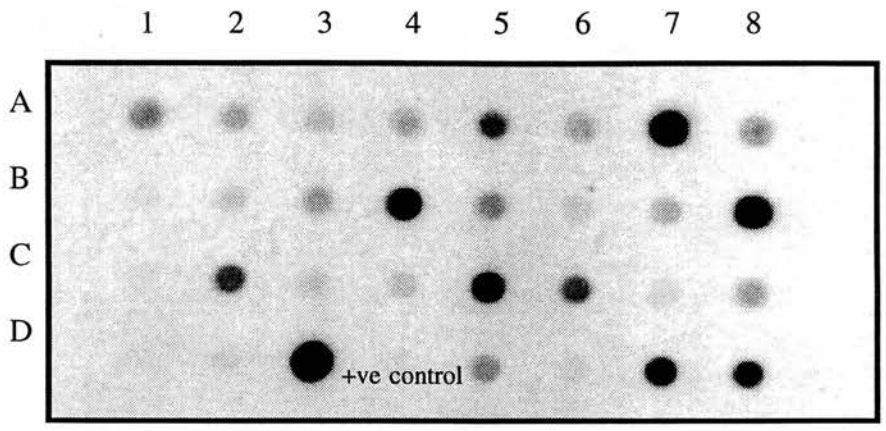


Figure 5.1 Immunoreactivities of culture supernatants from hybridomas generated by immunising mice with P1 fraction using dot-blots against P1 proteins. Immunoreactivities were detected by using ECL followed by exposure to autoradiography film.

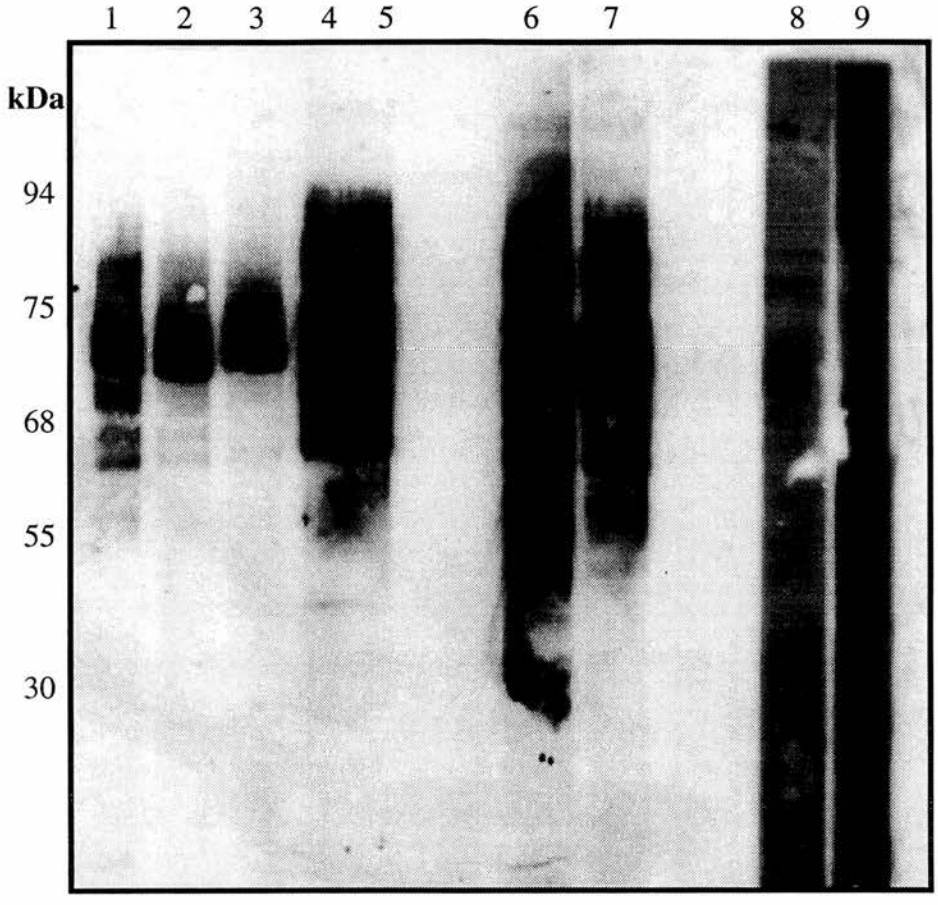


Figure 5.2 Immunoblot showing the immunoreactivities of culture supernatants from hybridomas generated by immunising mice with P1 proteins against chromaffin granule membrane proteins. Immunoreactivities were detected using enhanced chemi-luminescence(ECL).

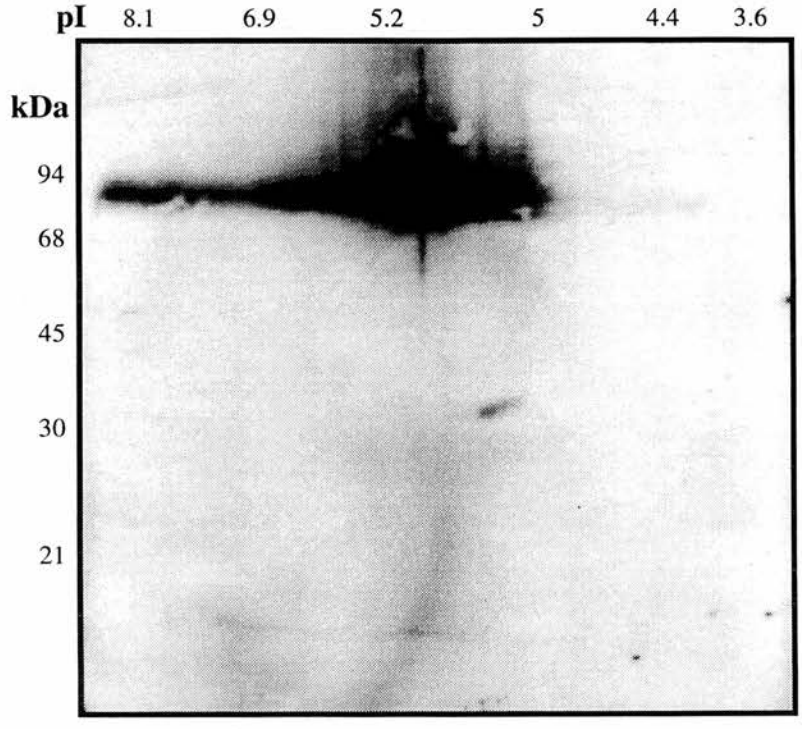


Figure 5.3 Two-dimensional immunoblot showing the immunoreactivity of culture supernatant from hybridomas generated by immunising mice with P1 proteins (secreted by cell line 7) against chromaffin granule membrane proteins

.

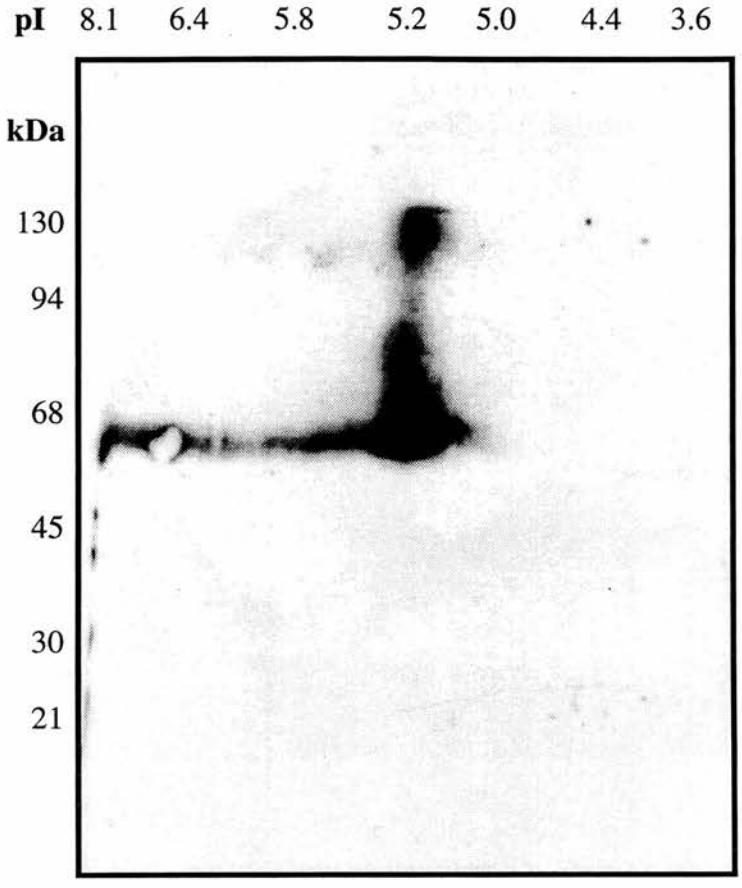


Figure 5.4 Two-dimensional immunoblot showing the immunoreactivity of culture supernatant from hybridomas generated by immunising mice with P1 proteins (secreted by cell line 12) against chromaffin granule membrane proteins.

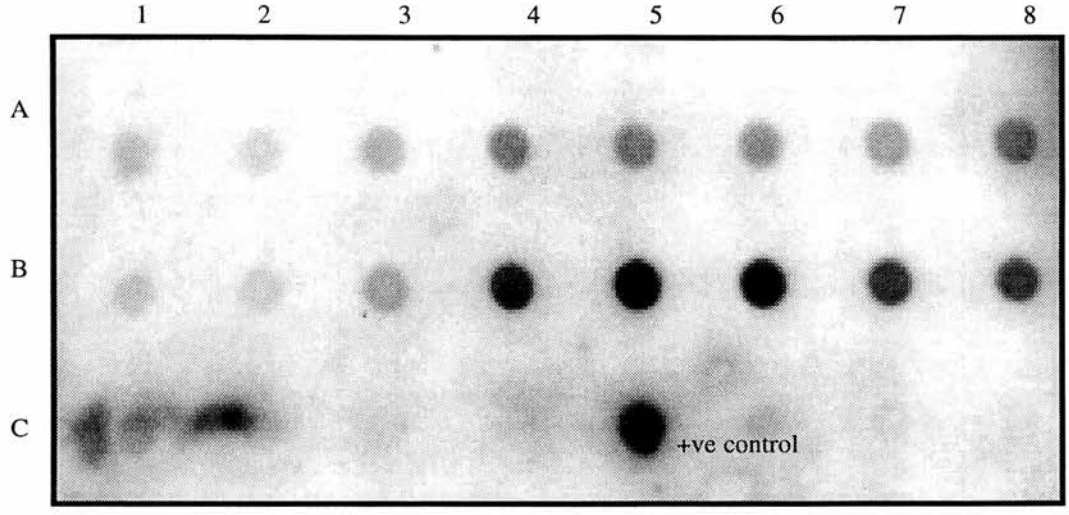


Figure 5.5 Immunoreactivities of culture supernatants from hybridomas generated by immunising mice with proteins eluted from a Con A-agarose column using dot-blots against P1 proteins. Immunoreactivities were detected by using ECL

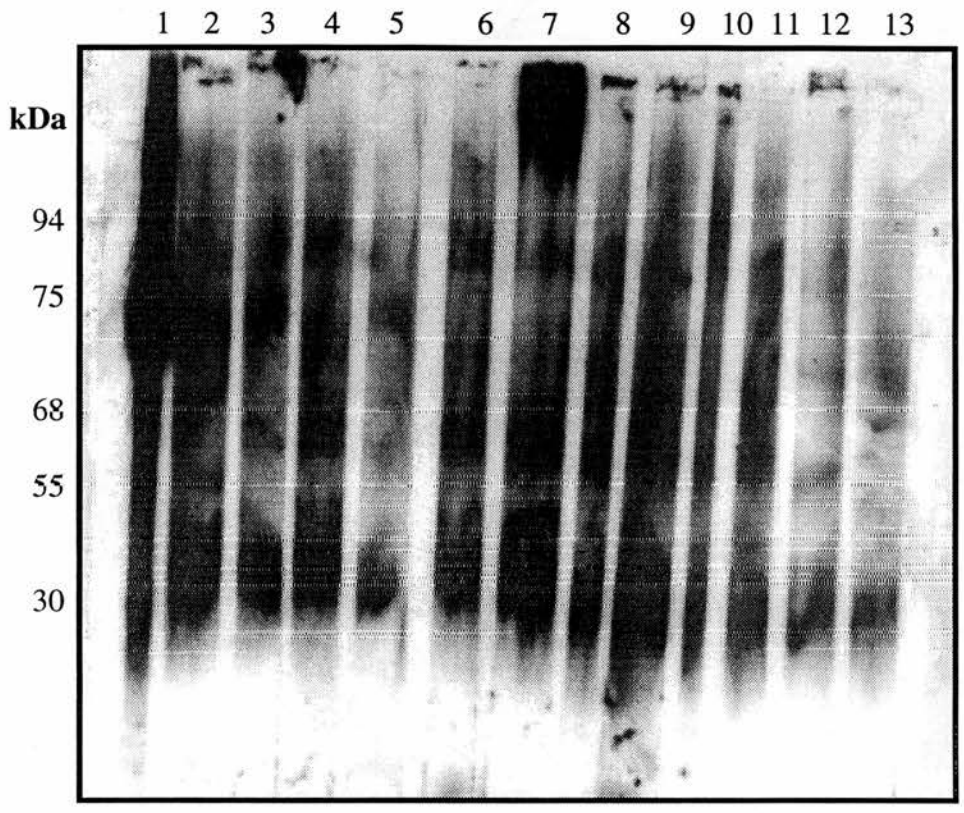


Figure 5.6 One-dimensional immunoblot (9% gel) showing the immunoreactivities of culture supernatants from hybridomas generated by immunising mice with proteins eluted from a Con A-agarose column against P1 proteins.
Lane1: positive control (dopamine β-monooxygenase).
Lanes 2-13: hybridomas supernatants.

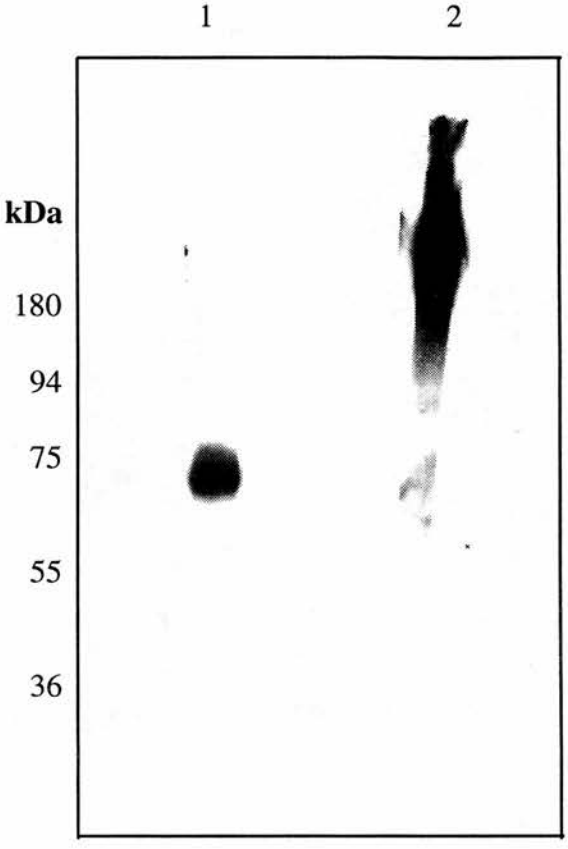


Figure 5.7 One-dimensional immunoblot (6% gel) of the P1 fraction showing the immunoreactivities of culture supernatants from hybridomas (cell lines number 14) generated by immunising mice with proteins eluted from a Con A-agarose column
Lane 1: dopamine β-monooxygenase.
Lane 2: culture supernatant of cell line 14.

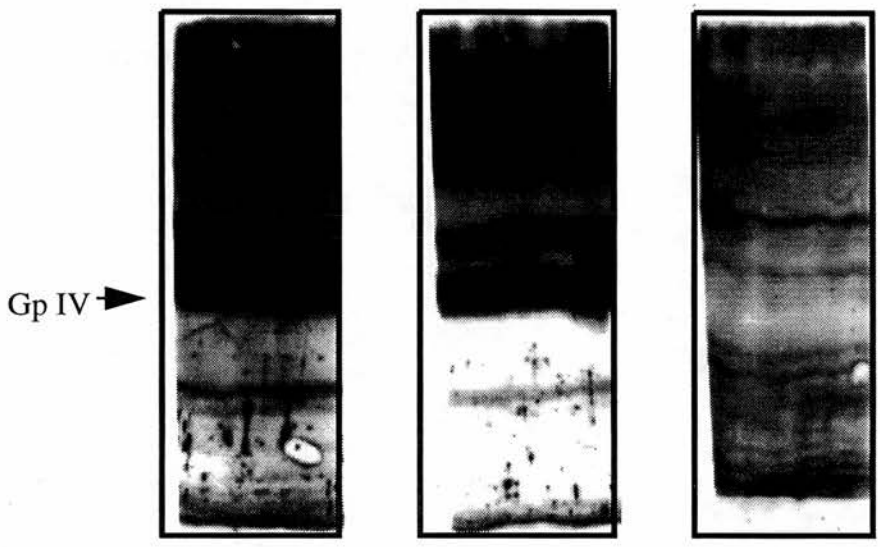


Figure 5.8 Immunblot of P1 fractions showing the immunoreactivities of the rabbit antiglycoprotein IV serum generated by immunising rabbits with electroeluted glycoprotein IV from SDS gels. Immunoreactivities were detected by using ECL
Lane a: antiglycoprotein IV serum at dilution 1 : 200
Lane b: antiglycoprotein IV serum at dilution 1 : 4000
Lane c: preimmune serum.

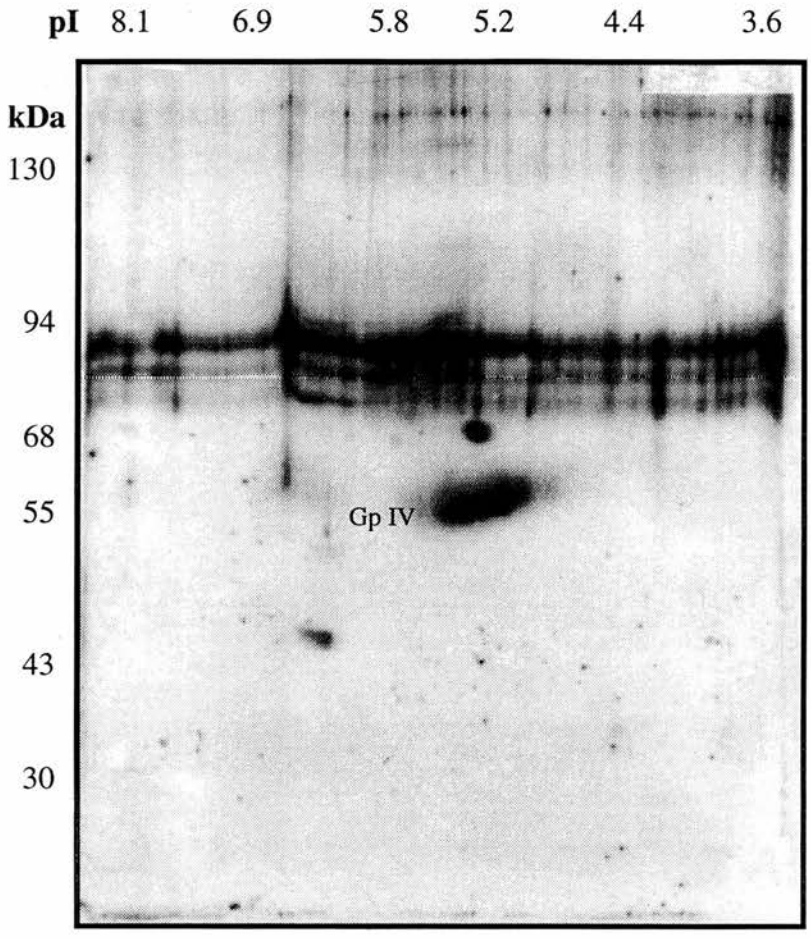


Figure 5.9 50µg of chromaffin granule membranes were separated by two-dimensional gel electrophoresis. proteins were transferred onto nitrocellulose and decorated with antiglycoprotein IV serum. Immuno-reactivities were detected by using enhanced chemi-luminescence (ECL).

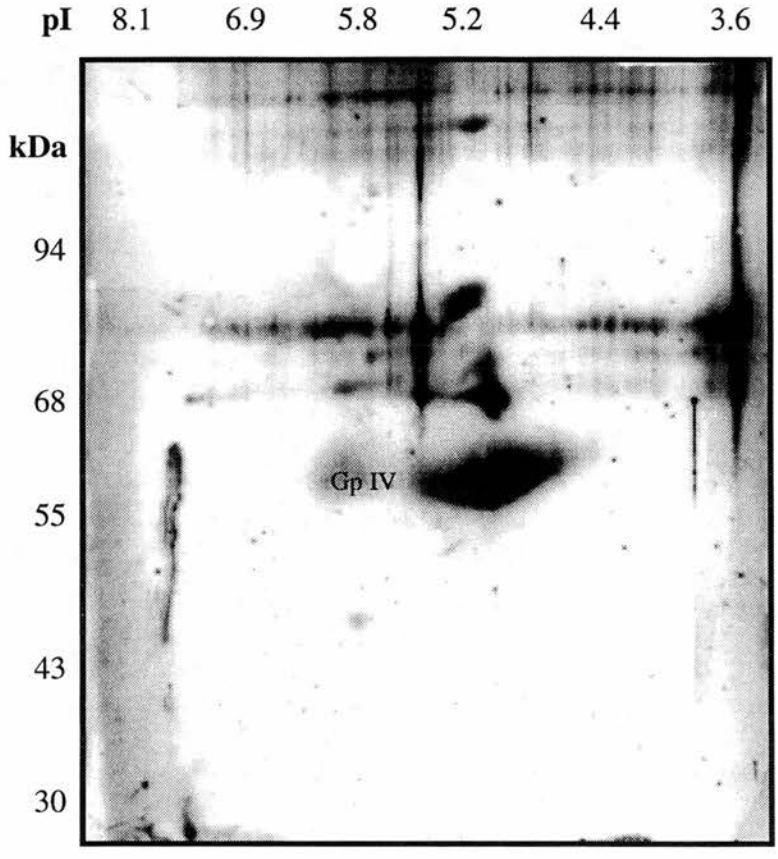


Figure 5.10 50µg of P1 proteins were separated by two-dimensional gel electrophoresis. proteins were transferred onto nitrocellulose and decorated with antiglycoprotein IV serum. Immunoreactivities were detected by enhanced chemi-luminescence (ECL).

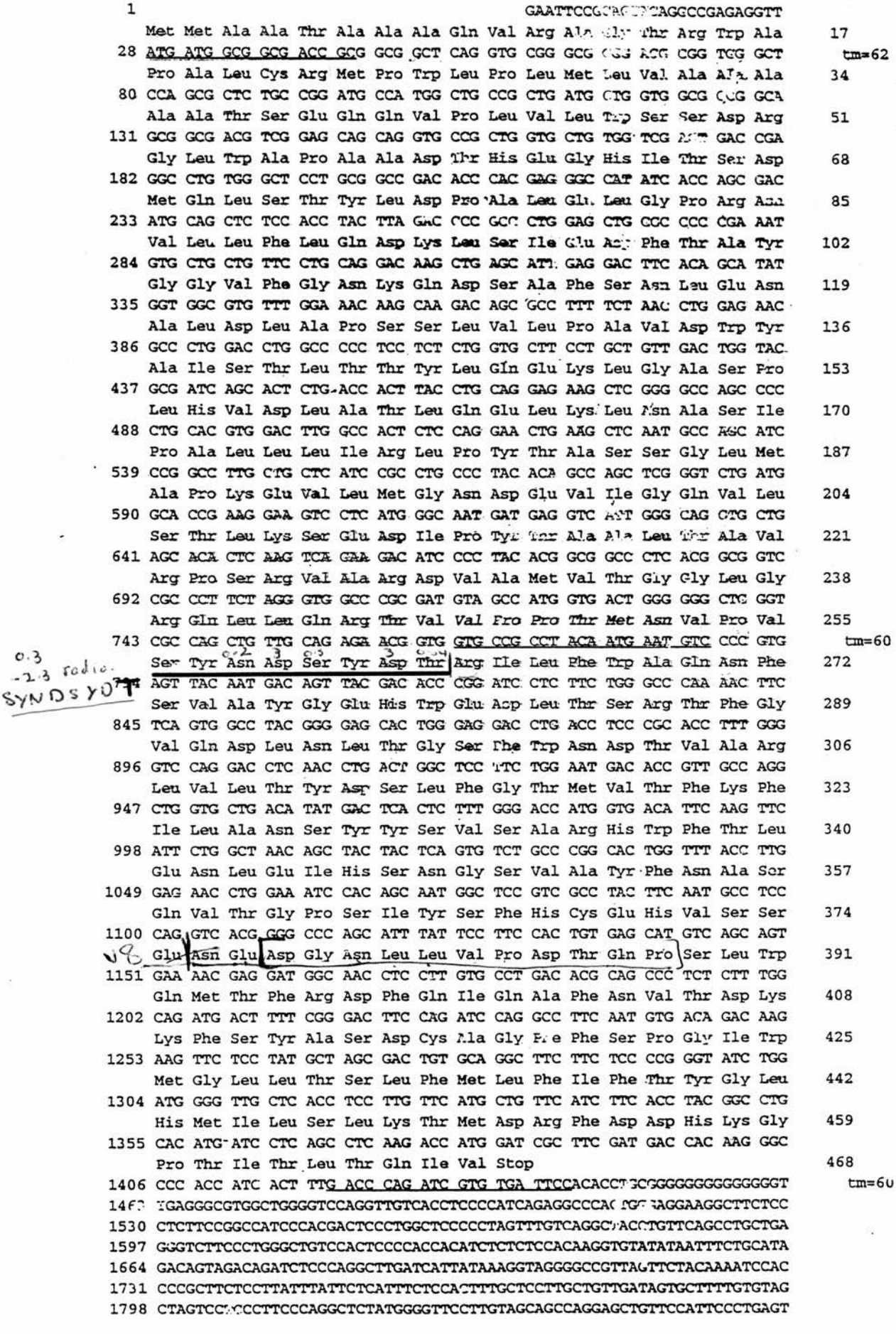


Figure 5.11 Ac45 aminoacid sequence [Supek et al.,1994]

Figure 5.12

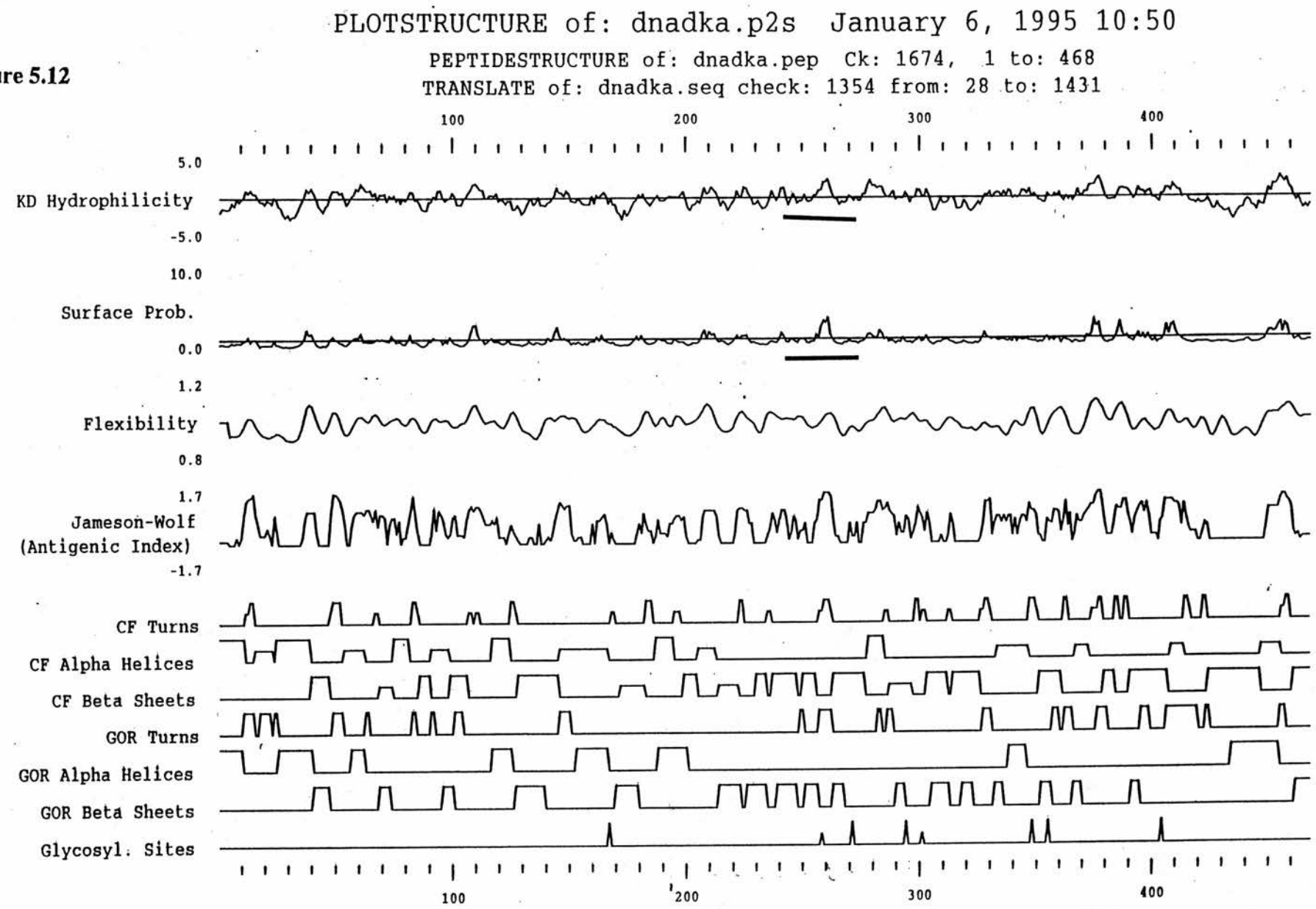
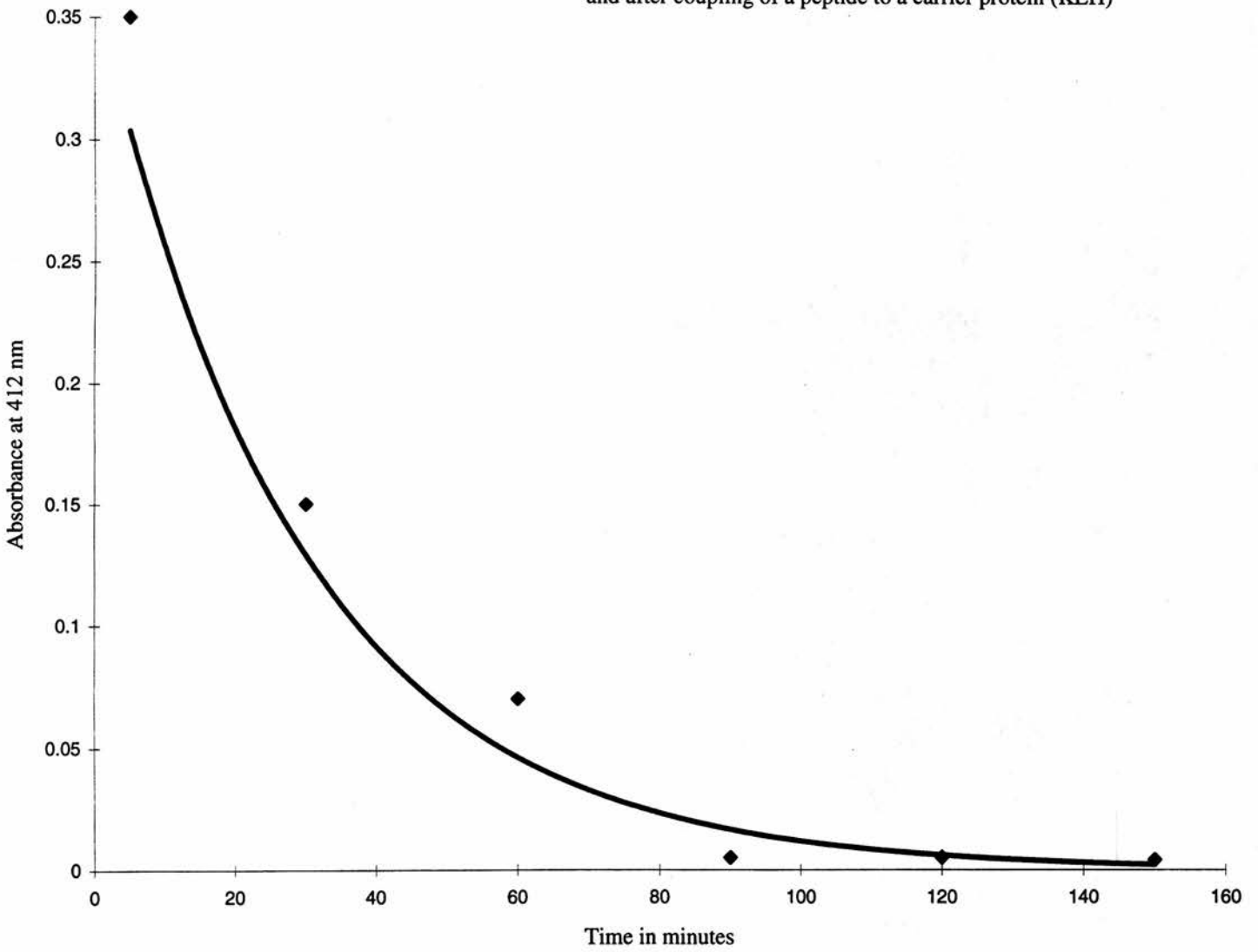

PLOTSTRUCTURE of: dnadka.p2s January 6, 1995 10:50
PEPTIDESTRUCTURE of: dnadka.pep Ck: 1674, 1 to: 468
TRANSLATE of: dnadka.seq check: 1354 from: 28 to: 1431
100
200
300
400
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
100
200
300
400

Figure 5.13 The curve shows the total number of sulfhydryls present befor and after coupling of a peptide to a carrier protein (KLH)



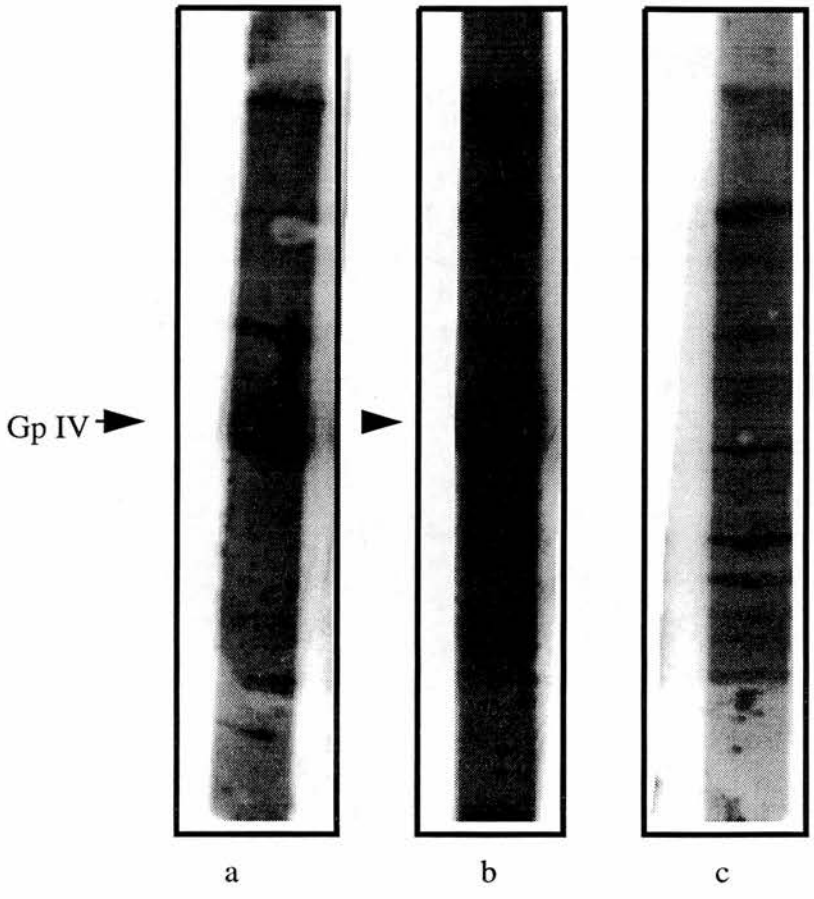


Figure 5.14 Immunoblot of P1 fraction showing the immunoreactivities of antiglycoprotein IV serum generated by immunising rabbits with conjugate formed from coupling thepeptide to KLH against P1. Immunoreactivities were detected using ECL.
Lane a: antiglycoprotein IV serum at dilution 1:200
Lane b: antipeptide serum at dilution 1:200
Lane c: preimmune serum of antipeptide serum at dilution 1:200

Chapter 6
Characterisation of bovine chromaffin granule membrane glycoprotein IV

6.1 Glycoprotein IV is a subunit of the chromaffin granule membrane H^+-ATPase

6.1.1 Introduction

Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) is a new technique that was developed by Schägger and von Jagow (1991) to analyse the protein complexes of the mitochondrial inner membrane, and is found to be suitable for the separation of water-soluble and membrane protein complexes in the range 100 to 1000 kDa, its resolution in the range 10 to 100 kDa being significantly lower. It is a charge shift method, in which the electrophoretic mobility of proteins is affected by binding of negatively charged Coomassie blue G dye. Even basic proteins that bind Coomassie blue migrate to the anode at the running pH of 7.5, and it can be used for the isolation of enzymatically active membrane proteins solubilized from biological membranes for clinical diagnosis, analysis of purity, molecular mass estimation, and for the determination of subunit composition and degree of homogeneity. The principle of this technique is based on the separation of large proteins and protein complexes on the basis of their molecular mass under non-denaturing conditions by electrophoresis in the first-dimension. These protein complexes can be further resolved into their component polypeptides when run in second dimensional electrophoresis under denaturing conditions. Blue Native gel electrophoresis therefore offers a new technique for analysis of chromaffin granule membrane protein complexes, in particular the V-ATPase complex. Heart mitochondria respiratory complexes can be used as molecular mass markers. Apart from isolating the V-ATPase complex from other chromaffin granule membrane proteins, Blue Native PAGE also offers a good means of separating the two sectors of the enzyme in their native state, thus allowing both parts to be studied separately.

The antiserum raised against glycoprotein IV was used to probe blots of one- and two-dimensional Blue Native gels, in order to investigate the association of glycoprotein IV with protein complexes of the chromaffin granule membrane.

6.1.2 Results and discussion

In this work solubilized chromaffin granule membrane and phospholipid-rich pellet (P1) proteins were solubilized in 2%(w/v) dodecyl maltoside and electrophoresed in the presence of Coomassie Blue G dye in Blue Native PAGE as described in section 2.2.4.3. Distinct sets of bands corresponding to the V-ATPase enzyme complex and other proteins in the membrane became visible during running. These sets of bands were better visualised after destaining of the gel in 10% acetic acid to remove the excess dye in the background. Figure 6.1.1 shows first-dimension Blue-Native gels of chromaffin granule membrane proteins, standard molecular mass markers (bovine heart mitochondria respiratory complexes) and partially-purified V-ATPase (P1fraction proteins). As shown in this Figure Blue native gel electrophoresis of chromaffin granule membrane (lane A) revealed two distinct bands identified as the holo-ATPase (V_oV_1) at 890kDa and the membrane sector (V_o) at 450kDa on the basis of the mobility of bovine heart mitochondria respiratory complexes (lane B). Three other proteins have also been identified in the first dimension gel of the chromaffin granule membrane: tetrameric dopamine β-monooxygenase (DBM), chromogranin A, and cytochrome b561. BN-PAGE of the P1 fraction from chromaffin granule membranes (lane C) gives a similar set of bands for holo enzyme but without any contamination with other membrane complexes such as DBM complex. To confirm the position of the holo-ATPase (V_oV_1) and membrane subcomplex (V_o), Blue-Native gels of chromaffin granule membrane proteins were analysed by immunoblotting using several antisera directed against H^+-ATPase subunits. Figure 6.1.2 shows an immunoblot using antisera raised against *Kalanchoe daigremontiana* V-ATPase subunit B (57 kDa) (lane a) and subunit A (72 kDa) (lane b) which confirmed the position of the holoenzyme (V_oV_1), while the position of the membrane sector (V_o) was confirmed by probing the blot of membrane with antisera directed against membrane sector subunits 116 kDa and 39 kDa from chromaffin granule V-ATPase subunits as shown in lanes c and d respectively.

After the position of the holo-ATPase (V_1V_o) and its subcomplex V_o were confirmed in the first dimension of BN-PAGE these complexes were further analysed by subjecting them to second-dimensional SDS-Tricine PAGE, to resolve the protein complexes into the constituent polypeptides. Figure 6.1.3 shows a two-dimensional SDS-Tricine PAGE of chromaffin granule membrane stained with Coomassie blue G. In this gel, the holo-ATPase (V_oV_1) was resolved into nine different subunits with apparent molecular masses of approximately 116kDa, 72kDa, 57kDa, 45kDa, 42kDa, 39kDa, 33kDa, 20kDa and 16kDa which agree with those reported previously [Percy and Apps, 1985; Moriyama and Nelson, 1987]. The membrane sector (V_o) was resolved into four different subunits with molecular masses of 116kDa, 45kDa, 39kDa, and 16kDa. DBM monomer, chromogranin A and cytochrom b561 were also identified in this gel. Figure 6.4.1 shows BN-PAGE of the P1 fraction from chromaffin granule membranes it shows the same number of bands are present in V_oV_1 and V_o as were found in chromaffin granule membranes, but there is no contamination with other membrane complexes such as DBM. To further confirm the position of these subunits, immunoblots of two-dimensional (BN-PAGE/SDS tricine) gels were probed using antisera directed against H^+-ATPase subunits of 39kDa and 116kDa as shown in Figure 6.1.5 and Figure 6.1.6 respectively. The positions of holo-ATPase (V_oV_1) and the membrane sector (V_o) were fully confirmed by these blots. Another two-dimensional blot of chromaffin granule membrane proteins was decorated with Con A and HRP as shown in Figure 6.1.7 and confirmed the position of chromaffin granule membrane proteins including glycoprotein IV. Since the locations of holo-ATPase (V_oV_1) and its subcomplex had been confirmed by one- and two-dimensional BN-PAGE, a one-dimensional blot of chromaffin granule membrane proteins was probed with antiglycoprotein IV serum, as shown in Figure 6.1.8. This blot shows that antiglycoprotein IV serum crossreacted with both subcomplexes, a narrow band appearing with holo-ATPase (V_oV_1) and a very strong band appeared with the membrane sector (V_o). Chromaffin granule membrane and P1 proteins were then analysed by two-dimensional with immunoblotting using antiglycoprotein IV serum as shown in

Figures 6.1.9 and 6.1.10 respectively. Glycoprotein IV comigrated with other H^+-ATPase subunits confirming that it is part of this enzyme complex. Taken together these results indicated that glycoprotein IV must be a part of the membrane sector of the H^+-ATPase. It is consistent with the known topography of H^+-ATPase since the catalytic subunit complex (V_1) is located on the cytoplasmic face of granule, whereas glycosyl chains are extracytoplasmic and must therefore be attached to subunits that are integral to the granule membrane [Apps *et al.,* 1989]. This alone suggests that glycoprotein IV must be a component of the membrane sector (V_o). The pattern of bands in Figure 6.1.8 is explained by the fact that chromaffin granule membranes contain V_o and V_1V_o in the ratio of about 11:1 [Webster and Apps, 1996].

Subunit Ac45 (now termed M45) was postulated to be part of the H^+-ATPase, since it co-sedimented through a glycerol density gradient with other H^+-ATPase subunits [Supek *et al.,* 1994], and was also confirmed as a subunit of membrane sector (V_o) of H^+-ATPase by using cold inactivation which leads to dissociation of the catalytic complex (V_1) from the membrane sector V_o [Moriyama *et al.,* 1989]. After this treatment Ac45 was found exclusively with the membrane sector (V_o) [Supek *et al.,* 1994]. Also Supek *et al.*, 1994 suggested that this polypeptide is present in stoichiometric amounts with other subunits, and there is at least one copy of Ac45 per V-ATPase complex . Now taken together the results obtained from N-terminal sequencing of glycoprotein IV, and the results obtained from BN-PAGE (immunobloting with antiglycoprotein IV as shown in Figures 6.1.8, 6.1.9 and 6.1.10) prove that glycoprotein IV and Ac45 (M45) are one and the same protein, and are a component of the membrane sector of the V-ATPase.

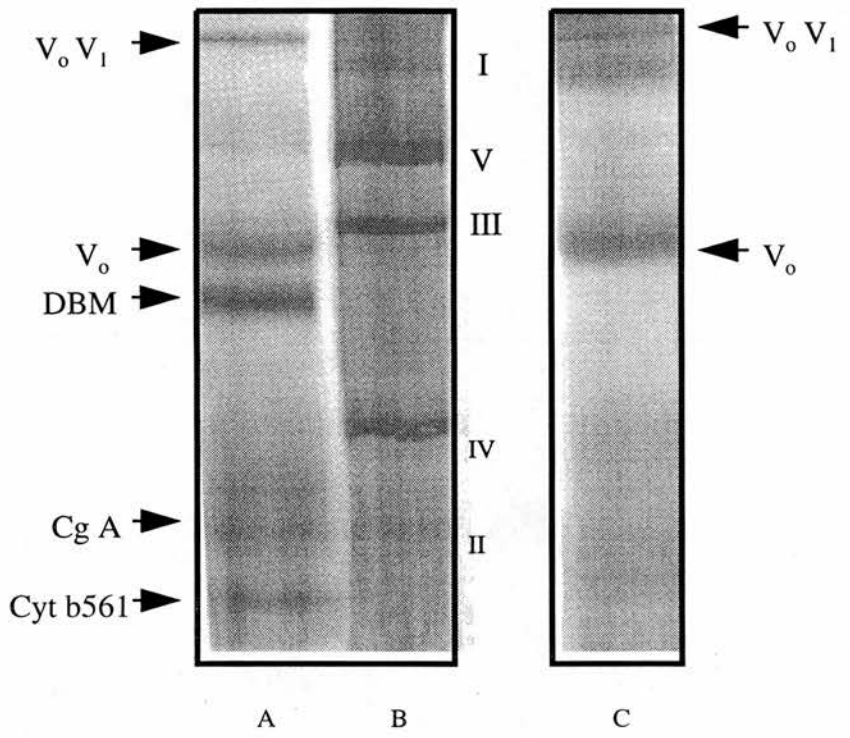


Figure 6.1.1 Chromaffin granule membrane proteins (A), mitochondrial proteins (B) and the P1 fraction from chromaffin granule membrane proteins (C) were separated in the first dimension of Blue Native PAGE using a 4-13% linear gradient gel. Bands were visualized after destaining the gels in 10% acetic acid.

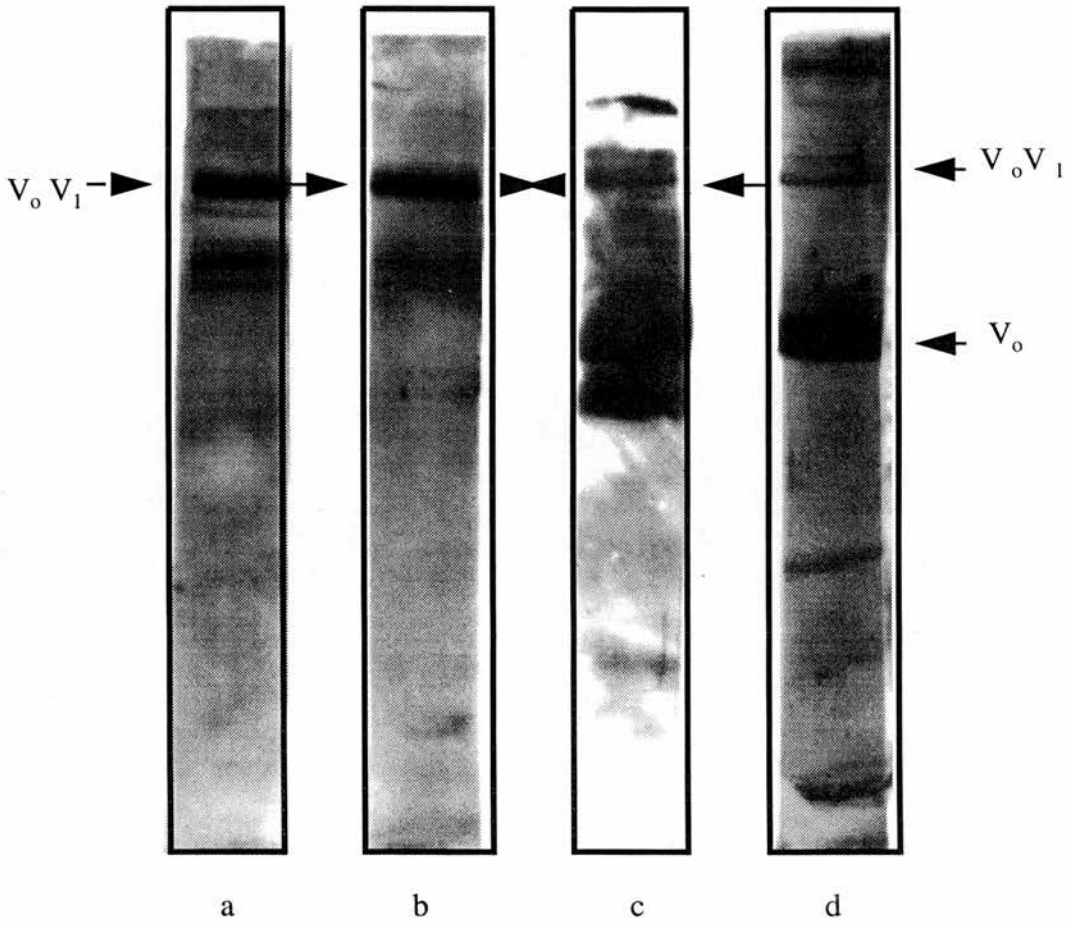


Figure 6.1.2 Chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE and immunoblotting was performed using antisera directed against the catalytic and membrane sectors of H^+-ATPase.
Lane a: against catalytic 57kDa subunit (subunit B of Kalanchoe tonoplast V-ATPase).
Lane b: against catalytic 72kDa subunit (subunit A of Kalanchoe tonoplast V-ATPase).
Lane c: against membrane sector 116kDa subunit (subunit M116 of chromaffin granule membrane V-ATPase).
Lane d: against membrane sector 39kDa subunit (subunit M39 of chromaffin granule membrane V-ATPase).

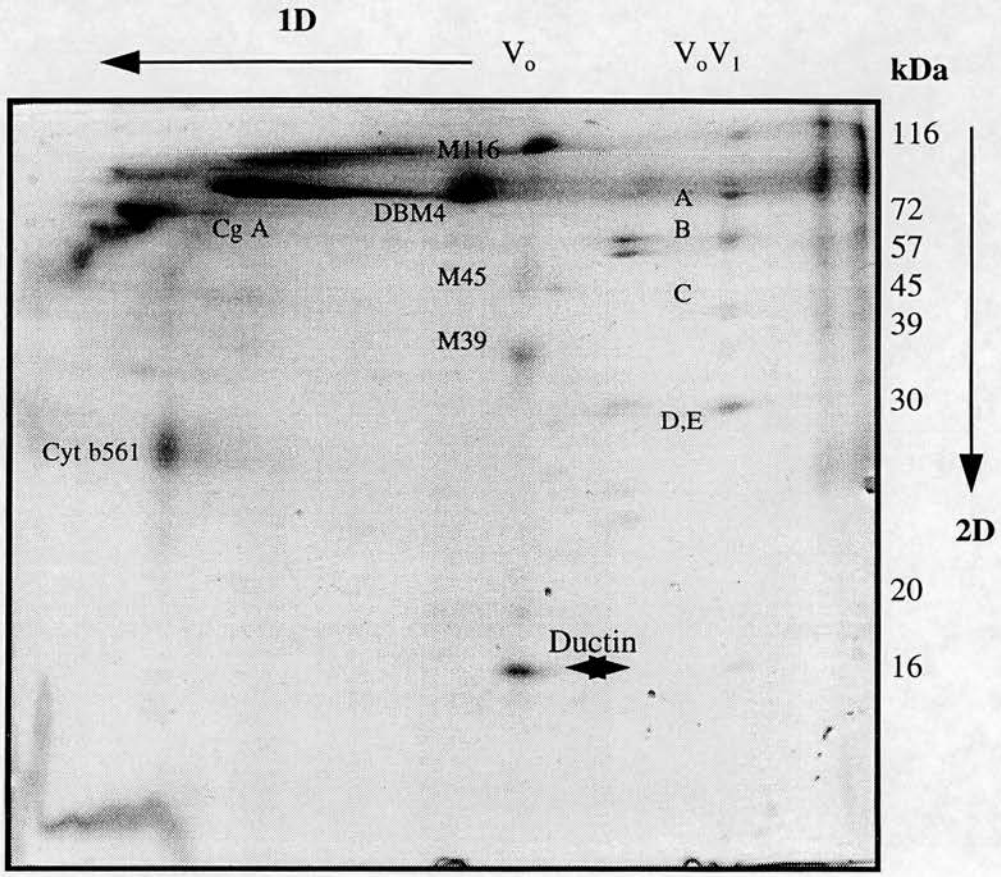


Figure 6.1.3 Chromaffin granule membrane proteins were separated by Blue Native PAGE in a 4-12% linear gradient gel (1D) followed by Tricine-SDS-PAGE in a 13% gel (2D). V_o V_1 shows the position of holo-ATPase complex, V_o shows the position of membrane sector. A, B, C, D and E are V_1 subunits and M116, M45, M39, and M16 are V_o subunits, DBM dopamine β-monooxygenase, Cyt b561 cytochrome b561 and CgA chromogranin A. The molecular mass values for the V-ATPase subunits are indicated on the right of the gel.

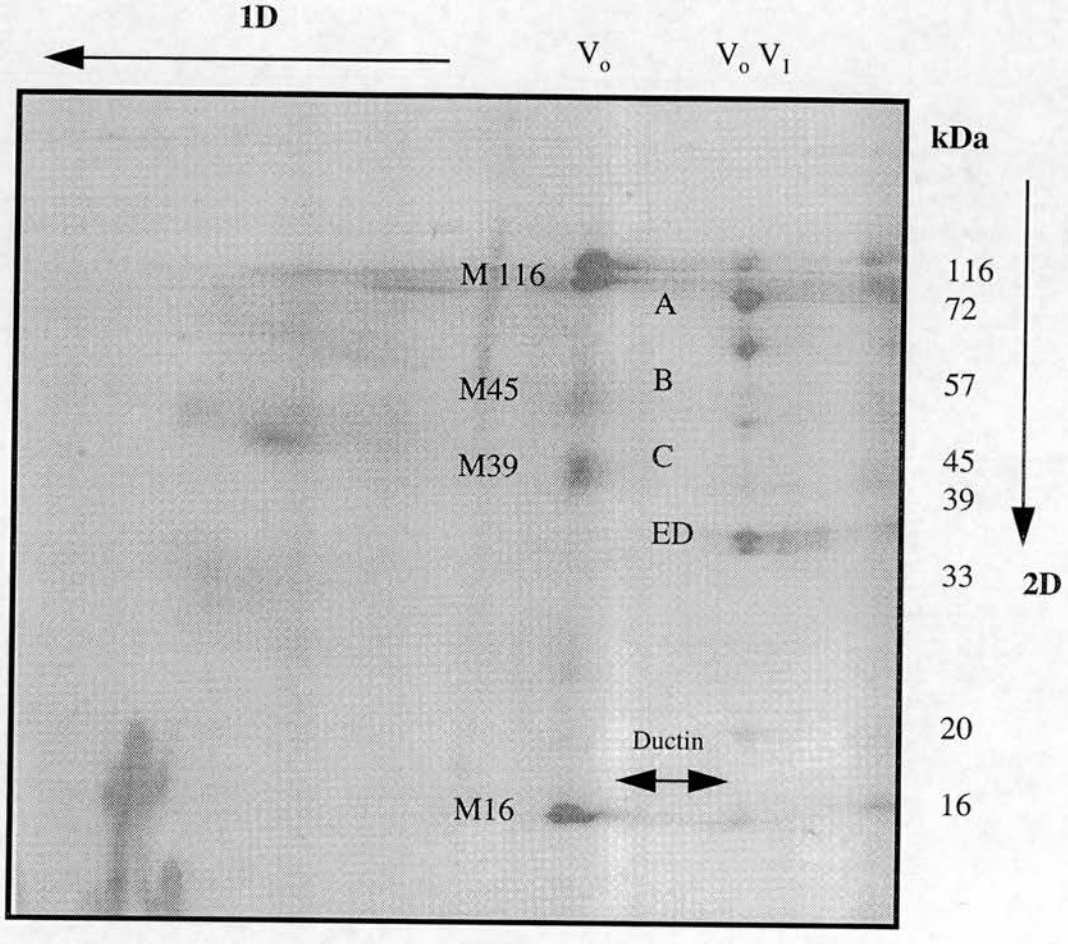


Figure 6.1.4 P1 fraction from chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE in a 4-12 linear gradient gel (1D) followed by tricine-SDS PAGE (2D). V_o V_1 shows the position of holo ATPase complex, V_o shows the position of membrane sector. A, B, C, D and F are V_1 subunits and M116, M45, M39 and M16 are V_o subunits. The molecular mass values for the V-ATPase subunit are indicated on the right of the gel

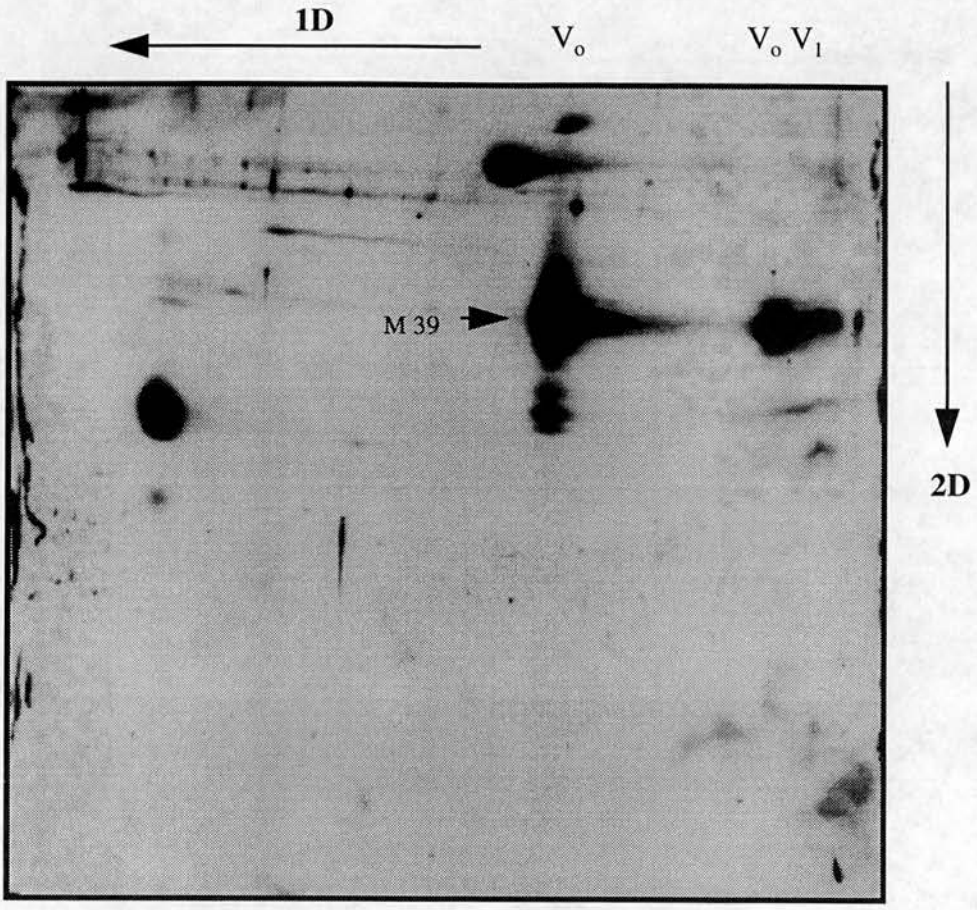


Figure 6.1.5 Chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE followed by SDS-tricine PAGE. immunoblotting was performed using antiserum directed against chromaffin granule membrane H^+-ATPase 39kDa subunit.

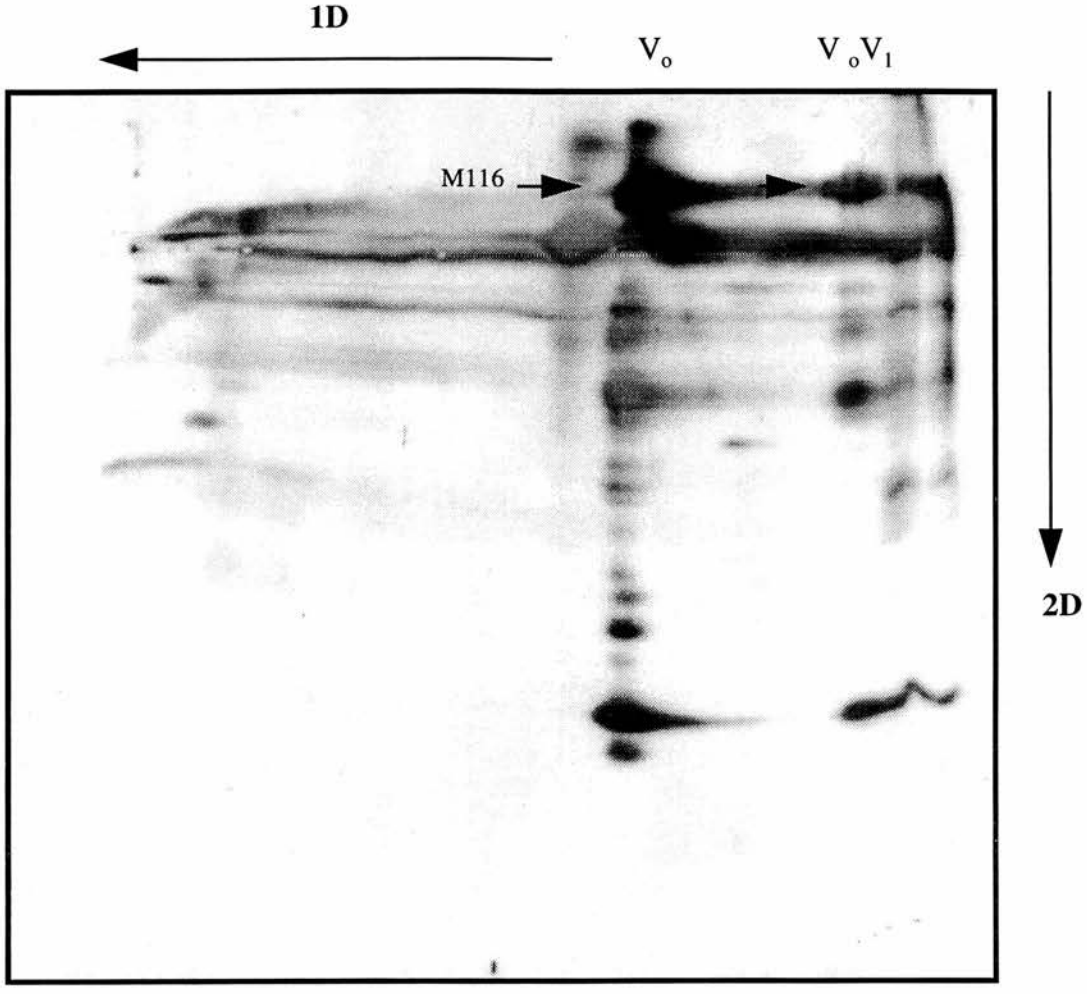


Figure 6.1.6 Chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE followed by SDS-tricine PAGE and immunoblotting was performed using antiserum directed against chromaffin granule membrane H^+-ATPase 116kDa subunit.

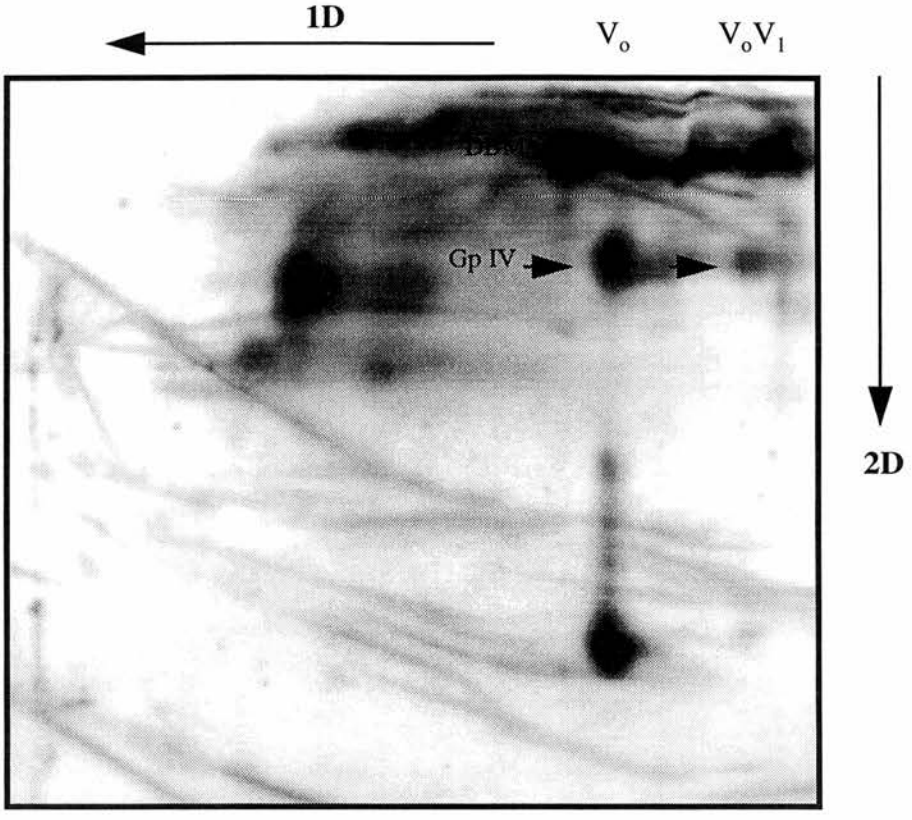


Figure 6.1.7 Chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE followed by SDS-tricine PAGE and immunoblotting was then performed using Con A and HRP.

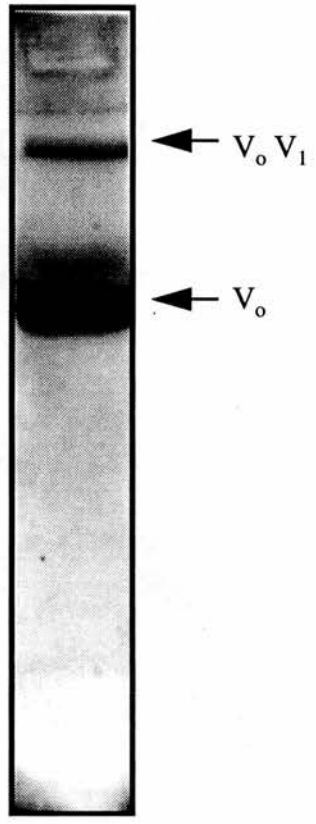


Figure 6.1.8 Chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE. Immunoblotting was then performed using antiserum directed against glycoprotein IV.

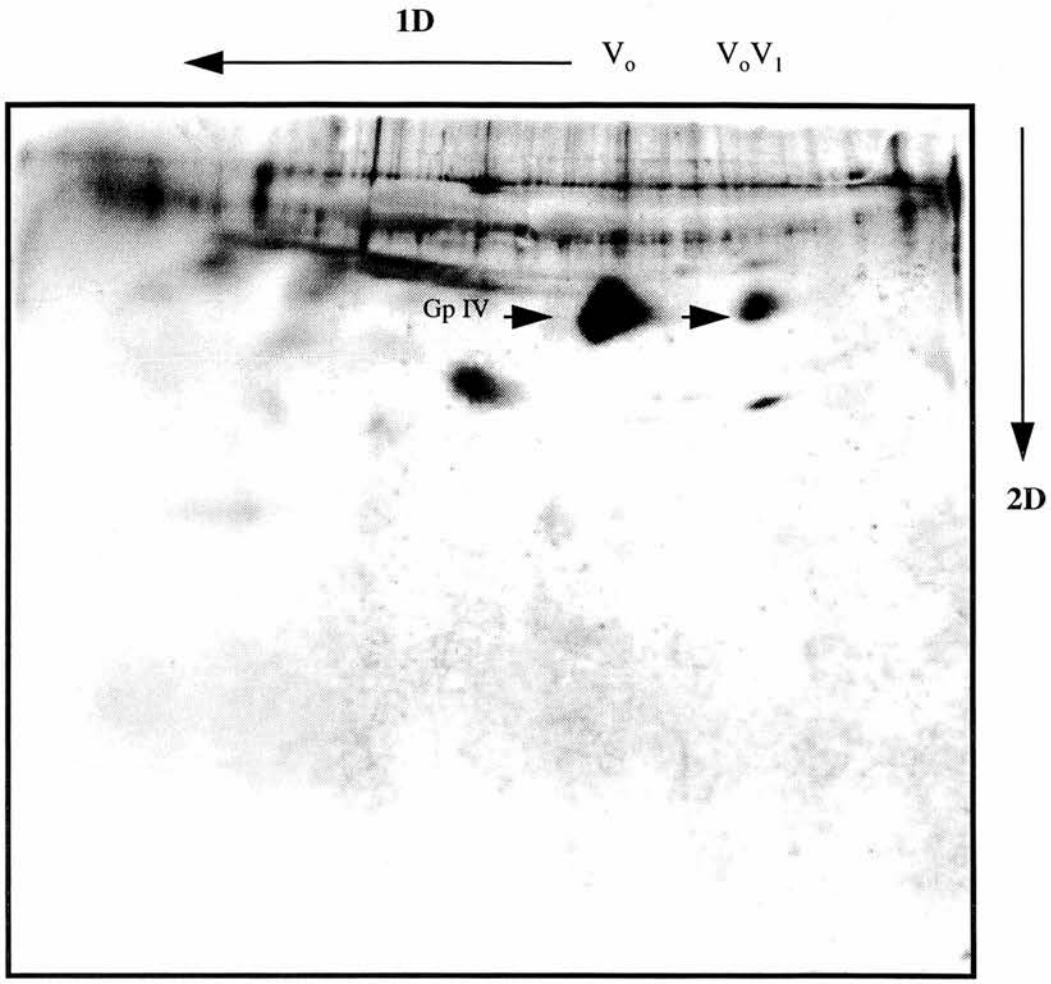


Figure 6.1.9 Chromaffin granule membrane proteins were separated by BN-PAGE followed by SDS-tricine PAGE and immunoblotting was then performed using antiserum directed against chromaffin granule membrane glycoprotein IV.

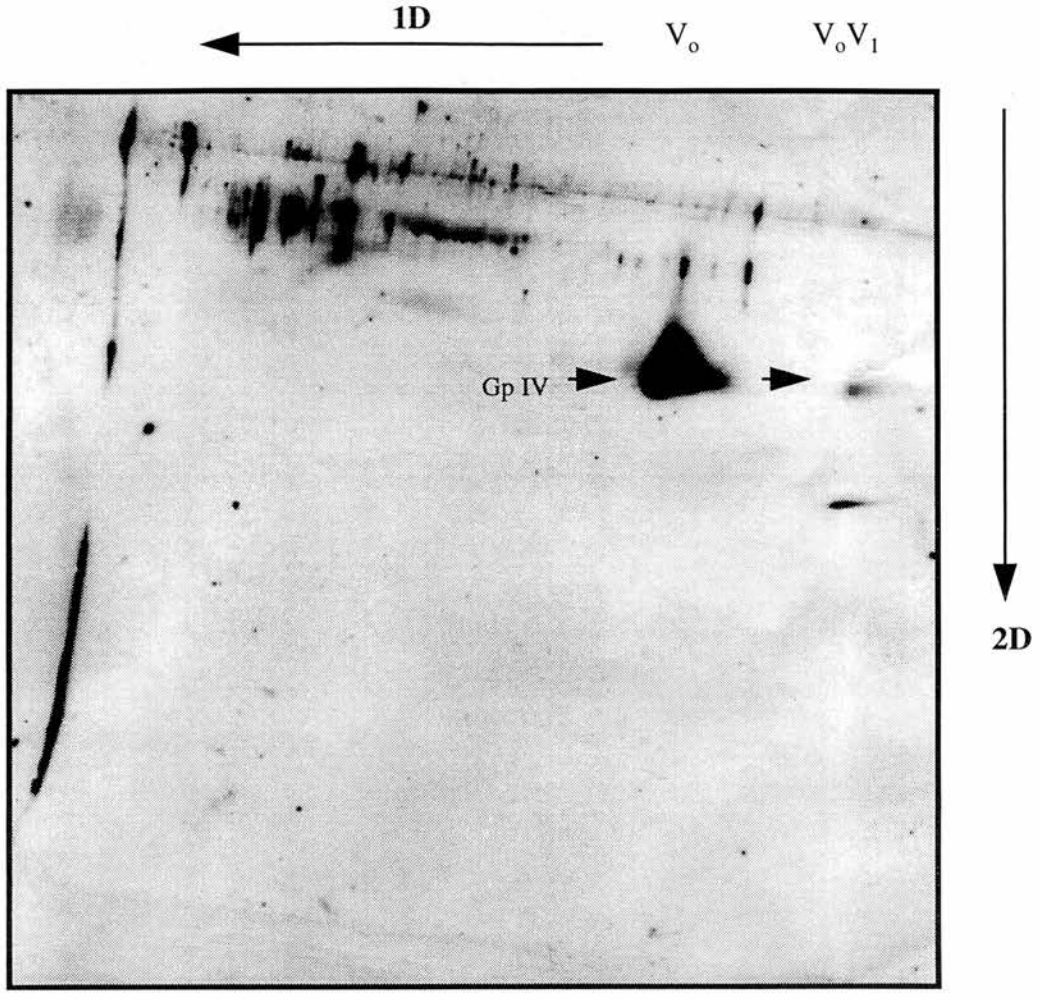


Figure 6.1.10 P1 fraction from chromaffin granule membrane proteins were separated by first dimension Blue Native PAGE followed by SDS-tricine PAGE. Immunoblotting was then performed using anti-glycoprotein IV serum.

6.2 Deglycosylation of chromaffin granule membrane glycoprotein IV

6.2.1 Introduction

Chromaffin granule membrane glycoprotein IV was reported to bind concanavalin A [Gavine *et al.,* 1984], which indicates the presence of N-linked oligosaccharide chains, as this lectin binds high mannose, hybrid, or bianntennary complex chains. Endoglycosidases and glycoamidases cleave intact oligosaccharides from proteins under mild conditions that preserve the structures of both the oligosacchrides and the protein. The enzymes most commonly used for digestion of intact glycoproteins and glycopeptides are endoglycosidase H (Endo H) (EC 3.2.1.96), endoglycosidase F2 (Endo F2) (EC 3.2.1.96) and peptide:N-glycosidase F (PNGase F) (EC 3.5.1.96). Endo H cleaves high-mannose-type and hybrid oligosaccharide chains, while Endo F2 cleaves biantennary complex-type oligosaccharides.

Peptide: N-glycosidase F has the broadest substrate specificity of the three; it cleaves tri- and tetraantennary complex-type chains as well as those cleaved by the other two enzymes. PNGase F is a glycoamidase that cleaves the bond between the asparagine residue of the protein and GlcNAc residue that joins the carbohydrate to the protein. Because it liberates nearly all N-liked oligosacchrides from glycoprotein, it is the preferred enzyme for complete removal of N-linked oligosaccharides chains. For this reason PNGase F was the enzyme of choice to use in deglycosylation analysis of membrane glycoprotein IV, in order to determine the approximate percentage of carbohydrate and the core polypeptide molecular mass of glycoprotein IV.

6.2.2 Results and discussion

Deglycosylation of membrane glycoprotein IV was performed on the insoluble, phospholipid-rich pellet (P1) which is enriched in glycoprotein IV and is produced by fractionation of membrane proteins with Triton X-114.

P1 was treated with peptide: N- glycosidase F overnight in the presence of a number of protease inhibitors and the reaction products were analyzed by one- and two-dimensional electrophoresis and immunoblotting with anti-glycoprotein IV serum. Figure 6.2.1a shows the one-dimensional SDS gel immunoblot after the enzymatic deglycosylation reaction, and indicate that PNGase F, which removes N-glycans of the high mannose and complex type from the intact glycoprotein IV, reduces the molecular mass by approximately 23 kDa, and leaves a core polypeptide of glycoprotein IV with a molecular mass of about 29 kDa (lane 1) as indicated by the molecular weight markers on the left side of the blot. Glycoprotein IV in the control (mock digested) sample remains in the normal position (45-50 kDa). To confirm that the carbohydrate core of glycoprotein IV had been removed another blot was analysed with Con A-HRP complex as shown in Figure 6.2.1b. It shows that glycoprotein IV after treatment with PNGase F does not bind Con A any more (lane 2) indicating complete removal of N-linked oligosaccharide chains. These results (Figures 6.2.1a and 6.2.1b) demonstrate that approximately 45% of the mass of glycoprotein IV is N-linked oligosaccharide.

For further characterisation of the products of enzymatic deglycosylation of GpIV with PNGase F, two-dimensional electrophoresis and immunoblotting blotting using anti-glycoprotein IV serum were carried out. Figures 6.2.2a and 6.2.2b respectively show the enzymatically deglycosylated and control (mock-digested) reaction of glycoprotein IV with PNGase F. As shown in Figure 6.2.2a, the products of enzymatic degradation of glycoprotein IV by PNGase F, which appear as a single band in the immunoblot of the one-dimensional gel (Figure 6.2.1a lane 1), were resolved into two components of the same molecular mass, but with a small difference in their pI, while glycoprotein IV in the control sample remains unaffected (Figure 6.2.2b).

The cDNA-deduced sequence of Ac45 encodes an open reading frame for a protein of 468 aminoacids with a postulated N-terminal membrane signal sequence of 35 amino acids, C-terminal membrane spanning domain and seven potential N-

glycosylation sites [Supek *et al.*, 1994]. Enzymatic deglycosylation yields a protein of apparent molecular mass 29 kDa (Figure 6.2.1a lane 1).

The aminoacid sequence results (Chapter 4) suggest that the processor of M45/glycoprotein IV is further processed by cleavage between two valines (residues 246 and 247) to a mature protein of only 222 amino acids. This mature form of the protein would have an unglycosylated molecular mass of 25390 Da; the difference between this and the apparent molecular mass of 29kDa is within the error expected in estimating the molecular mass of membrane proteins from their electrophoretic mobility. Expression of the cDNA encoding Ac45 *in vitro* yields a product of 52 kDa and an even larger glycosylated form in pancreatic microsomes. This consistent with translation of the entire sequence [Supek *et al.*, 1994]. The sequence of post-translational events in the adrenal medulla remains to be elucidated.

The result shown in the immunoblot of the two-dimensional gel indicates that glycoprotein IV of chromaffin granule membrane may have more than one isomer; however it is hard to exclude the possibility that the two forms arise through a chemical modification occurring during the prolonged digestion.

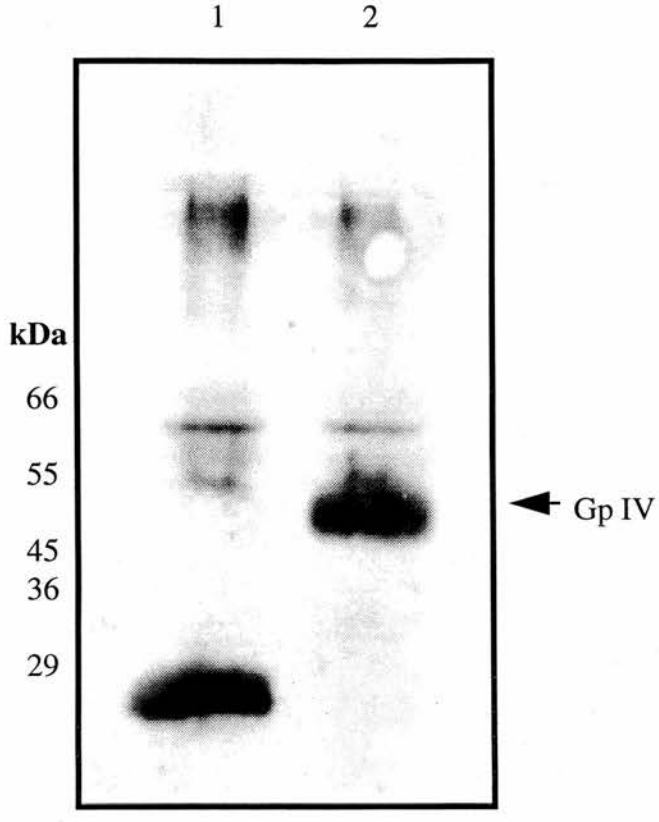


Figure 6.2.1a Partially purified V-ATPase (the P1 fraction from chromaffin granule membranes) was deglycosylated with peptide: N-glycosidase F, then the proteins were separated by SDS-PAGE followed by immunoblotting using anti-glycoprotein IV serum.
Lane 1: Enzymaticallydeglycosylated.
Lane 2: Control, mock digestion.
The scale on the left of the blot shows the position of molecular mass markers.

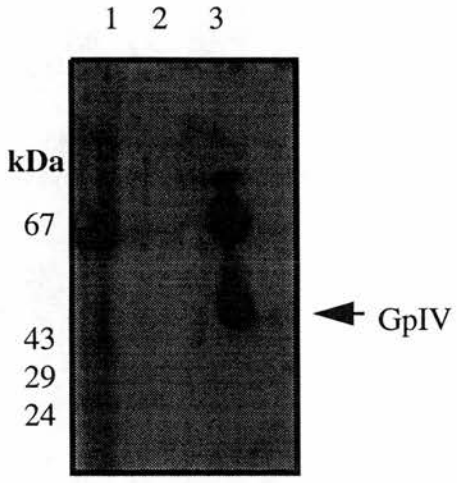


Figure 6.2.1b Partially purified V-ATPase (the P1 fraction from chromaffin granule membranes) was deglycosylated with peptide: N-glycosidase F, then the proteins were separated by SDS-PAGE followed by blotting using Con A-HRP complex.
Lane 1: molecular mass markers.
Lane 2: enzymaticallydeglycosylated.
Lane 3: Control, mock digestion.

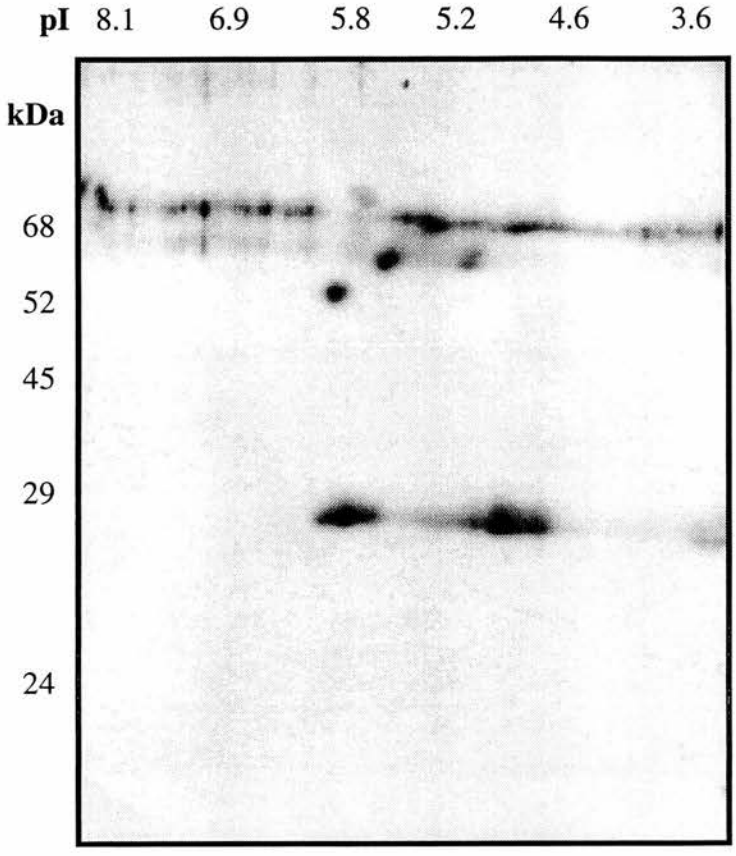


Figure 6.2.2 a The P1 fraction from chromaffin granule membranes was deglycosylated with peptide: N-glycosidase F, then the proteins were separated by electrofocusing in a first- dimension gel followed by second-dimension SDS-PAGE. Immunblotting was then performed using anti-glycoprotein IV serum.

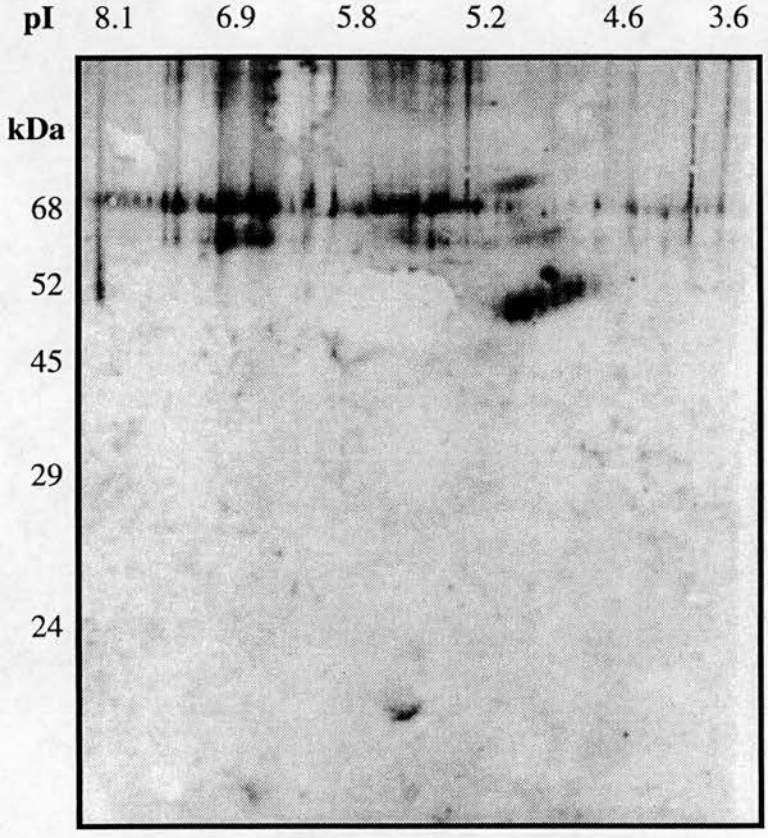


Figure 6.2.2b Control for Figure 6.2.2a the P1 fraction from chromaffin granule membranes was mock-digested (incubated without enzyme). Proteins were separated by electrofocusing in a first-dimensional gel followed by second-dimension SDS-PAGE. Immunblotting was then performed using antiglycoprotein IV serum.

6.3 Testing the glycosyl-phosphatidylinositol (GPI) anchor within membrane glycoprotein IV.

6.3.1 introduction

A variety of eukaryotic proteins are tethered to the plasma membrane by glycosyl-phosphatidyinositol (GPI) membrane anchors [Cross *et al.,* 1990; Ferguson 1991] consisting of a glycan bridge between phosphatidyl inositol and phosphoethanolamine; the phosphoethanolamine being in an amide linkage to the C-terminus of the protein. The phosphoethanolamine connects via a series of three mannose residues to non N-acetylated glycosamine residue which in turn links to a phosphatidylinositol molecule, the latter being the sole means of anchorage in the lipid bilayer . This structure is remarkably conserved throughout evolution, though the lipid moieties of GPIs are quite heterogeneous. This portion may consist of diacyl glycerol, lyso-acyl compounds or ceramide. The hydrocarbon chains also vary in length and degree of saturation, and may be present as mixtures of species [Ferguson *et al.,* 1984,1986 & 1991; Cross *et al.,* 1990].

Different protocols are designed to allow identification of GPI-anchored proteins without detailed structural analysis. Triton X-114 partitioning provides a first hint of GPI anchorage. When cellular material is extracted in Triton X-114 at low temperature, the detergent-rich solution contains amphiphilic proteins, including integral membrane proteins and species bearing GPI-anchors. Once GPI-anchor-containing proteins are released from the lipid component of the anchor, they will no longer partition into the detergent-enriched phase. This alteration of partition behaviour provides a rapid assay for the presence or absence of anchors [Brown and Rose, 1992].

Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) efficiently cleaves GPI-anchors [Low *et al.,* 1987 and 1988]. When an intact cell is digested with this enzyme or when acted on by an endogenous phosopholipase C, GPI-anchored proteins can often be released from the lipid bilayer.

After this incubation the cell mixture is centrifuged, and both the resulting pellet and supernatant are analyzed. Proteins released from GPI anchors are then found in the solution. Lack of release may indicate the absence of a GPI anchor, but a positive control is required in interpreting negative results. Among the proteins reported to be anchored on the cell surface via a GPI anchor is acetylcholinesterase [Robert *et al.,* 1988]. This enzyme was also reported to be present in chromaffin granules, in the matrix as well as in a membrane-bound form [Gratzl *et al.,* 1981]

6.3.2 Results and Discussion

In initial attempts at releasing soluble protein from chromaffin granule membranes, membranes were washed with 0.1M $Na_2 CO_3$ (pH11) in order to break open the vesicular structure and remove any extrinsic proteins [Howell and Palade, 1982, Higgins, 1984]. After washing chromaffin granule membranes were incubated with PI-specific phospholipase C as described in section 2.6. The digestion was carried out overnight, following by centrifugation; the resulting pellet and supernatant were both analyzed by immuno- and Con-A lectin blotting. Immunoblotting was carried out using specific antibodies directed against membrane dopamine β-monooxygenase, glycoprotein II, and synaptotagmin (p65), and Con A-HRP complex was used to analyze membrane glycoprotein IV.

Incubation of chromaffin granule membrane with PI-specific phospholipase C failed to yield a release of any soluble tested proteins into the supernatant fraction as shown in Figure 6.1.3 which show the effect of phospholipase C on membrane dopamine β-monooxygenase (A), glycoprotein II (B), synaptotagmin (p65) (C) and glycoprotein IV (D). That bovine membrane dopamine β-monooxygenase is not anchored via covalently-attached phosphatidyl inositol was reported previously [Stewart *et al .,* 1988]. This was based on measuring the activity of the membrane-bound enzyme following digestion with phospholipase C as well as chemical analysis of the insitol and phosphate content of the soluble form of the enzyme.

The efficiency of the PI-PLC reaction in this work was tested by measuring acetylcholinesterase activity according to the method of Ellman (1961) as described in section 2.3.7.

The activity of enzyme was measured in both pellet and supernatant fractions from the reaction mixtures (digested and control). The activity of this enzyme was found in the supernatant of digested reaction as well as in the pellet of control reaction, indicating the release of acetylcholinesterase from the membrane and the efficiency of the PI-phospholipase C.

Table 6.1 shows the measured activity in μmol/ml/min in the above samples.

Control reaction		Digestion reaction	
Pellet	Supernatant	Pellet	Supernatant
0.279	0.014	0.02	0.230

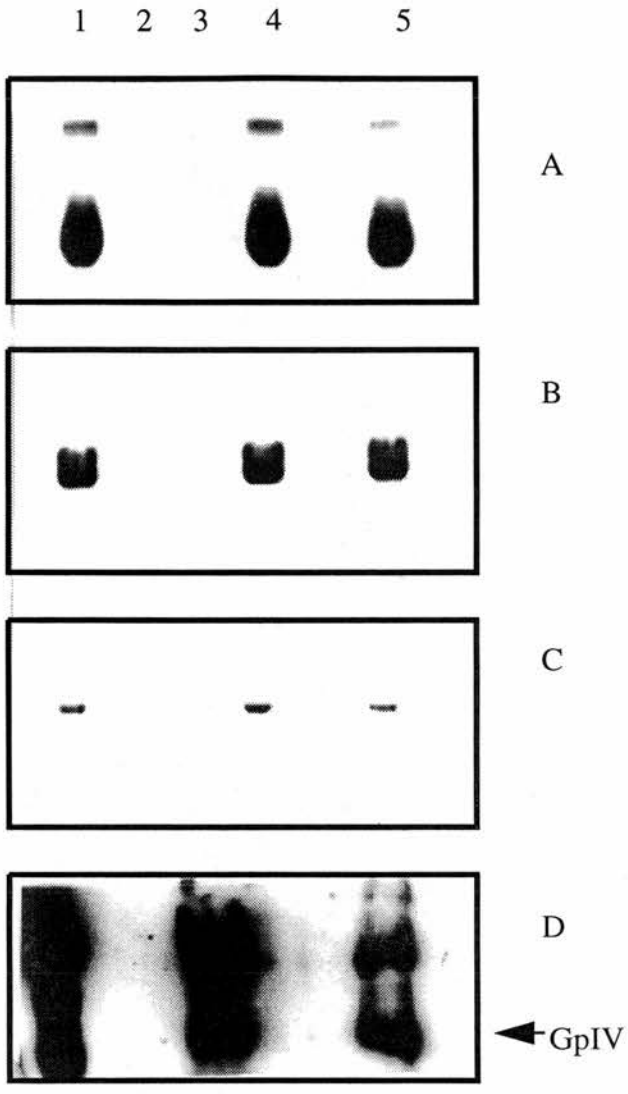


Figure 6.3.1 Chromaffin granule membranes were digested with phosphatidylinositol-specific phosoholipase C, then proteins were separated one-dimension SDS-PAGE. Blotting was then performed using antidopamineβ-monooxygenase (A), antiglycoprotein II (B), antisynaptotagmin (C), and Con A-HRP complex (D).
Lane 1: pellet of control.
Lane 2: supernatant of control.
Lane 3: supernatant of digestion.
Lane 4: pellet of digestion.
Lane 5: chromaffin granule membranes
(track 5 in D is P1 from chromaffin granule membrane).

6.4 Topography of glycoprotein IV within the chromaffin granule membrane

6.4.1 Introduction

Several studies have been made to investigate the topography of components within the chromaffin granule. Early studies of membrane protein topography indicated that a number of membrane proteins are glycosylated (Eagles *et al.,* 1975; Huber *et al.,* 1979; Cahill and Morris 1979], and use of galactose oxidase digestion followed by radioactive borohydride labelling suggested that their carbohydrate moieties are on the matrix side of the membrane [Huber *et al.,* 1979].

The organisation of some proteins within the membrane has been investigated using immunological techniques, specific antisera directed against these proteins being used to analyse the result of digesting intact granules and broken membranes with proteinases. The result of this study indicated that cytochrome b-561 is exposed on both surfaces (i.e it is a transmembrane protein), whereas dopamine β-monooxygenase is exposed only on the inner (matrix) surface of the granule membrane; also that part of glycoprotein II is exposed on the outside of chromaffin granule and can be degraded by exogenous proteinases, as was confirmed later by amino acid sequencing [Abbs and Phillips, 1980; Hieber *et al.,* 1993]. Similar studies have been done on synaptotagmin (p65) [Tugal *et al.,* 1991]: intact granules and broken membranes were digested with trypsin and investigated with monoclonal antibodies directed against synaptotagmin (p65), which indicated that this protein also has a transmembrane topography. Complement fixation experiments with antiglycoprotein III serum showed that this antiserum reacted strongly with broken membranes but not with intact granules, indicating that the antigenic determinants of glycoprotein III together with those of dopamine β-monooxygenase are exposed on the matrix surface of the membrane [Fischer-Colbrie *et al.,* 1984].

The topography of the subunits of chromaffin granule membrane ATPase I was also investigated by Apps *et al.,* (1989), who found that the subunits of 120, 72, 57, 41 and 17kDa in intact granules were accessible to N-hydroxysuccinimidylbiotin (SNHS-biotin) and were degraded by trypsin indicating that all of these subunits are exposed on the cytoplasmic side of the granule membrane. 120 and 17kDa subunits are insoluble during the washing of the membrane at pH11 which showed that these two subunits are intrinsic membrane proteins. Treatment with endoglycosidase F confirmed that only the subunit of 120kDa is glycosylated.

In the present work an attempt was made to study the organisation of glycoprotein IV within the chromaffin granule membrane and the location of this protein in other tissues.

6.4.2 Results and discussion

To establish the membrane topology of bovine glycoprotein IV, intact granules and broken membranes were incubated with the following proteinases: trypsin, papain, pronase, proteinase K and endopeptidase V8 at a range of concentrations up to 200µg/ml. Control granule or membrane samples were incubated in the absence of any proteinase. In the case of membranes the solubilized peptides were separated from membrane-bound peptides by centrifugation, but the in case of intact granules, these were purified by centrifugation through 1.8M sucrose to remove those which had lysed during incubation with proteinases, and membranes were then prepared from the proteinase-treated granules. All samples were suspended in 10mM Hepes pH 7.0 containing proteinase inhibitors and finally dissolved in electrophoresis sample buffer containing potassium biphthalate pH4.0 to inhibit any further proteinase activity and were analysed by one-dimensional SDS-gel electrophoresis and immunoblotting using antiglycoprotein IV serum. In the case of membranes treated with trypsin or pronase, membrane glycoprotein IV was unaffected by these two proteinases, and there were no proteolysis products, since a significant band of glycoprotein IV was still present even at 100 µg/ml proteinase as shown in figures 6.4.1 and 6.4.2.

The efficiency of proteolysis was tested by probing the blot with antibody directed against synaptotagmin (p65) (Figure 6.4.3). In the case of membranes treated with papain, glycoprotein IV was unaffected at concentrations of papain up to 50μg /ml but above that glycoprotein IV was completely degraded without producing any immunoreactive product in the supernatant (Figures 6.4.4a and b).

In the case of broken membranes treated with proteinase K a major soluble antibody-reactive peptide of 36 kDa was released at 0.01 and 0.2μg /ml, but above this concentration glycoprotein IV was completely degraded as shown in Figure 6.4.5a. In the case of broken membranes treated with endopeptidase V8, as shown in Figure 6.4.6a, a major soluble antibody-reactive peptide of 38 kDa was released at 0.01,0.2, 10, and 75 μg/ml V8 . After obtaining these results proteinase K and V8 were used to digest the intact granules. Although the major soluble peptide of 36kDa was released from membranes with as little as 0.01μg/mg proteinase K apperently by cleavage at a proteinase K-hypersensitive site, this peptide was not seen, nor were any other immunoreactive products, on treatment of granules with proteinase K.

Glycoprotein IV in intact granules was not affected by proteinase K since a significant band of full size is still present even at 200 μg/ml as shown in Figure 6.4.5b. On treatment of intact granules with V8 proteinase, no soluble products were released, and intact glycoprotein IV was still present even at 200μg/ml proteinase as shown in Figure 6.4.6b. These results indicate that most of the sequence of glycoprotein IV is located within the granules and that there is no proteinase-sensitive site on the cytoplasmic side of the granule membrane.

The hydropathy blot of the Ac45 (M45) subunit of V-ATPase from chromaffin granule membrane indicates the presence of a single transmembrane segment close to the C-terminus of the polypeptide. V8 cleavage of Ac45 gives a fragment containing only one potential glycosylation site very close to the transmembrane helix. It has been suggested that the protein is synthesised with a signal sequence that directs it into the ER and following cleavage and glycosylation, it is attached to the membrane with the C-terminal helix, leaving the glycosylation site on the lumenal side of the membrane [Supek *et al.*, 1994].

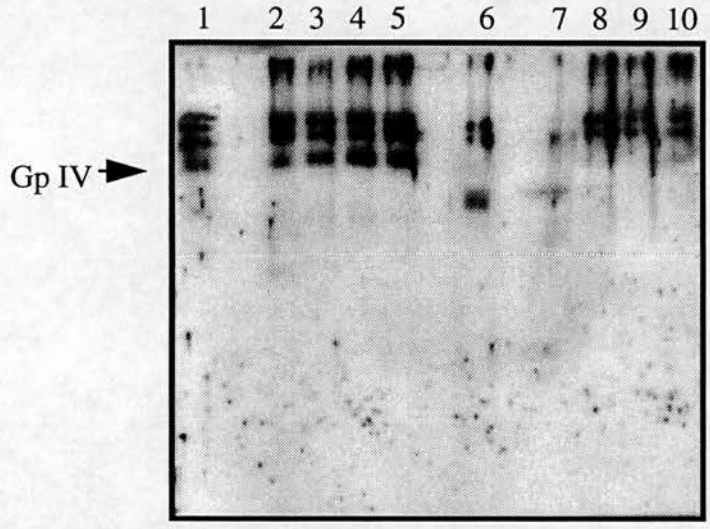


Figure 6.4.1 Chromaffin granule membranes were incubated with a range of trypsin concentrations (0.01-100μg). The soluble and insoluble fractions were examined separately by immunoblotting with antiglycoprotein IV serum.
Lane 1 is the control incubation without trypsin.
Lanes 2-5 are membrane-bound fractions from membrane incubated with 0.01, 2, 5 and 100μg/ml trypsin respectively.
Lanes 7-10 are the soluble fraction from the same incubation.
Lane 6 contained the molecular weight markers.

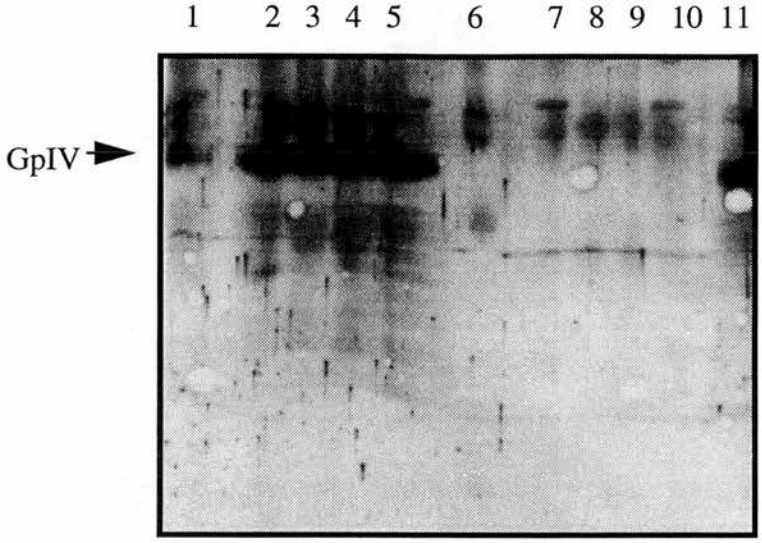


Figure 6.4.2 Chromaffin granule membrane were incubated with a range of pronase concentrations (0.01-100µg). The soluble and insoluble fractions were analysed separately by immunoblotting using anti-glycoprotein IV serum.
Lanes 1 and 11 are controls without pronase.
Lanes 2-5 are membrane-boundfractions incubated with 0.01, 2, 5 and 100µg/ml pronase respectively.
Lanes 7-10 are the soluble fractions from the same incubations

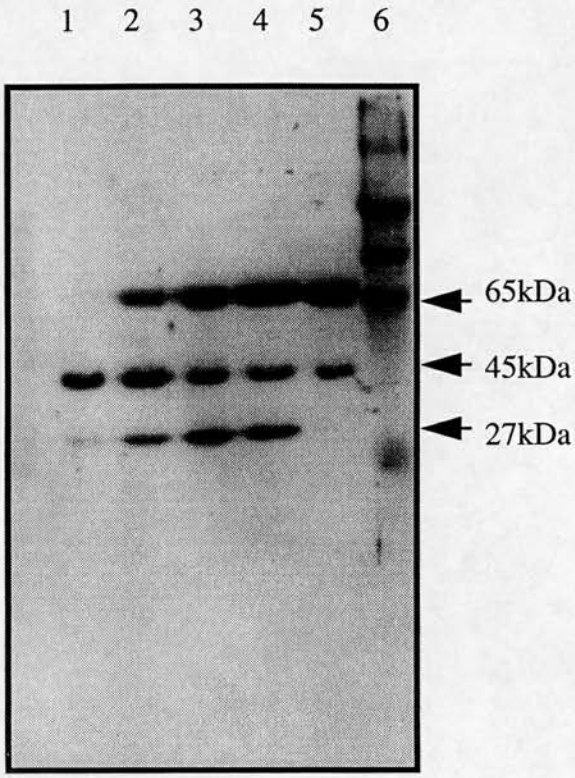


Figure 6.4.3 Chromaffin granule membranes were incubated with a range of trypsin concentrations 0.1-100µg/ml. The proteins were then separated by one-dimension SDS-PAGE followed by immunoblotting using antisynaptotagmin (p65) monoclonal antibody.
Lane 1: 100µg/ml
Lane 2: 5.0µg/ml
Lane 3: 2.0µg/ml
Lane 4: 0.1µg/ml
Lane 5: 0.0µg/ml
Lane 6: molecular mass markers.

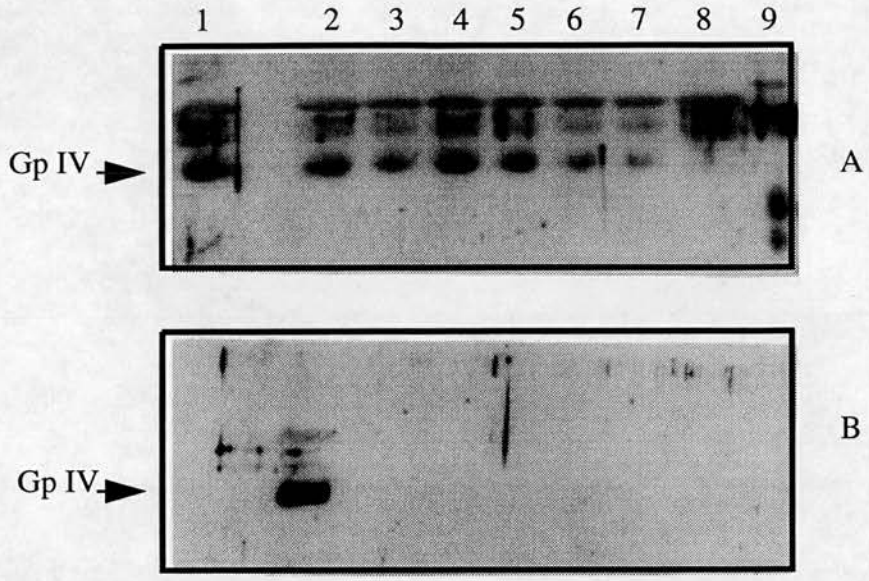


Figure 6.4.4 Chromaffin granule membranes were incubated with a range of papain concentrations (0.01-200μg/ml). The soluble and insoluble fractions were examined separately by immunoblotting with antiglycoprotein IV serum.
Blot A:
Lane 1 control incubation without papain.
Lanes 3-8 are membrane-bound fractions from membrane incubated with 0.01, 0.2, 0.5, 50, 100 and 200μg/ml papain.
Blot B:
Lane 1 control incubation without papain.
Lanes 3-9 are soluble fractions from the same incubation (blot A)

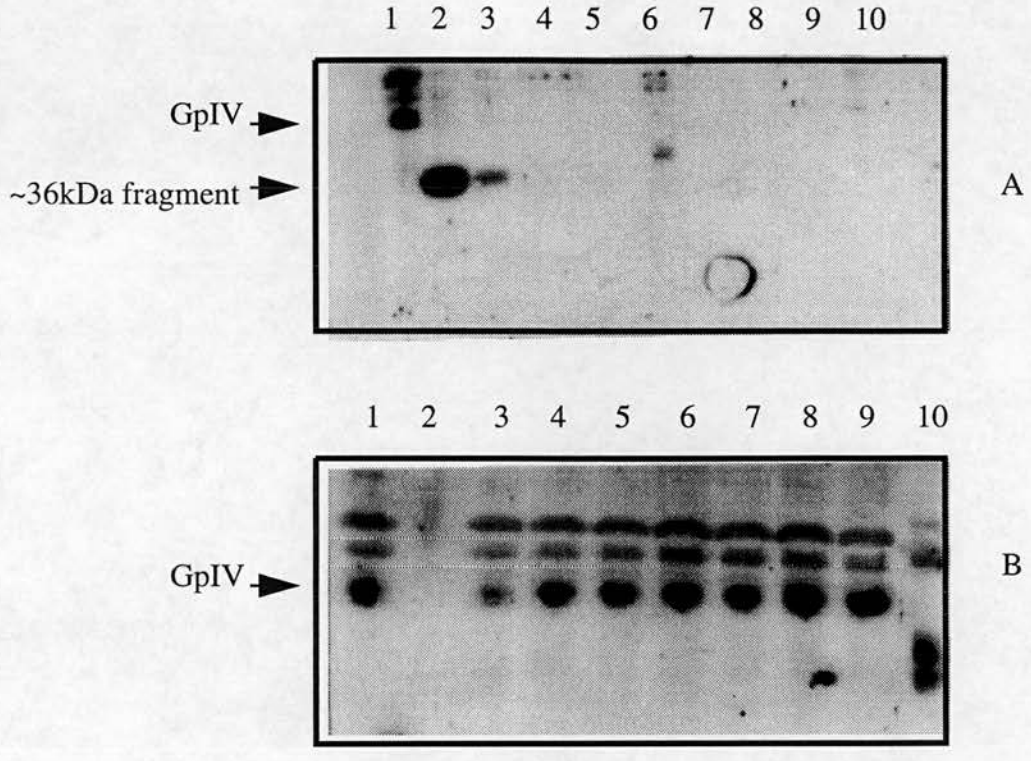


Figure 6.4.5 Chromaffin granule membranes (A) and intact granules (B) were incubated with a range of proteinase K concentrations (0.01-200 µg/ml). Soluble and insoluble fractions were examined separately by immunoblotting with antiglycoprotein IV serum.
Blot A:
Lane 1 control incubation without enzyme,
Lanes 2-5 are soluble fractions from membrane incubated with 0.01, 2, 5 and 100 µg/ml proteinase K respectively,
Lane 6 is molecular mass marker, and
Lanes 7-10 are insoluble fractions from the same incubation.
Blot B:
Lane 1 is control incubation without enzyme,
Lanes 3-9 are insoluble fractions from intact granules incubated with 0.01, 0.05, 1, 2, 5, 100, 200 µg/ml proteinase K respectively and
Lane 10 molecular mass marker.

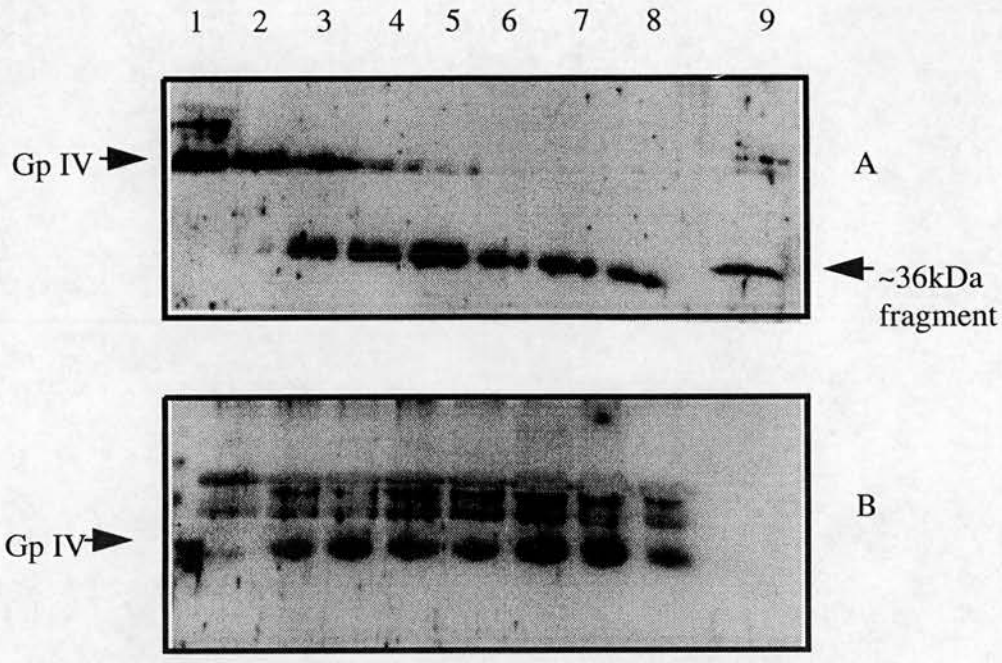


Figure 6.4.6 Chromaffin granule membranes and intact granules were incubated with a range of V8 proteinase concentrations (0.01-200μg/ml). The products were examined by immunoblotting using anti-glycoprotein IV serum.
Blot A (broken membranes):
Lane 1: control digestion without V8
Lanes 2-8 are the products from membrane incubated with 0.01, 0.25, 5, 50, 100, 200 and 500 μg/ml V8.
Lane 9: molecular mass markers.
Blot B intact granules incubated with the same V8 proteinase concentrations.

6.5 Localisation of glycoprotein IV

6.5.1 Introduction

A number of chromaffin granule membrane proteins have been found in tissues other than the adrenal medulla following of immunological techniques. For example cytochrome b651 was found in both large dense core and small dense core vesicles of sympathetic neurones as well as in the secretory granules of the anterior and posterior pituitar [Duong *et al.*, 1984; Prus s and shepard, 1987]. Glycoprotein II has been found in the membrane of kidney lysosomes [Weiler *et al.*, 1990] and glycoprotein III has also been found in several endocrine tissues and in the brain, kidney, liver and serum [Laslop *et al.*, 1993] (for more details see introduction, Chapter I). Glycoprotein IV was investigated in chromaffin granules of horse and pigs by PAS staining which seemed to indicate that this protein is absent in these organelles [Huber *at al.*,1979]. In this present work another attempt was made to investigate the localisation of glycoprotein IV using immunological techniques. Since the identification of glycoprotein IV as one of the subunits of chromaffin granule membrane H^+-ATPase was made using BN-PAGE/Tricine SDS PAGE. This technique was used to investigate glycoprotein IV in partially purified kidney microsomal V-ATPase and in the ATPase of chromaffin granules isolated from human phaeochromocytoma, also one-dimensional SDS-PAGE followed by immunoblotting using antiglycoprotein IV serum was used with membrane from other sources in this work.

6.5.2 Results and discussion

Membrane proteins obtained from chromaffin granules isolated from human phaeochromocytoma were analysed using two-dimensional BN-PAGE followed by immunoblotting using antiglycoprotein IV serum as shown in Figure 6.5.1.

Antiglycoprotein IV serum cross- reacted with a protein which apparently has the same molecular mass as glycoprotein IV from bovine chromaffin granule

membranes and present in the V_o component of the ATPase (as well as V_oV_1 , the holoenzyme) The protein is also found in human neuroblostoma cells (Figure 6.5.4, lane 5).

Bovine kidney microsomal membranes were prepared as described in section 2.2.2, and fractionated with Triton X-114 to give a P1 fraction enriched in renal tubule H^+-translocating ATPase [Gillespie *et al.,* 1991]. The P1 fractions was analysed using one dimensional SDS-PAGE followed by immunoblotting using antiglycoprotein IV serum as shown in figure 6.5.2, lane 4. Kidney membranes were also investigated by BN-PAGE/ SDS-Tricine PAGE followed by immunoblotting using antiglycoprotein IV serum as shown in Figure 6.5.3. Antiglycoprotein IV serum did not crossreact with any protein similar to bovine glycoprotein IV in the one-dimensional gel. Although there was apparent reactivity of the serum with a band of about 55kDa, this was much sharper than is usually found for glycoprotein IV, and the characterisitic spot is not seen in the position of V_oV_1 or V_o in the two-dimensional BN-PAGE/ Tricine SDS-PAGE electrophoretograms.

Recently the V_o sector of V-ATPase (subunits Ac39, Ac116 and subunit c) was shown to be associated with other proteins such as SV2 synaptobrevin and synaptotagmin in rat synaptic vesicles [Galli *et al.,* 1996]. Western blotting was used to look for glycoprotein IV in these organelles. A sample of rat brain synaptosomes was analysed with one-dimensional SDS-PAGE and immunoblotting as shown in Figure 6.5.2 lane 5 and this did not cross-react with antiserum against glycoprotein IV, which may indicate that this protein is absent from V-ATPase of rat synaptic vesicles. As shown in Figure 6.5.1 the antiserum reacted with membrane from AtT-20 cells (transformed cell of mouse anterior pituitary, lane 3) and PC-12 (rat phaeochromocytoma cell line, lane 4) so the lack of crossreactivity is not due to the species-specificity of the antiserum. It is noteworthy that Supek *et al.,* (1994) also failed to detected GpIV (Ac45) in the mouse brain synaptic vesicles, although, in contrast to the present work, these researchers reported Ac45 to be present in bovine kidney V-ATPase.

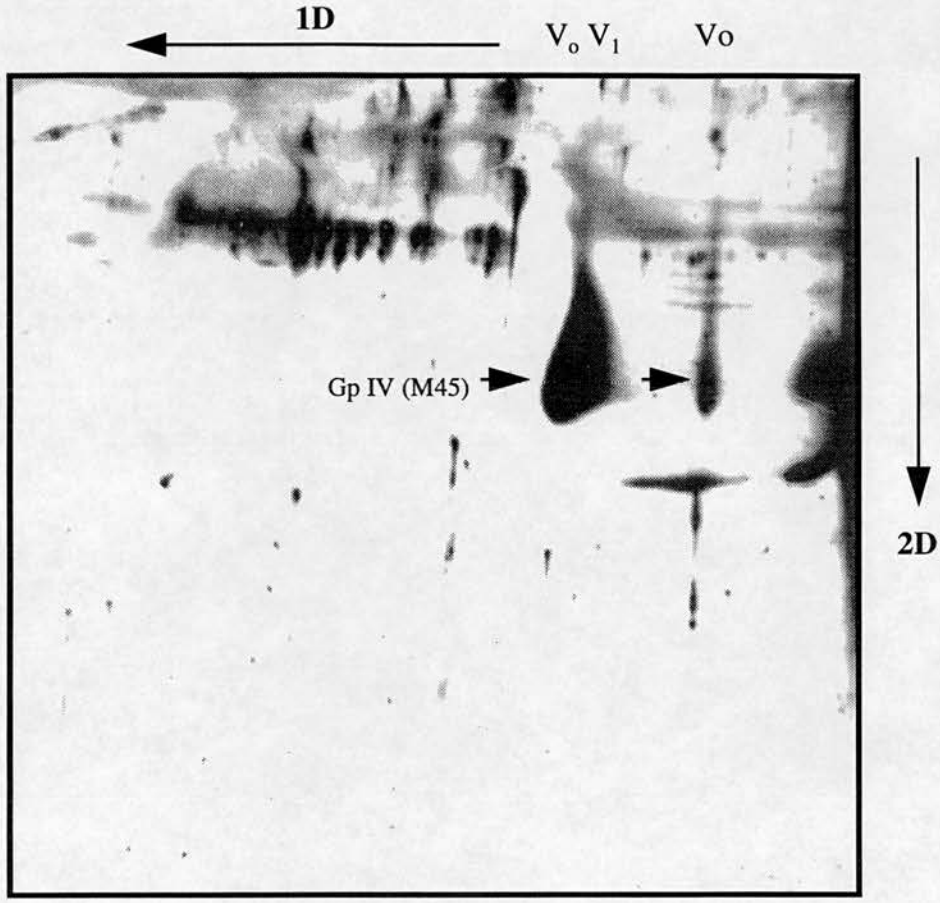


Figure 6.5.1 Chromaffin granule membrane proteins from human phaeochromocytoma were separated by BN-PAGE followed by SDS-tricine PAGE. Immunoblotting was then performed using antiserum directed against bovine chromaffin granule membrane glycoprotein IV.

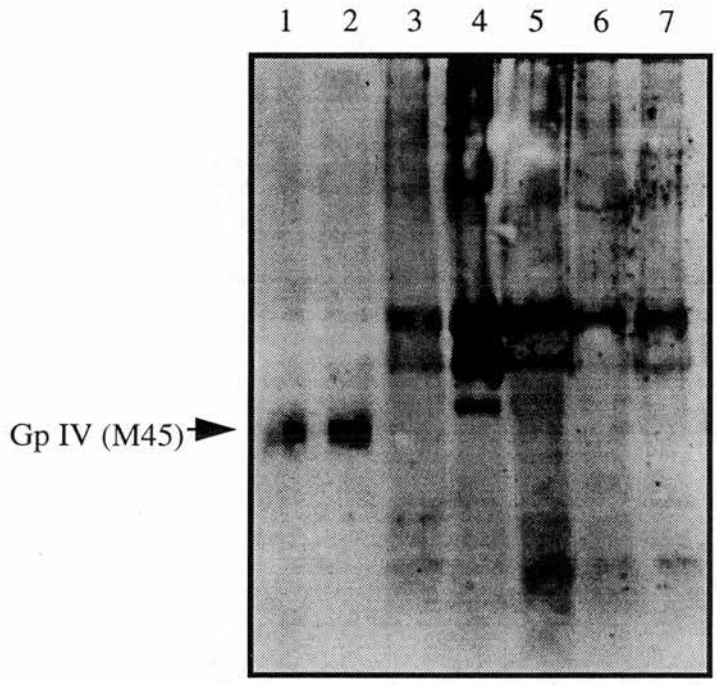


Figure 6.5.2 Proteins from different secretory cell membranes were separated by SDS-PAGE. Immunoblotting was then performed using anti-glycoprotein IV serum. Immunoreactivities were detected using ECL.
Lanes 1 and 2 : bovine chromaffin granule membranes,
Lane 3 : P1 fraction from rat insulinoma granules,
Lane 4 : P1 fraction from bovine kidney microsomal membranes,
Lane 5 : Rat brain synaptosomes.
In each case approximately 20 μg proteins was loaded

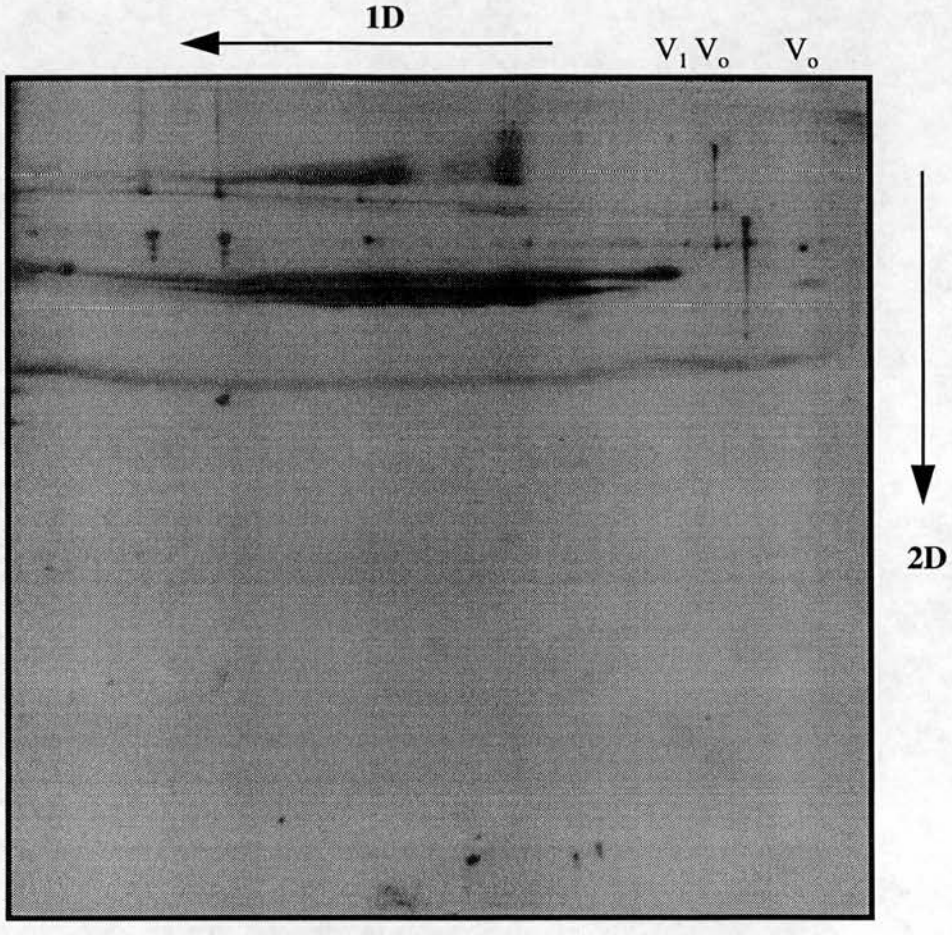


Figure 6.5.3 Kidney microsomal membrane proteins were separated by Blue Native PAGE followed by SDS-tricine PAGE. Immunoblotting was then performed using antiserum directed against bovine chromaffin granule membrane glycoprotein IV.

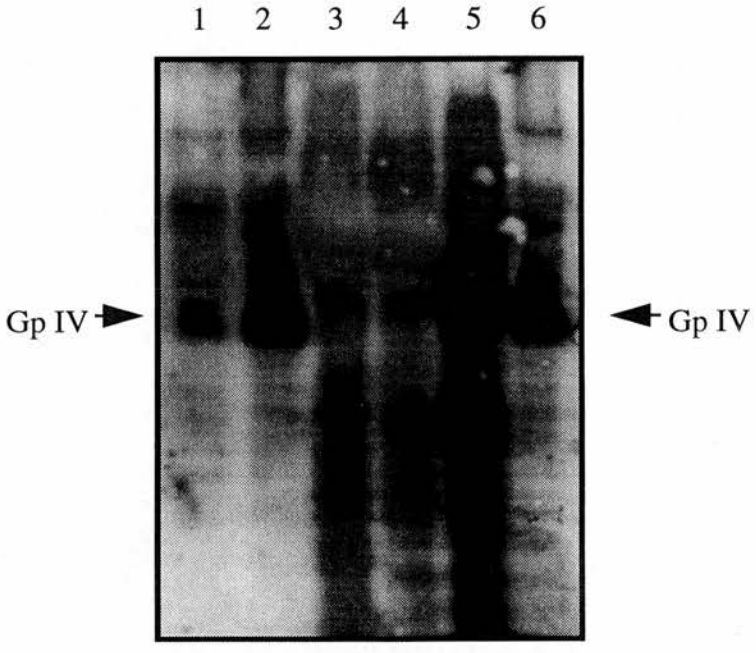


Figure 6.5.4 Proteins from different secretory cells were separated by SDS-PAGE. Immunoblotting was then performed using anti-glycoprotein IVserum. Immunoreactivities were detected using ECL.
Lane 1: bovine chromaffin granule membranes,
Lanes 2 and 6: P1 fraction bovine chromaffin granule membranes,
Lane 3: P1 fraction from mouse AtT-20,
Lane 4: P1 fraction from Rat PC-12,
Lane 5: P1 fraction from human neuroblastoma granule membranes (SH-SY5Y).

Chapter 7
Amplification of the Ac45 gene

7.1 Introduction

The theoretical basis of the polymerase chain reaction (PCR) was proposed in the 1970s, however, its practical use started only in the mid-1980s when Kary Mullis and co-workers developed PCR as a technique which could be used to generate large amounts of single-copy gene from genomic DNA [Mullis *et al.*,1986; Saiki *et al.*, 1985]. This technique rapidly found many applications and its use is still growing.

The simplicity of the reaction, as well as its speed and sensitivity, make it ideally suited to a wide variety of applications such as direct cloning from genomic DNA or cDNA, genetic fingerprinting of forensic samples, diagnosis and characterization of infectious and genetic diseases (including cancer), analysis of allelic sequence variation, analysis of RNA transcript structure, direct nucleotide sequencing of genomic DNA and cDNA, genomic footprinting, and site-directed mutagenesis [Lee *et al.*, 1987; Kawasaki *et al.*, 1988; Brisson *et al.*, 1989].

Basically, the PCR reaction is performed using double-stranded DNA containing the region to be amplified, and two single-stranded oligonucleotide primers which are complementary to sequences flank that region. In addition, there is a protein component (heat-stable DNA polymerase), deoxynucleoside triphosphates (dNTPs), buffer and salts.

The first step involves denaturing the template by heating in the presence of a large molar excess of each of the two oligonuclotide primers and four deoxynucleoside triphosphates. Annealing between the oligonuclotide primers and their target sequences occurs when the reaction mixture is cooled. After that the annealed primers are extended with DNA polymerase. The cycle of denaturing, annealing and synthesis of DNA is then repeated, so that 30 cycles should possibly result in a 10^7 fold-amplification of the desired product. Various PCR methods for site-directed mutagenesis have been described and these methods have simplified the introduction of sequence alterations into plasmid DNAs by eliminating the necessity for a single-stranded circular DNA intermediate and multiple subcloning steps.

Previous chapters of this thesis reported three lines of evidence about the relation of glycoprotein IV to Ac45. These are N-terminal amino acid sequence data from glycoprotein IV, immunological crossreactivity and the demonstration that glycoprotein IV is a subunit of chromaffin granule membrane H^+-ATPase (Chapters 4, 5and 6).

In this present work a PCR approach was used to test whether glycoprotein IV and Ac45 are derived from the same gene product. Accordingly three primers were designed corresponding to the Ac45-N-terminal, glycoprotein IV-N-terminal and Ac45-C-terminal as shown in Figure 7.1. PCR reactions were performed using these primers with bovine adrenal medulla cDNA library as a template.

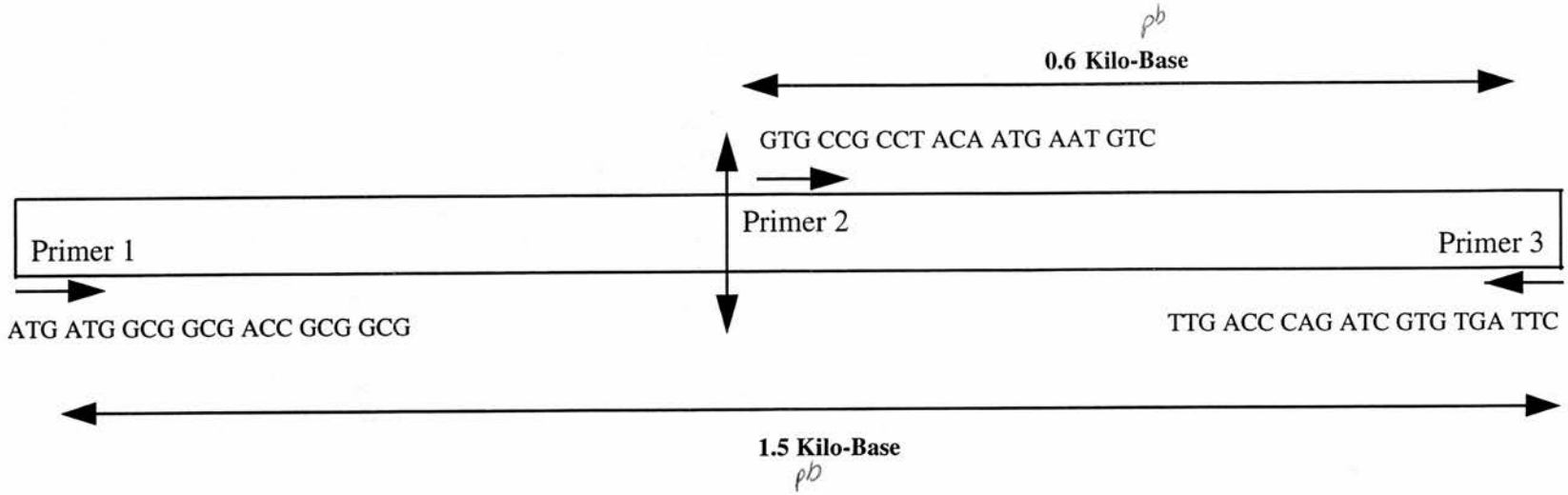


Figure 7.1 PCR reaction was carried out by designing three primers corresponding to:
primer 1: Ac45 N-terminal,
primer 2: Glycoprotein IV N-terminal,
primer 3: Ac45 C-terminal.

7.2 PCR to characterize immunoreactive clones

PCR was performed using bovine adrenal medulla cDNA library (10ng) which served as a template, the reactions being primed with degenerate synthetic oligonucleotides (listed below) in a total volume of 100µl. Two PCR reaction were set up:

reaction 1 contains primers 1+3

reaction 2 contains primers 2+3

1- ATG ATG GCG GCG ACC GCG GCG primer 1 Tm = 62 °C

2- GTG CCG CCT ACA ATG AAT GTC primer 2 Tm = 60 °C

3- TTG ACC CAG ATC GTG TGA TTC primer 3 Tm = 60 °C

Reactions were carried out with

2µl template (1ng)

10µl dNTPS (8mM each)

2µl each primer (10µM)

10µl Taq (10x) polymerase buffer

1µl Taq polymerase (2.5 units/µl)

6µl $MgCl_2$ (25mM)

58 µl water

100µl mineral oil was layered on the top of the reaction mixture. After 30 cycles of denaturation (94°C, 2min), annealing (55-60 °C, 2min), and polymerisation (72°C, 1.5min), the reaction products were separated on 1%agarose gels and visualized with ethidium bromide.

7.2.1 Agarose gel electrophoresis

1% agarose was melted in 100ml of TBE buffer, and ethidium bromide was added to a final concentration of 0.5 µg ml^{-1}. The molten gel was cast in a BRL mini-gel system. PCR product samples (500ng) were loaded onto the gel with standard 1Kilo-base DNA markers. A potential of 100V was applied until the bromophenol blue migrated to the appropriate distance through the gel. DNA bands were then examined by UV light.

7.2.2 Purification of DNA from agarose gels

After separation of reaction products by gel electrophoresis, the bands of interest were located using a short wave UV light transilluminator for the minimum length of time to avoid damage to the DNA. Bands were cut out of the gel using a sterile scalpel blade and placed into a sterile microfuge tube prior to starting DNA purification using the QIAEX II kit according to the manufacturer's instructions.

The quality of prepared DNA was determined by measuring the ratio of A_{260} to A_{280}. For pure DNA, the ratio should be higher than 1.5. According to the assumption that a DNA solution of 50 μg ml^{-1} has an A_{260} value of 1, the concentration of the prepared plasmid DNA can be determined.

7.2.3 Ligation and transformation

The purified PCR product was subcloned into PGEM-T vector according to the manufacturer's protocol (Promega). The molar mass ratio for DNA molecules was estimated using the following formula;

[ng vector x insert size (kb) / vector size (kb)] x molar ratio of insert = ng of vector required.

4μl (26ng) DNA was used and the ligation reaction was initiated by the addition of 1μl (50ng) PGEM-T vector, 1μl T4 DNA ligase, 1μl T4 (10x) ligase buffer and the reaction volume was made up to 10μl. A control sample was run without DNA. Ligation was carried out at 4°C overnight. 100μl competent NM522 cells (prepared using the calcium choride method outlined in Sambrook *et al.*,1989) were added to the entire ligation mix and stored on ice for 30 minutes.

The transformation mix was subjected to heat shock (42°C for 3 minutes) and then 5 ml LB added. The transformed cells were incubated at 37° C for 1 hour, harvested by centrifugation (2,000 rpm,10min, 4°C) and resuspended in 100μl LB. The resuspended cells were divided into 10μl, 30μl and 60μl aliquots, which were spread onto X-gal plates (LB agar containing 50μg/ml X-gal, 50μg/ml IPTG and 100μg /ml ampicillin).

The plates were incubated overnight at 37°C. Recombinant and non-recombinant transformants were distinguished by the hydrolysis of the chromogenic substrate X-gal. Transformants containing non-recombinant PGEM-T were visualized as blue colonies. The blue colour derives from the fact that the NM522 cells encode the N-terminal region of β-galactosidase which complements the C-terminal portion encoded by PGEM-T to produce an active β-galactosidase which hydrolyses X-gal to produce the blue dye. Recombinant transformants contain a disrupted C-terminal region of the β-galactosidase and so they fail to produce a functional β-galactosidase and remain white.

7.2.4 Analysis of recombinants by plasmid isolation and restriction

Colonies obtained from selective plates must be analysed to ensure that they result from transformation with the desired plasmid. Individual colonies were selected from transformation plates and used to inoculate 5ml LB containing 100μg/ml ampicillin. The cells were grown overnight at 37°C and subjected to the plasmid miniprep method outlined in Sambrook *et al.*,1989. Aliquots of the plasmid preparation were digested with a range of restriction enzymes. All restriction enzyme cleavages were performed according to manufacturer's instructions. The desired amount of DNA was digested with 1μl of diluted restriction enzyme (corresponding to at least 1 unit. μg^{-1} of DNA) and 2 μl of restriction buffer. The reaction was made up to 20μl with sterile distilled water and incubated at 37°C for 2-3 hours and the resultant restriction fragments were analyzed by agarose gel electrophoresis.

7.3 Results and Discussion

Preliminary PCR reactions were carried out as an attempt to see whether Ac45 and glycoprotein IV are encoded from the same gene product by post-translational processing. Bovine adrenal medulla cDNA library was used as a template and degenerate oligonucleotides (listed in section 7.2) as primers. It was predicted that primers 1 and 3 would anneal to the sequence coding for Ac45 and primers 2 and 3 would anneal to the sequence coding for mature GpIV. Following 30 cycles of PCR, the reaction mixture containing primers 1and 3 should in theory contain products of 1.5kb, while the reaction mixture containing primers 2 and 3 should in theory contain a product of 0.6kb as shown in diagram 7.1. Analysis of the PCR products by agarose gel electrophoresis revealed the production of cDNA species in each reaction as shown in Figure 7.2.

One of the products of the reaction containing primer 1 and 3 is of 1.5kb and a definitive way to characterize this PCR product would be to subclone and sequence it. Five PCR reactions were set up and analysed by agarose gel electrophoresis. After analysis all PCR products of 1.5kb were pooled in a 1.5 ml microfuge tube and then subjected to agarose electrophoresis. The band of 1.5kb was excised from the agarose gel and purified using QIAEX II kit according to the manufacturer's instructions. The purified DNA was ligated into the pGEM-T vector, then the entire ligation mixture was used to transform competent cells using *E. coli* NM522 as a host strain. The transformants containing recombinant DNA were detected by the presence of white colonies. Individual white colonies were used for a plasmid miniprep. Plasmids isolated from single colonies were digested with Sal I and SacII restriction enzymes and analysed by gel electrophoresis as shown in Figure 7.3.

From this work the digestion product from a recombinant plasmid should be around 1.5Kb (1.5Kb cDNA insert + 0.1Kb) as can be seen from Figure 7.4 There is a Sac II site in the gene for Ac45 but it is close to the pGEM-T/AC45 junction so will not reduce the fragment size substantially (Figure 7.4) . However as shown in Figure 7.3 the digestion does not produce any fragment of this size.

After obtaining this result the Ac45 nucleotide sequence was analysed on a computer program to provide additional restriction site information for further restriction analysis (Figure 7.5) of the 1.5kb product from the PCR. The computer analysis of Ac45 showed that a digest with Bam H1and Hinc II should give the fragments of 0.42kb and 0.81kb respectively. But the digestion of the 1.5 kb PCR product with these enzymes showed no fragments of the predicted size as shown in Figure 7.6. A control digestion was performed with these enzymes and they were found to be fully active. This suggests that the 1.5kb PCR product was not Ac45. This result was not expected since the primers were designed using the bovine Ac45/GpIV sequence, however the PCR reactions gave either no product or many products. This may be explained by the fact that the library used in this work had been amplified, and it is possible that some of the cloned cDNAs contained in it had been lost during this amplification procedure. Also it was not known whether primers 1 and 3 would amplify a product at all, while primers 2 and 3 should definitely have amplified the gene encoding the glycoprotein IV which had been sequenced in this work, but no product of expected product (0.6 kb) was produced. It is therefore probably that the cDNA encoding glycoprotein IV was not represented in this library and future work should focus on using a new library.

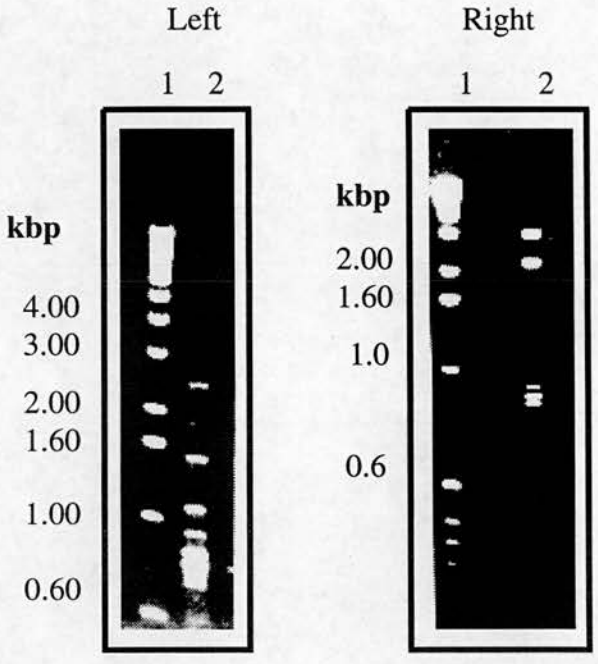


Figure 7.2 PCR was performed using bovine adrenal medulla cDNA library an primed with degenerated synthetic oligonucleotides listed in section 7.2. The reaction products were separated in 1% agarose gel and visualized with ethidium bromide under UV light.
Lanes 1 and 3 : 1 kilo-base molecular mass marker,
Lane 2 : products from reaction mixture containing primers 1 and 3,
Lane 4 : products from reaction mixture containing primers 2 and 3.

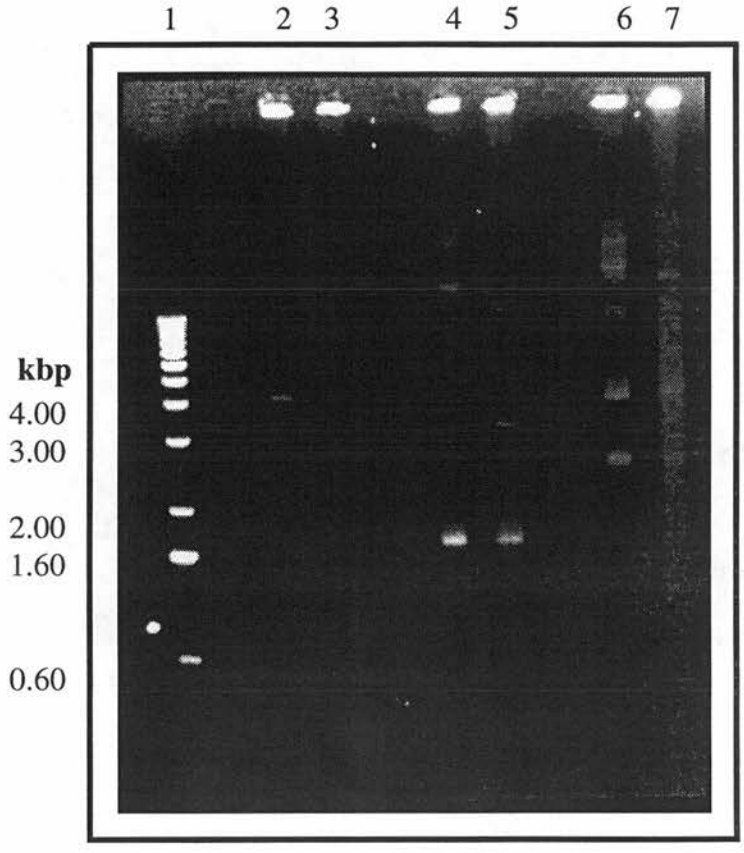


Figure 7.3 Plasmid PGEM-T vector preps were digested with restriction enzymes and the resultant of restriction products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide under UV light.
Lane 1: 1 kilo-base molecular mass markers,
Lanes 3, 5 and 7: plasmid preps of three of white colonies digested with restriction enzymes Sal I and Sac II.
Lanes 2, 4 and 6: control digestion for the above reactions.

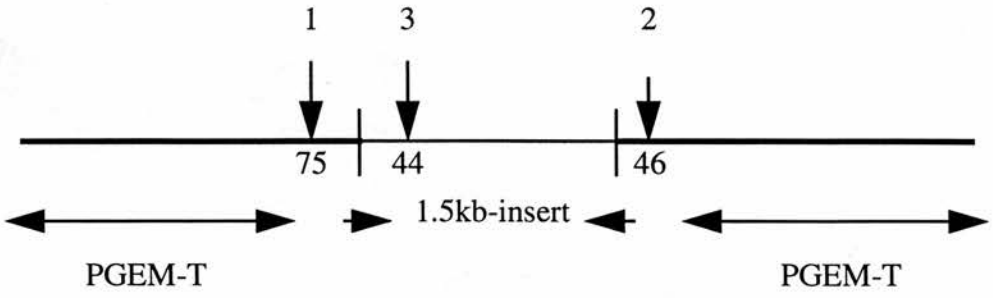


Figure 7.4 The restriction map of the 1.5 insert in plasmid PGEM-T vector
number 1 Indicates the restriction site on PGEM-T vector of one side
number 2 Indicates the restriction site on PGEM-T vector of other side
number 3 Indicates one of the restriction sites on the Ac45 insert (see Figure 7.5).

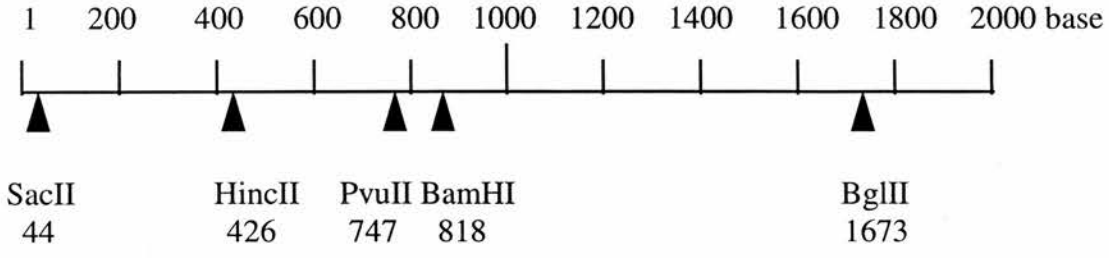


Figure 7.5 A map of the Ac45 gene showing the position of restriction sites.

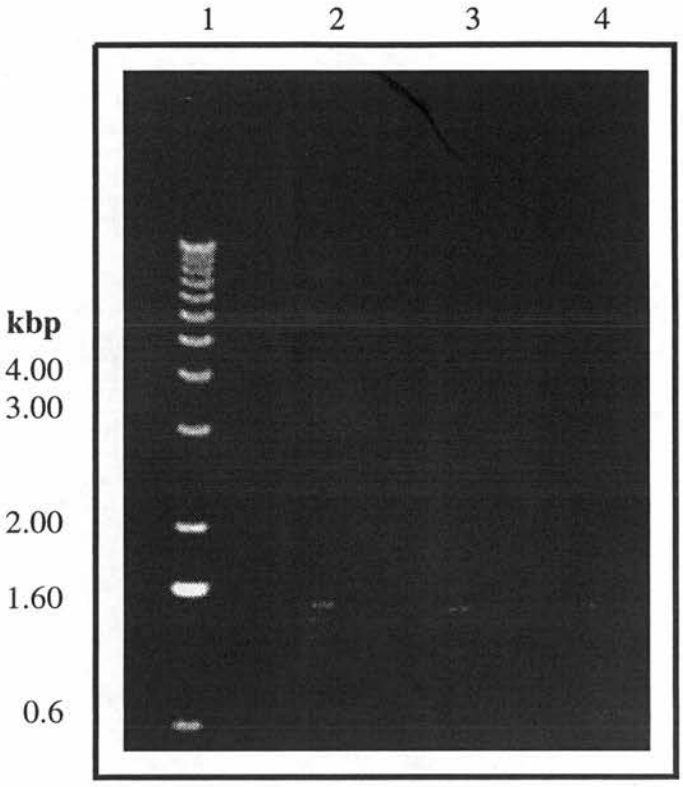


Figure 7.6 PCR products from reaction containing primers 1 and 3 wer digested with HincII and BamH1 restriction enzymes. The products were analyzed with gel agarose electrophoresis and visualized with ethidium bromide under UV light.
Lane 1: 1 kilo-base molecular mass markers,
Lane 2: PCR product was used as control for restriction reaction,
Lane 3: PCR products digested with restiction enzyme Hinc II,
Lane 4: PCR products digested with restiction enzyme Bam H1.

Chapter 8
General conclusions and future work

8.1 Conclusion

The aim of this work was the characterization of glycoprotein IV of bovine chromaffin granule membranes, a protein previously recognized only in lectin blots as a heterogeneous glycoprotein of pI range 4.8-5.3 and with an apparent molecular mass of 45-50 kDa. Although it binds concanavalin A very well and is also the most characteristic glycoprotein of the phospholipid-rich pellet phase after fractionation of membrane proteins with Triton X-114, this glycoprotein has not been previously characterized by amino acid sequencing, immunological or structural studies.

In this study glycoprotein IV was extracted from bovine adrenal chromaffin granule membranes by fractionation of the membrane with Triton X-114 and purified by affinity chromatography on Con A- agarose followed by electroelution from one-dimensional gel electrophoresis. The electroeluted GpIV was judged to be pure by electrophoresis, when the gels were stained with Coomassie Blue or when blots of the gels were decorated with Con A. This procedure provides considerable enrichment allowing us to visualize this glycoprotein for the first time using Coomassie Blue staining of one-dimensional polyacrylamide gels.

Antisera were raised against glycoprotein IV in rabbits and used for Western blotting of P1 fractions and chromaffin granule membrane proteins which had been run on one- and two-dimensional gels as well as blue-Native gels. These experiments showed conclusively that glycoprotein IV is a component of the membrane sector (V_o) of vacuolar H^+-ATPase. This result also indicated that the anti-glycoprotein IV serum recognized the same component as an antiserum directed against subunit M45 of the protein-translocating adenosine triphosphatase in the granule membrane, and confirmed that glycoprotein IV and M45 are identical proteins. Deglycosylation with Peptide:N-glycosidase F demonstrated that glycoprotein IV has a backbone with a molecular mass of 29kDa which indicated that, approximately 50% of the GpIV consists of N-linked oligosaccharides although the composition of the oligosaccharide chains has not been determined. Two-dimensional electrophoresis of the deglycosylated protein suggested heterogeneity, nut it is not known whether this represents the existence of natural isoforms, or is an artefact of the purification or deglycosylation procedure.

Study of the topography of membrane glycoprotein IV gave no evidence for the exposure of this glycoprotein on the outside of chromaffin granules and suggested that glycoprotein IV is membrane anchored as it is protected from proteolysis in intact granules but not in lysed granules. Glycoprotein IV was also tested for the existence of GpI anchor as a means of attachment to the granule membrane. Such a structure does not seem likely since glycoprotein IV was not solubilized from granule membranes by treatment with phosphatidylinositol phospholipase C.

To further characterize glycoprotein IV, the purified form of this protein was subjected to direct N-terminal amino acid sequencing. The eight amino acids obtained from this sequence (VPPTMNVP) were identified as an internal sequence (amino acids 247-254) within the published sequence of M45.

The DNA-deduced sequence of M45 encodes an open reading-frame for a protein of 468 amino acids claimed to have potential N-terminal signal sequence of 35 amino acids, a C-terminal membrane-spanning domain and seven potential N-glycosylation sites. Now our results suggest that this is further processed by cleavage between two valines (residue 246 and 247) to a mature protein of only 222 amino acids which would have an unglycosylated molecular mass of 25390 Da.

Enzymatic deglycosylation of glycoprotein IV yields a protein with a molecular mass of 29 kDa: the difference is no greater than is expected in the estimating the molecular mass of membrane proteins by electrophoresis.

Using a human cDNA clone termed CF2 as probe for Northern blotting, Yokoi *et al.*, (1994) detected transcripts in a number of tissues although the level of protein expression was not determined. Sequence comparison (Figure 8.1) shows that CF2 encodes human M45: the sequence of a similar rat cDNA is also available in the database. An open reading frame encoding a protein of just 281 amino acids was proposed, but comparison with the bovine sequence shows that the human DNA is truncated, beginning some 87 codons downstream from the start of the pre-proM45 open reading frame. Moreover the degree of sequence identity upstream from the proposed cleavage site is 93% (compared to 81% within the M45 sequence itself), which suggested that this sequence is not an untranslated leader, but is likely to be that of a functional protein.

Interestingly, the greatest sequence mismatches between the human and bovine forms of glycoprotein IV/M45 occur around the proposed cleavage site (amino acids 144-164 of prepro-M45). Human M45 appears in gels to be significantly smaller than the bovine protein(although the difference in mobility could be completely or partly due to difference in the amino acid composition). So cleavage of prepro-M45 may occur at different points in human and bovine chromaffin cells; alternatively mature M45 may be the result of an endoproteolytic cleavage followed by exoproteolysis. Study of the site of proteolysis, and characterization of the enzymes(s) involved, is an obvious development of this project. A first step would be N-terminal sequencing of M45 from human and rat. Since the N-terminal part of prepro-M45 (amino acids 1-246) has not yet been detected in chromaffin granules this could now be sought with suitable antibodies, that could be raised against defined aminoacid sequences or fusion proteins. One possibility is that this protein or a smaller polypeptide derived from it, is so far undiscovered component of the H^+-ATPase itself.

Two-dimensional Blue-Native gel electrophoresis showed that M45 is a component of the membrane sector of the H^+-ATPase, but it does not indicate the function of this protein. The anti-glycoprotein IV serum was used to study the localization of this protein in other secretory cells. This work revealed that glycoprotein IV is present in human chromaffin granule membranes, human neuroblastoma (SH-SY5Y) cells, mouse pituitary (AtT-20) cells, and rat phaeochromocytoma (PC-12) cells. This subunit has not been reported to be present in other V-ATPases, either from other mammalian tissues or from other organisms. However, as noted above, Yokoi *et al.,* (1994) detected transcript of the M45 gene in heart, brain, placenta, lung, liver, skeletal muscle and pancreas and Supek *et al.,* (1994) detected the protein in several regions of bovine brain by Western blotting.

In the present work, M45 could not be unambiguously detected either in kidney or rodent brain. There is therefore a real question about the tissues distribution of M45, which now requirs careful study using antibodies raised against M45-derived fusion proteins. Although M45 appears to be expressed in many mammalian tissues, as revealed by Northern blotting, searching the databases revealed no counterpart of

M45 in the genome of *Saccharomyces cerevisiae*. The conclusion is there is no counterpart of the M45 subunit in yeast V-ATPase (as is borne out by biochemical analysis), and this leads to the suggestion that M45 may play a role in regulating or targeting of the V-ATPase rather than ATPase activity. Several studies of yeast mutants suggest that the assembly of the enzyme starts with its membrane sector, but there is an enigma of how the V-ATPase assembles correctly in the numerous organelles in mammalian cells that contain it. The question of what directs V-ATPase to such diverse organelles as Golgi saccules, secretory vesicles, lysosomes, endosomes or plasma membrane has no current answer. V-ATPases in different membrane locations could required unique subunits responsible for proper sorting to the target membrane. The membrane sector of V-ATPase in chromaffin granules consists of a very conserved polypeptide called the proteolipid (ductin) as well as two less conserved polypeptides (M115 and M39) present in all known V-ATPases of eukaryotic cells (although tissue-specific isofforms of M115 have been reported). Consequently, specific membrane proteins may be required for the correct assembly of the different membrane sectors in different organelles. Since M45 was discovered as a membrane sector subunit in bovine chromaffin granule membranes V-ATPase and still there is a real question about the tissue distribution of M45, a possible function for M45 is to target the membrane sector to its specific location, and thereafter to aid in the assembly of the catalytic sector. However this role is entirely speculative, and the first test this postulate would be a careful investigation of the presence of M45 in different subcellular locations. In particular it would be interesting to discover whether M45 occurs in any plasma-membrane located V-ATPase, such as that of osteoclasts. It would be particularly interesting if M45 were confined to the V-ATPase of endocrine secretory vesicles, since the association of the subunit of the membrane sector of V-ATPase with synapobrevin-synaptophysin complex formation [Galli *et al.,* 1995] has been taken to suggeste that this interaction may play a role in recruiting the proton pump into synaptic vesicles. This might be tested by ''knockout''experiments, either in transgenic animals or cells transfected so as to express antisense DNA, or treated with antisense oligonucleotides.

```
bov  MMAATAAAQV  RAGTRWAPAL  CRMPWLPLML  VAAAAATSEQ  QVPLVLWSSD
rat  MMAATVVSRI  RTGTRWAPVL      WLLLSL  VAAAAVAAEQ  QVPLVLWSSD

bov  RGLWAPAADT  HEGHITSDMQ  LSTYLDPALE  LGPRNVLLFL  QDKLSIEDFT
hu                                                ECW  REELSIEDFT
rat  RDLWAPVADT  HEGHITSDMQ  LSTYLDPALE  LGPRNVLLFL  QDKLSIEDFT

bov  AYGGVFGNKQ  DSAFSNLENA  LDLAPSSLVL  PAVDWYAIST  LTTYLQEKLG
hu   AYGGVFGNKQ  DSAFSNLENA  LDLAPSSLVL  PAVDWYAIST  LTTYLQEKLG
rat  AYGGVFGNKQ  DSAFSNLENA  LDLAPSSLVL  PAVDWYAIST  LTTYLQEKLG

bov  ASPLHVDLAT  LQELKLNASI  PALLLIRLPY  TASSGLMAPK  EVLMGNDEVI
hu   ASPLHVDLAT  LRELKLNASL  PALLLIRLPY  TASSGLMAPR  EVLTGNDEVI
rat  ASPLHVDLAT  LKELKLNASL  PALLLIRLPY  TASSGLMAPR  EVLTGNDEVI
                                                    ▼
bov  GQVLSTLKSE  DIPYTAALTA  VRPSRVARDV  AMVTGGLGRQ  LLQRTVVPPT
hu   GQVLSTLKSE  DVPYTAALTA  VRPSRVARDV  AVVAGGLGRQ  LLQKQPVSPV
rat  GQVLSTLESE  DVPYTAALTA  VRPSRVARDV  AMVAGGLGRQ  LLQTQVASPA

bov  MNVPVSYNDS  YDTRILFWAQ  NFSVAYGEHW  EDLTSRTFGV  QDLNLTGSFW
hu   IHPPVSYNDT  AP RILFWAQ  NFSVAYKDQW  EDLTPLTFGV  QELNLTGSFW
rat  IHPPVSYNDT  AP RILFWAQ  NFSVAYKDEW  KDLTSLTFGV  ENLNLTGSFW

bov  NDTVARLVLT  YDSLFGTMVT  FKFILANSYY  SVSARHWFTL  ENLEIHSNGS
hu   NDSFARLSLT  YERLFGTTVT  FKFILANRLY  PVSARHWFTM  ERLEVHSNGS
rat  NDSFAMLSLT  YEPLFGATVT  FKFILASRFY  PVSARYWFTM  ERLEIHSNGS

bov  VAYFNASQVT  GPSIYSFHCE  HVSSENEDGN  LLVPDTQPSL  WQMTFRDFQI
hu   VAYFNASQVT  GPSIYSFHCE  YVSSLSKKGS  LLVARTQPSP  WQMMLQDFQI
rat  VAHFNVSQVT  GPSIYSFHCE  YVSSLSKKGS  LLVTNV PSL  WQMTLHNFQI

bov  QAFNVTDKKF  SYASDCAGFF  SPGIWMGLLT  SLFMLFIFTY  GLHMILSLKT
hu   QAFNVMGEQF  SYASDCASFF  SPGIWMGLLT  SLFMLFIFTY  GLHMILSLKT
rat  QAFNVTGEQF  SYASDCAGFF  SPGIWMGLLT  TLFMLFIFTY  GLHMILSLKT

bov  MDRFDDHKGP  TITLTQIV
hu   MDRFDDHKGP  TISLTQIV
rat  MDRFDDRKGP  TITLTQIV
```

Figure 8.1 Alignment of the cDNA-derived sequence of bovine prepro-M45 [Supek *et al.*, 1994] with the partial human sequence [Yoloi *et al.*, 1994] and rat sequence. The proposed cleavage site in the bovine prepro-M45 is indicated by an arrow
Accession numbers of DNA sequences are:
Bovine: U10039
Human: D16469
Rat: AF035387

Appendix II

Aminoacid codes and data

Amino Acid	Abbreviations		Mw	Property
Alanine	Ala	A	89	Aliphatic
Arginine	Arg	R	174	Basic
Asparagine	Asn	N	132	Amide
Aspartate	Asp	D	133	Acid
Cysteine	Cys	C	121	Sulphydryl
Glutamine	Gln	Q	146	Amide
Glutamate	Glu	E	147	Acidic
Glycine	Gly	G	75	Aliphatic
Histidine	His	H	155	Basic
Isoleucine	Ile	I	131	Aliphatic
Leucine	Leu	L	131	Aliphatic
Lysine	Lys	K	146	Basic
Methionine	Met	M	165	Aliphatic
Phenylalanine	Phe	F	165	Aromatic
Proline	Pro	P	115	Amide
Serine	Ser	S	105	Aliphatic alcohol
Threonine	Thr	T	119	Aliphatic alcohol
Tryptophan	Trp	W	204	Aromatic
Tyrosine	Tyr	Y	181	Aromatic alcohol
Valine	Val	V	117	Aliphatic

Bibliography

Abbs MT, Phillips JH (1980) Organization of the proteins of chromaffin granule membranes. *Biochim. Biophys. Acta.* **595:**200-211.

Aberer W, Kostron H, Huber E, Winkler H (1978) A characterisation of the nucleotide uptake by chromaffin granules of bovine adrenal medulla. *Biochem. J.* **172:**353-360

Adachi I, Puopolo K, Marquez-Sterling N, Arai H, Forgac M (1990) Dissociation, cross-linking and glycosylation of the coated vesicle proton pump *J. Biol. Chem.* **265:**967-973

Aebersold RH, Teplow DB, Hood IE, Kent SB (1986) Electroblotting onto activated glass. High efficiency preparation of proteins from analytical sodium dodecyl sulfate-polyacrylamide gels for direct sequence analysis. *J. Biol Chem.* **261:**4229-4238

Anderson S, De Brujin MHL, Coulson AR, Eperon IC, Sanger F, Young IG (1982) Complete sequence of bovine mitochondrial DNA. *J. Mol. Biol.* **156:**683-717

Apps DK, Boisclair MD, Gavine FS, Pettigrew GW (1984) Unusual redox behaviour of cytochrome b561 from bovine chromaffin granule membranes. *Biochim. Biophys. Acta.* **764:**8-16

Apps DK, Percy JM, P`erez-Castiñeira JR (1989) Topography of a vacuolar-type H^+-translocating ATPase: chromaffin-granule ATPase I. *Biochem. J.* **263:**81-88

Apps DK, Pryde JG, Phillips JH (1980) Cytochrome b561 is identical with chromomembrane B, a major polypeptide of chromaffin granule membranes. *Neurosci* **5:**2279-2287

Apps DK, Phillips JH, Purves F (1985) Glycoproteins of the chromaffin granule matrix: the use of lectin blotting to distinguish several separate classes. *Neurosci.* **16:**77-487
→ Apps DK, Schatz.

Arai H, Terres G, Pink S, Forgac M (1988) Topography and subunit stoichiometry of the coated vesicle proton pump. *J. Biol. Chem.* **263:**879-802

Asamer H, Hörtnagl H, Winkler H (1971) Immunohistochemical demonstration of a membrane protein of chromaffin granules in adrenal medulla and sympathetic nerves. *Naunyn-Schmiedeberg's Arch. Pharmacal.* **270:**87-89

Ash DE, Papadopoulos NJ, Colombo G, Villafranca JJ (1984) Kinetic and

spectroscopic studies of the interaction of copper with dopamine β-hydroxylase. *J. Biol. Chem.* **259:**3395-3398

Ausubel FM, Brent R, Kingston R, Moore DD, Seidman JG, Smith JA, Strubl K (1987) Endoglycosidase and glycoamidase release of N-linked oligosaccharides In ''Current protocols in Molecular Biology''. **3:**1-16

Azaryan AV, Schiller M, Mende-Mueller L, Hook VYH (1995) Characteristics of the chromaffin granule aspartic proteinase involved in proenkephalin processing. *J. Neurochem.* **5:**1771-1779

Bajjalieh SM, Frantz GD, Weimann JM, Mc Connell SK, Scheller RH (1994) Differential experssion of synaptic vesicle protein 2 (SV2) isoforms. *J. Neurosci* **14:**5223-5235

Banerjee S, Margolis RU (1982) Peptide mapping studies of the chromogranins and of two chromaffin granule proteoglycans. *J. Neurochem.* **39:**1700-1703

Barasch J, Kiss B, Prince A, Saiman L, Gruenert D, Al-Awqati Q (1991) Defective acidification of intracellular organelles in csytic fibrosis. *Nature* **352:**70-73

Bastin JM, Kirkley J, McMichael AJ (1982) Production of monoclonal antibodies: a practical guide. in 'Monoclonal antibodies in clinical medicine' (Mc Michael AJ and Fabre JW. eds) 503-517 Academic Press, London.

Baumert M, Maycox PR, Navone F, De Camilli P, John R (1989) Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* **8:**379-384

Bartel A, Campbell D (1959) Some immunochemical differences between associated and dissociated hemocyanin. *Arch. Biochem. Biophys.* **82:**233-236

Bauw G, De Losse M, Inze D, Van Montagu M, Vanderchove J (1987) Alterations in the phenotype of plant cells studied by amino-terminal amino acid-sequence analysis of proteins electroblotted from two-dimensional gel-separated total extracts. *Proc. Natl. Acad. Sci. USA.* **84:**4806-4811

Beltran C, Nelson N (1992) The membrane sector of vacuolar H^+-ATPase is by itself impermeable to protons. *Acta. Physiol. Scand.* **146:**41-47

Benedum UM, Bauerle PA, Konecki DS, Frank R, Powell J, Mallet J, Huttner WB (1986) The primary structure of bovine chromogranin A: a representative of a class of

acidic secretory proteins common to a variety of peptidergic cells. *EMBO J.* **5:**1495-1502

Bieger SC, Henkle AW, Unsicker K (1995) Localization of basic fibroblast growth factor in bovine adrenal chromaffin cells. *J. Neurochem.* **64:**1521-1527

Birch NP, Davies AD, Christie DL (1986) Identification of a 27-kDa enkephalin-containing protein associated with bovine adrenal medullary chromaffin granule membranes by immunoblotting. *FEBS lett.* **197:**173-178

Bjerrum OJ, Bock E (1979) Immunochemically identical hydrophilic and amphiphilic forms of the bovine adrenomedullary dopamine-β-hydroxylase. *J. Biochem.* **188:**231-237

Blaschko H, Comline RS, Schneider FH, Silver M, Smith AD (1967) Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature* **215:**58-59

Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256:**1604-1607

Bowman BJ, Allen R, Wechser MA, Bowman EJ (1988) Isolation of genes encoding the *Neurospora crassa* vacuolar ATPase. Analysis of *vma-2* encoding the 57-kDa polypeptide and comperison to *vma-1*. *J. Biol. Chem.* **263:**14002-14007

Bowman BJ, Dschida WJ, Harris T, Bowman EJ (1989) The vacuolar ATPase of *Neurospora crassa* contains an F_o-like structure. *J. Biol. Chem.* 264:15606-15612

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248-254

Bradbury AE, Finnie MDA, Smyth DG (1982) Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* **298:**686-688

Briles EB, Kornfeld S (1978) Lectin-resistant cell surface variants of eukaryotic cells. *Trends Biochem. Sci.* **3:**223-227

Brown WE, Wold F (1973) Alkyl isocyanates as active site specific reagents for serine proteases. Reaction properties. *Biochemistry* **12:**828-286

Buckland RM, Radda GK, Shennan CD (1978) Accessibility of phospholipids in the chromaffin granule membranes. *Biochim. Biophys. Acta.* **513:**321-337

Buckley KM, Floor E, Kelly RB (1987) Cloning and seqence analysis of cDNA encoding p38, a major synaptic vesicle protein. *J. Cell Biol.* **105:**2447-2456

Buckley KM, Kelly RB (1985) Identification of a trans-membrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol* **100:**1284-1294

Bucsics A, Saria A, Lembeck F (1981) Substance P in the adrenal gland: origin and species distributions. *Neuropeptides* **1:**329-341

Bulenda D, Gratz LM (1985) Matrix free calcium in isolated chromaffin vesicles. *Biochemistry* **24:**7760-7765

Burger MM, Goldberg AR (1967) Identification of a tumor-specific determinant on neoplastic cell surfaces. *Proc. Natl. Acad. Sci. USA.* **57:**359-366

Burgoyne RD (1991) Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta.* **1071:**174-202.

Burgun C, Martinez de Muñoz D, Aunis D (1985) Osmotic fragility of chromaffin granules prepared under isoosmotic or hyperosmotic conditions and localisation of acetylcholinesterase. *Biochim. Biophys. Acta.* **839:**219-227

Cahill AI, Morris SJ (1979) Soluble and membrane lectin-binding glycoproteins of the chromaffin granule. *J. Neurochem.* **32:**855-867

Chamberlain LH, Henry J, Burgoyne RD (1996) Cysteine-string proteins are associated with chromaffin granules. *J. Biol. Chem.* **271:**19514-19516

Christie DL, Batchelor DC, Palmer DJ (1991) Identification of kex2-related proteases in chromaffin granules by partial amino acid sequence analysis. *J. Biol. Chem.* **266:**15679-15683

Christie DL, Palmer DJ (1990) Identification and characterization of glycoproteins after extraction of bovine chromaffin granule membranes with Lithium di-iodosalicylate. *Biochem. J.* **270:**57-61

Clegg JCS (1982) Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to adenovirus and flavavirus proteins. *Anal. Biochem.* **127:**389-394

Coupland RE (1968) Determining size and distributions of spherical bodies such as

chromaffin granules in tissue sections. *Nature* **217:**84-388

Coupland RE, Hopwood D (1966) The mechanism of the differential staining reaction for adrenaline and noradrenaline-storing granules in tissue fixed with glutaraldehyde. *J. Anat.* **100:**227-243

Coupland RE, Pyper AS, Hopwood D (1964) A method of differentiating between noradrenaline- and adrenaline-storing cells in the light and electron microscope. *Nature* **201:**1240-1242

Cunningham BA, Wand JL, Myron J, Waxdal MJ, Edelman GM (1975) The covalent and three-dimensional structure of concanavalin A. *J. Biol. Chem.* **250:**1503-1512

Da Prada M, Pletscher A, Tranzer JP (1972) Lipid composition of membranes of amine-storage organelles. *Biochem. J.* **127:**681-683

Davey MW, Sulkowski E, Carter WA (1976) Binding of human fibroblast interferon to Concanavalin A-agarose. Involvement of carbohydrate recognition and hydrophobic interaction. *Biochemistry* **15:**704-712

Dean GE, Nelson PJ, Rudneck G (1986) Characterization of native and reconstituted hydrogen ion pumping adenosinetriphosphatase of chromaffin granules. *Biochemistry* **25:**4918-4925

Degli Esposti M, Kamensky YUA, Arrutjunian AM, Konstantinov AA (1989) A model for the molecular organization of cytochrome b561in chromaffin granule membranes. *FEBS lett.* **254:**74-78

Denda K, Konishi J, Hajiro K, Oshima T, Date T, Yoshida M (1990) Structure of an ATPase operon of an acidothermophilic archaebacterium *Sulfolobus acidocaldarius*. *J. Biol. Chem.* **265:**21509-21513

de Oliveira-Filgueiras OM, Van den Besselaar AMHP, Van den Bosch H (1979) Localization of lysophosphatidylcholine in bovine chromaffin granules. *Biochim. Biophys. Acta.* **558:**73-84

Diliberto EJ, Allen PL (1980) Semidehydroascorbate as a product of the enzymatic conversion of dopamine to norepinephrine. Coupling of semidehydroascorbate reductase to dopamine β-hydroxylase. *Mol. pharmacol.* **17:**421-426

Diliberto EJ, Viveros OH, Axelrod J (1976) Subcellular distributions of protein carboxymethylase and its endogenous substrates in the adrenal medulla: possible role in excitation-secretion coupling. *Proc. Natl. Acad. Sci. USA* **73:**4050-4054

Drapeau GR (1980) Sustrate specificity of a proteolytic enzyme isolated from a mutamt of *Pseudomonase fragi.* *J. Biol. Chem.* **255:**839-845

Dreyfus H, Aunis D, Harth S, Mandel P (1977) Gangliosides and phospholipids of the membranes from bovine adrenal medullary chromaffin granules. *Biochim. Biophys. Acta.* **489:**89-97

Dumont M, Day R, Lemaire S (1983). Distinct distribution of immunoreactive dynorphin and leucine enkephalin in various populations of isolated adrenal chromaffin cells. *Life Sci.* **32:**287-294

Dunbar B, Wilson SB (1994) A buffer exchange procedure giving enhanced resolution to polyacrylamide gels prerun for protein sequencing. *Anal. Biochem.* **216:**227-228

Duong LT, Fleming PJ, Russell JT (1984) An identical cytochrome b561 ie present in bovine adrenal chromaffin vesicles and posterior pituitary neurosecretory vesicles. *J. Biol. Chem.* **259:**4885-4889

Eagles PAM, Johnson LN, Horn CV (1975) The distribution of concanavalin A receptor sites on the membrane of chromaffin granules. *J cell Sci* **19:** 33-47

Eckerskorn C, Mewes W, Goretzki H, Lottspeich F (1988) A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. *Eur. J. Biochem* **167:**509-513

Edelman GM, Cunningham BA, Reeke GN, Becker JW, Waxadal MJ, Wang JL (1972) The covalent and three-dimensional structure of concanavalin A *Proc. Nat. Acad. Sci. USA* **69:**2580-2584

Eipper BA, Mains RE. Glembot Ski CG (1983) Identification in pituitary tissue of a peptide α–amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. *Ptoc. Natl. Acad. Sci. USA* **80:**5144-5148

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetycholinesterase activity. *Biochem. Pharmacol.* **7:**88-95

Emini EA, Hughes JV, Perlow HD, Boger J (1985) Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J. Virol.* **55:**836-839

Erikson J, Eiden L, Hoffman B (1992) Expression cloning of a reserpine-sensitive

vesicular monoamine transporter. *Proc. Natl. Acad. Sci. USA* **89:**10993-10997

Etzler ME, Kabat EA (1970) Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from *Dolichos biflorus. Biocehmistry* **9:**869-877

Falkensammer G, Fischer-Colbrie R., Winkler H (1985) Biogenesis of chromaffin granules: incorporation of sulphate into chromogranin B and into proteoglycan. *J. Neurochem.* **45:**1475-1480

Feany MB, Lee S, Edwards RH, Buckley KM (1992) The synaptic vesicle protein SV2 is a novel type of transmembrane transporter. *Cell* **70:**861-867

Ferguson MAJ, Duszenko M, Lamont GS, Overath P, Cross GAM (1986) Biosynthesis of *Trypanosoma brucei* variant surface glycoprotein: N-glycosylation and addition of a phosphatidylinositol membrane anchor. *J. Biol. Chem.* **261:**356-362

Fernandez J, DeMott M, Atherton D, Mische SM (1992) Internal protein sequence analysis: enzymatic digestions for less than 10μg of protein bound to polyvinylidene difluoroide or nitrocellulose membrane. *Anal. Biochem.* **201:**255-264

Fischer-Colbrie R, Diez-Guerra J, Emson PC, Winkler H (1986) Bovine chromaffin granules: Immunological studies with antisera against neuropeptideY, [Met] enkephalin and bombesin. *Neurosci.* **18:**167-174

Fischer-Colbrie R, Frischenschlager I (1985) Immunological characterisation of secretory proteins of chromaffin granules: chromogranin A, chromogranin B and enkephalin-containing peptides. *J. Neurochem.* **44:**1854-1861

Fischer-Colbrie R, Schachinger M, Zangerle R, Winkler H (1982) Dopamine β-hydroxylase and other glycoproteins from the soluble content and membranes of adrenal chromaffin granules: isolation and carbohydrate analysis. *J. Neurochem.* **38:**725-732

Fischer-Colbrie R, Schober M (1987) Isolation and characterization of chromogranins A, B and C from bovine chromaffin granules and a rat pheochromocytoma. *J. Neurochem.* **48:**262-270

Fischer-Colbrie R, Zangerle R, Frischenschlager I, Weber A, Winkler H (1984) Isolation and characterization of a glycoprotein from adrenal chromaffin granules. *J .Neurochem.* **44:**1008-1016

Foran P, Lawrence G, Dolly JO (1995) Blockade by botulinum neurotoxin B of

catecholamine release from adrenochromaffin cells correlates with its cleavage of synaptobrevin and a homologue present on the granules. *Biochemistry* **34:**5494-5503

Fournier S, Trifaró JM (1988) A similar calmodulin-binding protein expressed in chromaffin, synaptic and neurohypophyseal secretory vesicles. *J. Neurochem* **50:**27-37

Franson RC, Van den Bosch H (1982) Lysophospholipase activity of bovine adrenal medulla. *Biochim. Biophys. Acta.* **711:**75-82

Fricker LD, Evans CJ, Esch ES, Herbert E (1986) Cloning and sequence analysis of cDNA for bovine carboxypeptidase E. *Nature* **323:**461-464

Fricker LD, Snyder SH (1982) Enkephalin Convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. *Proc. Natl. Acad. Sci. USA* **79:**3886-3890

Futai M, Naumi T, Maeda M (1989) ATP synthase (H^+-ATPase): results of combined biochemical and molecular biological approaches. *Annu. Rev. Biochem.* **58:**111-136

Fujino Y, Fujii T, DaimonT (1994) Presence of Laminin B chain-like protein in bovine and rat adrenal chromaffin granules. *J. Biochem.* **115:**615-621

Garcwal HS.

Gavine FS, Pryde JG, Deane DL, Apps DK (1984) Glycoproteins of the chromaffin granule membrane: separation by two-dimensional electrophoresis and identification by lectin blotting. *J. Neurochem.* **43:**1243-1252

Geissler D, Martinek A, Margolis RU, Margolis RK, Skrivanek JA, Ledeen R, König P, Winkler H (1977) Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. *Neuroscience* **2:**685-693

Gerdes HH, Rosa P, Phillips E, Baeuerle PA, Argos P, Huttner WB (1989) The primary structure of human secretogranin II, a widespread tyrosine-sulphated secretory protein that exhibits low pH- and calcium-induced aggregation. *J. Biol. Chem.* **264:**12009-12015

Getlawi F, Laslop A, Schägger H, Ludwig J, Haywood J, Apps D (1996) Chromaffin granule membrane glycoprotein IV is identical with Ac45, a membrane-integral subunit of the granule's H^+-ATPase *Neurosci Lett.* **219:**13-16

Gillespie J, Ozanne S, Tugal B, Percy J, Warren M, Haywood J, Apps DK (1991) The vacuolar H^+-translocating ATPase of renal tubules contains a 115 kDa glycosylated subunit. *FEBS Lett.* **282:**96-72

Goedert M, Reynolds GP, Emson PC (1983) Neurotensin in the adrenal medulla *Neurosci. Lett.* **35:**155-160

Goldstein IJ, Hollerman CE, Smith EE (1965) Protein-carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. *Biochemistry* **4:**876-883

Goldstein IJ, Reichert CM, Misaki A (1973) Protein-carbohydrate interaction. II Inhibition studies on the interaction of concanavalin A with polysaccharides. *Ann. N.Y. Acad. Sci.* **234:**283-295

Goldstein IJ, So LL (1965) Interaction of concanavalin A with model substrates. *Arch. Biochem. Biophys* **111:**407-414

Gorr SU, Hamilton JW, Cohn DV (1991) Sulfated secreted forms of bovine and porcine parathyroid chromogranin A (secretory protein-I). *J. Biol. Chem.* **266:**5780-5784

Gowda DC, Hogue-Angeletti R, Margolis RK, Margolis RU (1990) Chromaffin granule and PC-12 cell chondroitin sulphate proteoglycans and their relation to chromogranin A. *Arch. Biochem. Biophys.* **281:**219-224

Graham LA, Hill KJ, Stevens TH (1994) VMA7 encodes a novel 14 kDa subunit of the *Saccharomyces cerevisiae* vacuolar H^+-ATPase complex. *J. Biol. Chem.* **269:**25974-25977

Gratzl M (1984) Distribution of chromaffin secretory vesicles, acetylcholinesterase and lysosomal enzymes in sucrose and percoll gradients. *Anal. Biochem.* **142:**148-154

Gratzl M, Krieger-Bauer H, Ekerdt R (1981) Latent acetylcholinesterase in secretory vesicles isolated from adrenal medulla. *Biochim. Biophys. Acta.* **649:**355-366

Gräf R, Lepier A, Harvey WR, Wieczorek H (1994) A novel 14 kDa V-ATPase subunit in the tobacco hornworm midgut. *J. Biol. Chem.* **269:**3767-3774

Grider BP, Andersen P, White AE, Zhout Z, Li X, Mattsson JP, Lundberg L, Keeling DJ, Xie X-S, Stone DK, Peng S-B (1997) Subunit G of the vacuolar proton pump. Molecular characterization and functional expression. *J. Biol. Chem.* **272:**10721-10728

Grigoryan NA, Nalbandyan RM, Bunatian HCh (1981) An extremely acidic copper-containing protein from chromaffin granules. *Biochem. Biophys. Res. Commun.* **100:**921-928

Griswold DE, Antell L, Bender PE, Hanna N, Poste G (1985) Induction of supressor cells, interleukin-2 production and mitogenesis with monomeric concanavalin A: different actions of tetrameric and monomeric concanvalin A. *Mol Immunol.* **22:**1311-1316

Gross GAM (1990) Glycolipid anchoring of plasma membrane proteins. Annu. Rev. Cell Biol. **6:**1-3

Gunther GR, Wang JL, Yahara I, Cunnigham BA, Edelman GM. (1973) Concanvalin A derivatives with altered biological activities. *Proc. Nalt. Acad. Sci. USA.* **70:**1012-1026

Hagn C, Klein RL, Fischer-Colbrie R, Douglas BH,Winkler H (1986a). An immunological characterization of five common antigens of chromaffin granules and of large dense-cored vesicles of sympathetic nerve. *Neurosci. Lett.* **67:**295-300

Hagn C, Schmid KW, Fischer-Colbrie R, Winkler H (1986b) Chromogranin A, B and C in human adrenal medulla and endocrine tissues. *Lab. Invest.* **55:**405-411

Haigh JR, and Phillips JH (1989) A sodium/proton antiporter in chromaffin granule membranes. *Biochem. J.* **257:**499-507

Hammaratröm S, Kabat EA (1969) Purification and characterization of blood-group A reactive hemagglutinin from the snail *Helix Pomatia* and a study of its combining site *Biochemistry* **8:** 2696-2705

Hardman KD, Ainsworth CF (1972) Structure of concanavalin A at 2.4° A resolution. *Biochemistry* **11:**4910- 4919

Harlow E, Lane D (1988) Antibodies: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York. 56-100

Harrington MG (1990) Elution of proteins from gels in 'Methods in enzymology' (Deutscher MP), **182:**488-495 Academic press, London.

Hawkes R (1982) Identification of concanavalin A binding proteins after SDS-gel electrophoresis and protein blotting. *Anal. Biochem* **123:**143-146.

Helle KB, Reed RK, Pihl KE, Serck-Hanssen G (1985a) Osmotic properties of the chromogranin and relation to osmotic pressure in catecholamine storage granules. . *Acta. physiol. Scand.* **123:**21-33

Helle KB, Pihl KE, Serck-Hanssen G (1985b) Effects of calcium and limited proteolysis on membrane-bound and releasable dopamine β-hydroxylase in adrenomedullary catecholamine granules. *Acta. physiol. Scand.* **125:**423-427

Henry JP, Botton D, Sagne C, Issambert MF, Desnos C, Blanchard V, Raizman V (1994) Biochemistry and molecular biology of the vesicular monoamine transport from chromaffin granules. *J. Exp. Biol.* **196:**251-262

Hieber AD, Christie DL (1993) Characterization of glycoprotein II from bovine adrenal medullary chromaffin granules. *J. Biol. Chem.* **268:**11073-11078

Higgins JA (1984) The transverse distribution of phospholipids in the membranes of Golgi subfractiona of rat hepatocytes. *Biochem. J.* **219:**261-272

Hook VYH (1984) Carboxypeptidase B-like activity for the processing of enkephalin precursors in the membrane component of bovine adreno-medullary chromaffin granules. *Neuropeptides* **4:**117-126

Hook VYH, Eiden LE, Brownstein MJ (1982) A carboxypeptidase processing enzyme for enkephalin precursors. *Nature* **295:**341-342

Hook VYH, Mezey E, Fricker LD, Pruss RM, Siegel RE, Brownstein MJ (1985) Immunochemical characterization of carboxypeptidase B-like peptide-hormone-processing enzyme. *Proc. Natl. Acad. Sci. USA* **82:**4745-4749

Hörtnagl H, Winkler H, Lochs H (1972) Membrane proteins of chromaffin granules: Dopamine β-hydroxylase, a major constituent. *Biochem. J.* **129:**187-195

Hopp TP, Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* **78:**3824-3828

Houmard J, Drapeau GR (1972) Staphylococcal protease: a proteolytic enzymes specific for glutamoyl bonds. *Proc. Nalt. Acad. Sci. USA.* **69:**3506-3512

Howard IK, Sage HJ, Stein MD, Young NM, Leon MA, Dyckes DF (1971) Studies on a phytohemagglutinin from the lentil. *J. Biol. Chem.* **246:**1590-1595

Howell KE, Palade GE. (1982) Heterogeneity of lipoprotein particles in hepatic Golgi fractions. *J. Cell Biol* **92:**822-832

Howell JI, Lucy JA. (1969) Cell fusion induced by lysolecithin. *FEBS lett.* **4:**147-150

Huber E, König P, Schuler G, Aberer W, Plattner H, Winkler H (1979) Characterisation and topography of the glycoproteins of adrenal chromaffin granules. *J. Neurochem.* **32:**35-47

Hunkapiller MW, Kent M, Caruthers, Dreyer W, Firca J, Giffin C, Hotvath S, Hunkapiller P, Tempat P, Hood L (1984) A microchemical facility for the analysis and synthesis of genes and proteins. *Nature* **310:**105-107

Hunter A, Waldron K, Apps DK (1982) Determination of the proportion of sealed vesicles in a preparation of chromaffin granule membrane 'ghosts'. *FEBS Lett.* **144:**51-55

Hutton JC, Davidson HW, Grimaldi KA, Peshavaria M (1987a) Biosynthesis of betagranin in pancreatic β-cells. *Biochem. J.* **244:**449-456

Hutton JC, Davidson HW, Peshavaria M. (1987b) Proteolytic processing of chromogranin A in purified insulin granules. Formation of a 20 kDa N-terminal fragment (betagranin) by the concerted action of a Ca^{2+} -dependent endopeptidase and carboxypeptidase H (EC 3.4.17.10) *Biochem. J.* **244:**457-464

Iacangelo A, Affolter HU, Eiden LE, Herbert E, Grimes M (1986) Bovine chromogranin A sequence and distribution of its messenger RNA in endocrine tissues. *Nature* **323:**82-86

Iacangelo AL, Fischer-Colbrie R, Koller KJ, Brownstein MJ, Eiden LE (1988) The sequence of porcine chromogranin A messenger RNA demonstrates chromogranin A can serve as the precursor for the biologically active hormone, pancreastatin *Endocrinology* **122:**2339-2341

Johnston PA, Cameron PL, Slukenbrok H, John R, De Camilli P, Südhof TC (1989) Synaptophysin is targetted to similar microvesicles in CHO and PC12 cells. *EMBO J.* **8:**2863-2872

Johnson RG, Scarpa A (1974) Catecholamine equilibrium gradient of isolated chromaffin vesicles induced by ionophore X-537 A. *FEBS lett.* **47:**117-121

Johnson RG, Scarpa A (1976) Intenral pH of isolated chromaffin vesicles. *J. Biol Chem.* **251:**2189-2191

Kawasaki ES, Clark SS, Coyne MY, Smith SD, Champlin R, Witte ON, McCormick FP (1988) Diagnosis of chronic myeloid and acute leukemias by detection of leukemia-specific mRNA sequences amplified *in vitro* *Proc. Natl. Acad. Sci. USA* **85:**5698-5702

Kelly RB (1995) Synaptotagmin is just a calcium sensor. Current Biology **5:**257-259

Kent UM, Fleming PJ (1990) Cytochrome b561 is fatty acylated and oriented in the chromaffin granule membrane with its carboxylterminus cytoplasmically exposed. *J. Biol. Chem.* **265:**16422-16427

Kiang WL, Krusius T, Finne J, Margolis RU, Margolis RK (1982) Glycoproteins and proteoglycans of the chromaaffin granule matrix. *J. Biol. Chem.* **257:**1651-1659

Kilpatrick L, Gavine F, Apps DK, Phillips J (1983) Biosynthetic relationship between the major matrix proteins of adrenal chromaffin granules. *FEBS Lett.* **164:**383-388

Kirshner N (1962) Uptake of catecholomines by a particulate fraction of the adrenal medulla. *J. Biol. Chem.* **273:**2311-2317

Kirchmair R, Gee P, Hogue-Angeletti R, Laslop A, Fischer-Colbrie R, Winkler H (1992) Immunological characterization of the endoproteases PC1 and PC2 in adrenal chromaffin granules and in the pituitary gland. *FEBS lett.* **297:**302-305

Klinman JP, Krueger M, Brenner M, Edmondson DE (1984) Evidence for two copper atoms/subunit in dopamine β-monooxygenase catalysis. *J. Biol. Chem.* **259:**3399-3402

Kohan SA, Pescatori M, Brecha NC, Mastrogiacomo A, Umbach JA, Gunderson GB (1995) Cysteine string protein immunoreactivity in the nervous system and adrenal gland of rat. *J. Neurosci.* **15:**6230-6238

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:**495-497

Kopell WN, Westhead EW (1982) Osmotic pressure of solutions of ATP and catecholamines relating to storage in chromaffin granules. *J. Biol. Chem.* **257:**5707-5710

Krieger TJ, Hook VYH (1991) Purification and characterization of a novel thiol protease involved in processing the enkephalin precursor. *J. Biol. Chem.* **266:**8376-8383

Krieglstein K, Unsicker K (1995) Bovine chromaffin cells release a transforming growth factor-like molecule contained within chromaffin granules. *J. Neurochem.* **65:**1423-1426

Kryvi H, Flatmark T, Terland O (1979) Comparison of adrenaline and noradrenaline storage granules of bovine adrenal medulla. *Eur. J. Cell Biol.* **20:**78-82

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685

Lamouroux A, Vigny A, Faucon Biguet N, Darmon MC, Franck R, Henry J-P, Mallet J (1987) The primary structure of human dopamine β-hydroxylase: insight into the relationship between the soluble and membrane-bound forms of the enzyme *EMBO J.* **6:**3931-3937

Laslop A, Steiner HJ, Egger C, Wolkersdorfer M, Kapelari S, Hogue-Angeletti R, Erickson JD, Fischer-Colbrie R, Winkler H (1993) Glycoprotein III (clusterin, sulfated glycoprotein 2) in endocrine, nervous and other tissues: immunochemical characterization, subcellular localization and regulation of biosynthesis. *J. Neurochem.* **61:**1498-1505

Laslop A, Fisher-Colbrie R, Hook V, Obendorf D, Winkler H (1986) Identification of two glycoproteins of chromaffin granules as the carboxypeptidase H. *Neurosci. Lett.* **72:**300-304

Laslop A, Fischer-Colbrie R, Kirscke H, Hogue-Angeletti R, Winkler H (1990) Chromogranin A-processing proteinases in purified chromaffin granules: contaminants or endogenous enzymes? *Biochim. Biophys. Acta.* **1033:**65-72

Lassman H, Hagn C, Fischer-Colbrie R, Winkler H (1986) Presence of chromogranin A, B and C in bovine endocrine and nervous tissues: a comparative immunohistochemical study. *Histochem. J.* **18:**380-386

Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA, (1987) Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification *Science* **237:**175-178

Leitch B, Finbow ME (1990) The gap junction-like form of vacuolar proton pump channel component appears not to be an artifact of isolation: An immunological localization study. *Exp. Cell Res.* **196:**218-226

Leist M, Wendel A (1996) A noval mecanism of murine hepatocyte death inducible by Concanavalin A. *J. Hepatology* **25:**948-959

Lemaire S, Day R, Dumont M, Chouinard L, Calvert R (1984) Dynorphin and enkephalins in adrenal paraneurones. Opiates in the adrenal medulla. *Can. J. Physiol. Pharmacol.* **62:**484-492.

Lerner RA (1984) Antibodies of predetermined specificity in biology and medicine. *Adv. Immunol.* **36:**1-44

Leube RE, Kaiser P, Seiter A, Zimbelmann R, Franke WW, Rehm H, Knaus P, Betz H, Reinke H, Beyreuther K, Weidenmann B (1987) Synaptophysin: Molecular organization and mRNA expressions as determined from from cloned cDNA. *EMBO J.* **6:**3261-3268

Levin EY, Levenberg B, Kaufman S (1960) The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J. Biol. Chem.* **235:**2080-2086

Li C, Ullrich B, Zhang JZ, Anderson RGW, Brose N, Südhof TC (1995) Ca^{2+}-dependent and -independent activities of neuronal and non-neuronal synaptotagmins *Nature* **375:**593-599

Lindberg I, Van der Hurk WH, Bui C, Batie CJ (1995) Enzymatic characterization of immunopurified prohormone Convertase 2: potent inhibition by a 782 peptide fragment. *Biochemistry* **34:**5486-5493

Lis H, Sela BA, Sachs L, Sharon N (1970) Specific inhibition by N-acetyl-D-galactosamine of the interaction between soy bean agglutinin and animal cell surface *Biochim. Piophys. Acta.* **211:**582-585

Liu Y, Peter D, Roghani A, Schuldiner S, Prive GC, Eisenberg D, Brecha N (1992) A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. *Cell* **70:**539-551

Low MG (1986) The glycosyl-phosphatidylinositol anchor of membrane proteins. *Biochim. Biophys. Acta.* **988:**427-454

Low MG, Saltiel AK (1988) Structural and function roles of glycosyl-phosphatidylinositol in membranes. *Science* **239:**268-275

Lynch DR, Stittmater SM, Venable JC, Snyder SH (1986) Enkephalin convertase: Localisation to specific neuronal pathways. *J. Neurochem.* **6:**1662-1675

Lynch DR, Stittmater SM, Venable JC, Snyder SH (1987) Enkephalin convertase in gastrointestinal tract and associated organs characterised and localised with [^{3}H-]-guanidinoethylmercaptosuccinic acid. *Endocrinol.* **121:**116-126

Lynch DR, Venable JC, Snyder SH (1988) Enkephalin convertase in the heart: similar disposition to atrial natriuretic factor. *Endocrinol.* **122:**2683-2691

Majame EA, Alho H, Kataoka Y, Lee CH, Yang H-YT (1985) Neuropeptide Y in bovine adrenal glands: distribution and characterization. *Endocrinol.* **117:**1162-1168

Mandel M, Moriyama Y, Hulmes JD, Pan Y-CE, Nelson H, NelsonN (1988) cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPase. *Proc. Natl. Acad. Sci. USA* **85:**5521-5524

Manolson MF, Ouellette BFF, Filion M, Poole RL (1988) cDNA sequence and homologies of the 57- kDa nucleotide-binding subunit of the vacuolar ATPase from *Arabidopsis*. *J. Biol. Chem.* **263:**17987-17994

Margolis RK, Finne J, Krusius T, Margolis RU (1984) Structural studies on glycoprotein oligosaccharides of chromaffin granule membranes and dopamine β-hydroxylase. *Archs. Biochem. Biophys.* **228:**443-449

Maron R, Stern Y, Kanner BI, Schuldiner S (1983) Functional assymetry of the amine transporter from chromaffin granules. *J. Biol. Chem.* **258:**11476-11481

Matsudaira P (1987) Sequence from picomole quanatities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262:**10035-10038

Matsudaira P, Janmey P (1988) Pieces in the actin-severing protein puzzle. *Cell* **54:**139-140

Matsumoto I, Osawa T (1970) Purification and characterization of a cytisus-type anti-H(O) phytohemaglutinin from *Ulex europeus seeds*. *Arch. Biochim. Biophys.* **140:**484-491

Matthew WD, Tsavaler L, Reichardt LF (1989) Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue *J. Cell Biol.* **91:**257-269

Merrifield RB (1963) Solid phase peptide synthesis I. The synthesis of a tetrapeptide. *Science* **85:**2149-2154

Mikaelyan MV, Grigoryan NA, Nalbandyan RM (1987) Similarities of physico-chemical and antigenic properties of neurocupreins and extremely acidic proteins from chromaffin granules. *Biochim. Biophys. Acta.* **924:**548-555

Mizobe F, Iwamoto M, Livett BG (1984) Parallel but separate release of catecholamines and acetylcholinesterase from stimulated adrenal chromaffin cells in culture. *J. Neurochem.* **42:**1433-1438

Moos M, Nguyen NY, Liu T-Y (1988) Reproducible, high-yield sequencing of proteins electrophoretically separated and transferred to an inert support. *J. Biol. Chem.* **263:**6005-6008

Moriyama Y, Nelson N (1987) Nucleotide binding sites and chemical modification of the chromaffin granule proton ATPase. *J. Biol. Chem.* **262:**14723-14729

Moriyama Y, Nelson N (1988) Purification and properties of a vanadate- and N-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. *J. Biol. Chem* **263:**8521-8527

Moriyama Y, Nelson N (1989) Cold inactivation of vacuolar proton-ATPases. *J. Biol.* Chem. **264:**3577-3582

Natori S. Huttner WB (1994) Peptides derived from the granins *Biochimie.* **76:**277-282

Nelson H, Mandiyan S, Nelson N (1995) A bovine cDNA and a yeast gene-VMA 8 encoding subunit D of the vacuolar H^+-ATPase. *Proc. Natl. Acad. Sci. USA* **92:**497-501

Nelson H, Mandiyan S, Nelson N (1994) The *Saccharomyces cerevisiae* VMA 7 gene encodes a 14-kDa subunit of the vacuolar H^+-ATPase catalytic sector. *J. Biol. Chem.* **269:**24150-24155

Nelson H, Nelson N (1989) The progenitor of ATP synthases was closely related to the current vacuolar H^+-ATPase. *FEBS Lett.* **247:**147-153

Nelson N (1992) Evolution of organellar proton-ATPases. *Biochim. Biophys. Acta* **1100:**109-124

Nguyen MH, Harbour D, Gagnon C (1987) Secretory proteins from adrenal medullary cells are carboxyl-methylated *in vivo* and released under their methylated form by acetylcholine. *J. Neurochem.* **49:**38-44

Njus D, Knoth J, Cook C, Kelly PM (1983) Electron transfer across the chromaffin granule membrane. *J. Biol. Chem.* **258:**27-30

Nicholls DG (1994) Monoamine and purine neurotransmitters. In ''Proteins, transmitters and synapses'' **11:** 200-221`

Obendorf D, Schwarzenbrunner U, Fischer-Colbrie R, Laslop A, Winkler H (1988) In adrenal medulla synaptophysin (p38) is present in chromaffin granules and in a special vesicle population. *J. Neurochem.* **51:**1573-1580

Ochs D (1983) Protein contaminants of sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.* **135:**470-474

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem* **250:**4007-4021

Palmer DJ, Christie DL (1990) The primary sequence of glycoproteins III from bovine adrenal chromaffin granules. Sequence similarity with human serum protein-40,40 and rat Sertoli cell glycoprotein 2. *J. Biol. Chem.* **265:**6617-6623

Pang DT, Wang JKT, Valto F, Benferati F, Greengard P (1988) Protein tyrosine phosphorylation in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **85:**762-766

Park DH, Kashimoto T, Ebstein RP, Goldstein M (1976) Purification and immunochemical characterization of dopamine β-hydroxylase from human pheochromocytoma. *Mol. Pharmacol.* **12:**73-81

Patzak A, Winkler H (1986) Exocytic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. *J. Cell Biol.* **102:**510-515

Paulova M, Entlicher G, Ticha M, Kostir JV, Kocourek J (1971) Studies on phytochemagglutinin. VII effect of Mn^{2+} and Ca^{2+} on hemagglutinination and polysaccharide precipition by phytohemagglutinin of *Pisum sativum. Bioch. Biophys. Acta.* **237:**513-518

Perin MS, Fried VA, Slaughter CA, Südhof TC (1988) The structure of cytochrome b561, a secretory vesicle-specific electron transport protein. *EMBO J.* **7:**2697-2703

Percy JM, Pryde JG, Apps DK (1985) Isolation of ATPase I, the proton-pump of chromaffin granule membranes. *Biochem. J.* **231:**557-564

Perez-Castierira JR, Apps DK (1990) Vacuolar H^+-ATPase of adrenal secretory protein complexes in enzymatically active form. *Anal. Biochem.* **271:**127-131

Perin MS, Fried VA, Stone DK, Xie X-S, Südhof TC (1991) Structure of the 116-kDa subunit of the clathrin-coated vesicle/ synaptic vesicle proton pump. *J. Biol. Chem.* **266:**3877-3881

Phillips JH (1973) Phosphatidylinositol kinase: a component of the chromaffin granule membranes. *Biochem. J.* **136:**579-587

Phillips JH (1982) Dynamics aspects of chromaffin granule structure. *Neurosci.* **7:**1595-1609

Pintor J, Rotlan P, Torres M, Miras-Portugal M-T (1992) Characterisation and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and sectretagogue-induced release. *Anal. Biochem.* **200:**296-300

Pluskal MG, Przekop MB, Kavonian MR, Vecoli C, Hicks DA (1986) Immobilon PVDF transfer membrane: a new membrane substrate for western blotting of proteins. *Biotechniques* **4:**272-274

Pohl TM, Phillips E, Song K, Gerdes HH, Huttner WB, Rüther U (1990) The organisation of the mouse chromogranin B (secretogranin I) gene. *FEBS lett.* **226:**219-224

Pryde JG, Phillips JH (1986) Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114: Application to subcellular fractions of the adrenal medulla. *Biochem. J.* **233:**525-533

Pruss RM, Shepard EA (1987) Cytochrome b561 can be detected in many neuroendocrine tissues using a specific monoclonal antibody. *Neurosci.* **22:**149-153

Rehm H, Weidenmann B, Betz H (1986) Molecular characterisation of synaptophysin, A major calcium-binding protein of the synaptic vesicle membrane. *EMBO J.* **5:**535-541

Reiffen FU, Gratzl M (1986) Ca^{2+} binding to chromaffin vesicle matrix proteins: Effect of pH, Mg^{2+} and ionic strength. *Biochemistry* **25:**4402-4406

Roda LG, Slater EP, Hogue-Angeletti RA (1980) Glycoproteins and glycopeptides in the chromaffin granule membranes. *Neurochem. Res.* **5:**1243-1250

Rodriguez C, Brayton KA, Brownstein M, Dixon JE (1989) Rat preprocarboxypeptidase H. *J. Biol. Chem.* **264:**5988-5995

Rodriguez del Castillo A, Torres M, Delicado EG, Miras-Portugal M-T (1988) Subcellular distribution studies of diadenosine polyphosphates AP4A- and AP5A- in bovine adrenal medulla: presence in chromaffin granules. *J. Neurochem.* **51:**1696-1703

Rosa P, Hille A, Lee RWH, Zanini A, De Camilli P, Huttner WB (1985) Secretogranins I and II: two tyrosine-sulphated secretory proteins common to a variety of cells secreting by the regulated pathway. *J. Cell. Biol.* **101:**1999-2011

Rosa P, Zanini A (1983) Purification of a sulphated secretory protein from the adenohypophysis. Immunochemical enhance that similar macromolecules are present in other glands. *Eur. J. Cell Biol.* **31:**94-98

Rosenberg RC, Lovenberg W (1977) Active dimers of dopamine β-hydroxylase in human plasma. *Mol. Pharmacol.* **13:**652-661

Sabban EL, Goldstein M (1984) Subcellular site of biosynthesis of the catecholamine biosynthetic enzymes in the bovine adrenal medulla. *J. Neurochem.* **43:**1663-1668

Sabban EL, Green LA, Goldstein M (1983) Mechanism of biosynthesis of soluble and membrane-bound forms of dopamine β-hydroxylase in PC12 phaeochromocytoma cells. *J. Biol. Chem.* **258:**7812-7818

Sakaguchi M, Mihara K, Sato R (1987) A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. *EMBO. J.* **6:**2425-2431

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of globin genomic sequences and restriction site analysis for diagnosis of sikle cell anemia *Science* **230:**1350-1354

Sambrook J, Fritsch EF, Maniantis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbour Labratory.

Saraste M, Sibbals PR, Whittinghofer A (1990) The P-Loop: A common motif in ATP and GTP binding proteins. *Trends biochem. Sci.* **15:**430-434

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to100 kDa. *Anal. Biochem.* **166:**368-379

Schägger H, Cramer WA, von Jagow G (1994) Analysis of molecular masses and oligomeric states of proteins complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **166:**368-379

Schneider E, Altendorf K (1987) Bacterial adenosine 5'-triphosphate synthase

(F_1-F_O) : purification and reconstitution of F_O complexes and biochemical and functional characterization of their subunits. *Microbiol. Rev.* **51:**477-497

Scherman D, Henry J-P (1983) Solubilization of the catecholamine carrier of chromaffin granule membranes in a form that binds substrates and inhibitors of uptake. *Biochemistry* **22:**2805-2810

Schuldiner S, Gabizon R, Maron R, Suchi R, Stern Y (1985) The amine transporter from bovine chromaffin granules. *Ann. NY Acad. Sci.* **456:**268-276

Schultzberg M, Lundberg JM, Hökfelt T, Terenius L, Brandt J, Elde RP, Goldstein M (1978) Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neurosci.* **3:**1169-1186

Seizinger BR, Liebisch DC, Gramsch C, Hertz A, Weber E, Evans CJ, Esch FS, Böhlen P (1985) Isolation and structure of a novel C-terminally amidated opioid peptide, amidorphin, from bovine adrenal medulla. *Nature* **313:**57-59

Serkine M, Ariga T, Miyatake T, Kuroda Y, Suzuki A, Yamakwa T (1984) Ganglioside composition of chromaffin granule membranes in bovine adrenal medulla. *J. Biochem.* **95:**155-160

Settleman T, Nolan J, Hogue-Angeletti R (1985) Chromogranin. An integral membrane protein. *J. Biol. Chem.* **260:**1641-1644

Senior AE (1990) The proton-translocating ATPase of *Escherichia coli*. *Ann Rev. Biophys. biophys. chem.* **19:**7-14

Senozan NM, Landrum J (1981) Hemocyanin of the giant keyhole limpet, *Megathura crenulata.* In invertebrate oxygen binding proteins: structure, active sites and function. Lamy J. LamyJ., Editorsa, Marcel Dekker, New tork 703-717

Sharon N (1993) Lectin-carbohydrate complexes of plants and animals: an atomic view. *Trends Biochem. Sci* **18:**221-226

Sharon N, Liz H (1972) Lectins: cell agglutinating and sugar-specific proteins. *Science* **177:** 949-959

Sharp RR, Richards EP (1977) Analysis of the Carbon-13 and proton NMR spectra of bovine chromaffin granules. Biochim. Biophys. Acta. **497:**14-28

Shen F-S, Roberts SF, Lindberg I (1989) A putative processing enzyme for

264:15600-15605

Siebert A, Lottspeich F, Nelson N, Betz H. (1994) Purification of the synaptic vesicle-binding protein physophilin. *J. Biol. Chem.* **269:**28329-28334

Simon J-P, Aunis D (1989) Biochemistry of the chromogranin A protein family. *Biochem. J.* **262:**1-13

Skotland T, Ljones T, Flatmark T, Sletten K (1977) NH-2 terminal sequence of dopamine β-hydroxylase from bovine adrenal medulla. *Biochim. Biophys. Acta.* **74:**1483-1489

Skotland T, Peterson L, Bäckström D LjonesT, Flatmark T, Ehrennberg A (1980) Electron paramagnetic resonance of the copper in dopamine β-monooxygenase. Rapid reduction by ascorbate, the steady-state redox level, chelation with EDTA and reactivation of the apoenzyme by added copper. *Eur. J. Biochem.* **103:**5-11

Smith AD, Winkler H (1967) Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J.* **103:**483-492

So LL, Goldstein IJ (1968) Protein-carbohydrate interaction. XVII on the number of combining sites on concanavalin A. The phytohemagglutinin of the jack bean. *Biochim. Biophys. Acta.* **165:**398-401

Stewart LC, Kliman JP (1988) Bovine membranous dopamine β-hydroxylase is not anchored via covalently attached phosphatidyl inositol. *J. Biol. Chem.* **263:** 12183-12186

Srivastava M, Gibson K, Pollard HB, Fleming PJ (1994) Human cytochrome b561: a revised hypothesis for conformation in membranes which reconciles sequence and functional information. *Biochem. J.* **303:**915-921

Strub J-M, Garcia-Sablone P, Lonning K, Taupenot L, Hubert P, van Dorsseloer A, Aunis D (1995) Processing of chromogranin B in bovine adrenal medulla. *Eur. J. Biochem.* **229:** 56-368

Südhof TC, Lottspeich F, Greenyard P, Mehl E, John R (1987) A synaptic vesicle protein with a noval cytoplasmic domain and four transmembrane regions. *Science* **238:**1142-1144

Sumner JB, Howell SF (1936) The identification of the hemagglutinin of the jack bean with concanavalin A. *J. Bacteriol.* **32:**227-237

ELSEVIER

Neuroscience Letters 219 (1996) 13–16

NEUROSCIENCE LETTERS

Chromaffin granule membrane glycoprotein IV is identical with Ac45, a membrane-integral subunit of the granule's H^+-ATPase

Fariha Getlawi[a], Andrea Laslop[b], Hermann Schägger[c], Jürgen Ludwig[c], Jeff Haywood[a], David Apps[a,*]

[a]*Department of Biochemistry, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9XD, UK*
[b]*Pharmakologisches Institut der Universität Innsbruck, Peter-Mayr-Strausse la, A-6020 Innsbruck, Austria*
[c]*Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt am Main, Germany*

Received 24 June 1996; revised version received 1 October 1996; accepted 8 October 1996

Abstract

Glycoprotein IV of bovine adrenal chromaffin granule membranes was purified by membrane fractionation with Triton X-114 and lectin affinity chromatography. An antiserum raised against this protein recognized the same component as one directed against subunit Ac45 of the proton-translocating adenosine triphosphatase in the granule membrane. Amino acid sequencing confirmed that glycoprotein IV and Ac45 are identical proteins, and also showed that they are derived from a larger precursor by removal of a 246-amino acid N-terminal sequence. Enzymatic deglycosylation indicated an apparent polypeptide molecular mass of 29 kDa for the mature Ac45/glycoprotein IV. Blue Native electrophoresis confirmed that this protein is a component of the membrane sector of the V-ATPase.

Keywords: Glycoprotein; Vacuolar; Adenosinetriphosphatase; Chromaffin

The membranes of adrenal chromaffin granules contain several glycoproteins, which were initially identified simply as lectin-binding components [1,2], but some of which have had functions ascribed to them subsequently [3]. Thus glycoprotein I is dopamine β-monooxygenase, and glycoproteins H, J and K are intragranular proteinases [4,5]. Glycoproteins II and III have been sequenced but are still of unknown function [6,7], and glycoproteins IV and V have not been studied at all.

A major component of the granule membrane is the H^+-translocating ATPase, the largest subunit of which (116 kDa) is a glycosylated transmembrane protein [8]. Recently, a second glycosylated subunit, named Ac45,was reported [9]; this, like glycoprotein IV, appears in electrophoretograms as a diffuse band of apparent molecular mass 45–50 kDa. We now report that glycoprotein IV and Ac45 are the same protein, and that this H^+-ATPase subunit is apparently produced by proteolytic processing of a much larger precursor.

Chromaffin granule membranes, isolated from bovine adrenal medullae, were resuspended to a protein concentration of 4 mg/ml by homogenization in 0.15 M KCl, 10 mM HEPES pH 7.4, 1 mM EDTA, 1 mM dithiothreitol. Triton X-114 was added to a final concentration of 2% (w/v); after 5 min at 0°C, the solution was centrifuged (10 min, g_{av} = 412 000), the supernatant discarded and the pellet washed in the same buffer by resuspension and recentrifugation. It was then solubilized with 2% (w/v) *n*-octyl-β-glucoside, diluted 4-fold with 0.02 M sodium phosphate, pH 7.4, then applied to a 2 ml Con A-agarose column, which was washed with 10 ml of 1 M NaCl, 1.7 M ethanediol, 0.02 M sodium phosphate, pH 7.4, 0.5% *n*-octyl-β-glucoside over 3 h, then eluted with 10% (w/v) α-methyl mannoside, in this buffer. Proteins eluted from the column were precipitated with trichloracetic acid and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Con A blotting.

To raise antibodies, glycoprotein IV was further purified by preparative polyacrylamide gel electrophoresis, electroeluted and injected into rabbits in multiple subcutaneous sites. A guinea-pig antiserum raised against an Ac45-containing fusion protein [9] was a gift from Dr. N. Nelson, Department of Biochemistry, Tel Aviv Univer-

* Corresponding author. Tel.: +44 131 6503722; fax: +44 131 6503711; e-mail: dka@srv1.med.ed.ac.uk

0304-3940/96/$12.00
PII S0304-3940(96)13151-7

sity, Israel. One and two-dimensional gel electrophoresis and blotting were performed as described elsewhere [2,10]. Blots were decorated with Con A and horseradish peroxidase (HRP) [11] or with antisera followed by HRP-conjugated second antibodies, and were developed by Enhanced Chemiluminescence (Amersham).

Enzymatic deglycosylation of glycoprotein IV was performed on an ATPase-enriched fraction produced by treatment of chromaffin granule membranes with 1.8% (w/v) Triton X-114 [12]. This pellet was resuspended at a final concentration of 1 mg protein/ml in 10 mM HEPES–NaOH, pH 7.2 containing 0.5% deoxycholate, 5 mM benzamidine, 100 μM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 50 μM TPCK and 50 μM TLCK. Endoglycosidase F (40 U/ml) was added and digestion carried out for 15 h at 37°C. Deglycosylated and control (mock-digested) samples were analyzed by electrophoresis and blotting with the anti-glycoprotein IV serum.

For protein sequencing, a 0.2 ml piece of gel was cut from the major Coomassie-stained band on an SDS-polyacrylamide gel of purified glycoprotein IV, crushed and 400 μl of 100 mM NH_4HCO_3 added. After addition of 0.1% SDS and incubation for 24 h, 0.6 ml of water was added, followed by a 100 mm^2 piece of Immobilon P membrane which had been wetted with methanol. This membrane bound the protein, and was used for N-terminal protein sequencing after washing with water and drying.

The V_0 sector of the H^+-ATPase was isolated by preparative Blue Native polyacrylamide gel electrophoresis [13], and the subunits resolved by tricine-SDS gel electrophoresis [14] using a 0.7 mm 13% acrylamide gel and a protein load of 60 μg/1.5 cm well. Electroblotting onto Immobilon P was performed as described [15].

Edman degradation was performed on a 473A gas-liquid protein sequenator (Applied Biosystems). Pieces of Immobilon P membrane (30–40 mm^2) with bound proteins were used for direct N-terminal sequencing. Identical pieces of membrane were also treated with 50 μl 80% HCOOH (16 h, 40°C) in order to specifically hydrolyse D-P bonds. Upon vacuum removal of HCOOH the proteins rebind and the membrane can be used for sequencing. Cytochrome b from bovine heart, which is present in *N*-formylated form [16] and contains a single D-P bond [17], was used as a control.

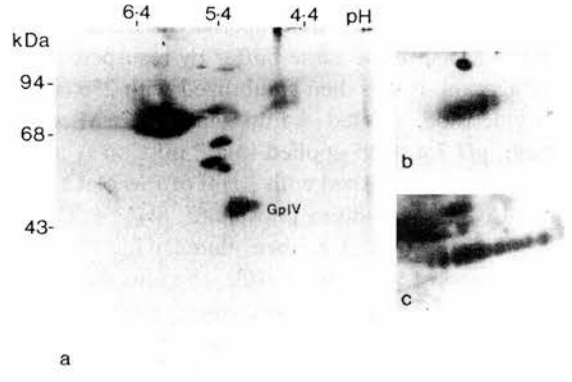


Fig. 1. Glycoprotein IV and Ac45 are closely similar in immune blots. (a) Lectin blot of a two-dimensional electrophoretogram of chromaffin granule membranes, decorated with Con A to visualize glycoprotein IV and other high-mannose glycoproteins. (b) Blot of the glycoprotein IV area of a similar gel, using an antiserum raised against purified glycoprotein IV. (c) Blot of the glycoprotein IV area of a similar gel, using an antiserum raised against a fusion protein containing part of the Ac45 sequence.

Two-dimensional separation of chromaffin granule membrane proteins by electrofocussing in urea/Triton X-100 in the first dimension and SDS-polyacrylamide gel electrophoresis in the second [5] produces characteristically-shaped spots for the glycoproteins, revealed by decoration of blots with Con A (see Fig. 1a). An antiserum directed against purified glycoprotein IV recognized a heterogeneous protein of apparent molecular mass 45–50 kDa and isoelectric point 4.8–5.3 (Fig. 1b), in a characteristic pattern. The same glycoprotein was recognized by the antiserum raised against a chimera [9] containing a 197-amino acid C-terminal fragment of Ac45 fused to *E. coli* maltose binding protein (Fig. 1c). This first hint of the identity of GpIV and Ac45 was confirmed by direct sequencing of the proteins, as described below.

Ac45 was postulated to be part of the H^+-ATPase, since it co-sedimented through a glycerol density gradient with other H^+-ATPase subunits [9]. Two dimensional electrophoresis of chromaffin granule membrane proteins, in which Blue native polyacrylamide gel electrophoresis (PAGE) was followed by SDS-tricine PAGE [13], confirms this. Native electrophoresis in the first dimension separates the holo-ATPase (V_1V_0) from the membrane sector lacking the catalytic subunits (V_0). The apparent molecular masses of these complexes are 850 and 460 kDa, respectively, using bovine heart mitochondrial respiratory complexes as standards [18]. The positions of V_1V_0 and V_0 were confirmed using antisera directed against V_1 (72 and 57 kDa subunits) and V_0 (116 and 39 kDa subunits) (results not shown). On second-dimension SDS-gel electrophoresis the individual V-ATPase subunits are resolved (Fig. 2a). Blotting with the anti-glycoprotein IV serum shows that Ac45/glycoprotein IV is present in both subcomplexes, and must therefore be a component of V_0 (Fig. 2b). This is consistent with the known topography of the H^+-ATPase, in that the catalytic subunit complex (V_1) is located on the cytoplasmic face of the granule, whereas glycosyl chains are extracytoplasmic, and attached to subunits that are integral to the granule membrane [10].

Direct N-terminal sequencing of glycoprotein IV, prepared by lectin affinity chromatography, and of Ac45, prepared by Blue Native polyacrylamide gel electrophoresis to isolate the V_0 sector and subsequent SDS-gel electrophoresis to separate the subunits, were performed. In each case the same sequence, VPPTMNVP, was obtained. This was identified as an internal sequence (amino acids 247–254) within the published Ac45 sequence [9]. It confirms that glycoprotein IV and Ac45 are identical proteins.

The DNA-deduced sequence of Ac45 encodes an open reading-frame for a protein of 468 amino acids, with a potential N-terminal signal sequence of 35 amino acids, a C-terminal membrane-spanning domain and seven potential N-glycosylation sites. Our results suggest that this is further processed by cleavage between two valines (residues 246 and 247) to a mature protein of only 222 amino acids. Presumably the apparent molecular mass of 45–50 kDa of this fragment is due to glycosylation (see below). Proteolytic degradation during purification can be excluded since the bands were transferred directly from the SDS gel to PVDF membranes, and then sequenced.

Nevertheless, we performed another experiment that excluded the presence of a larger, N-terminally blocked protein, with the full Ac45 sequence, within the transferred protein band. Acidolytic cleavage with 80% formic acid should split this protein once, since a single D-P bond is present in the DNA-deduced sequence [9] and the newly-generated N-terminal sequence PALELGPR should then be observed, in addition to VPPTMNVP. However no new

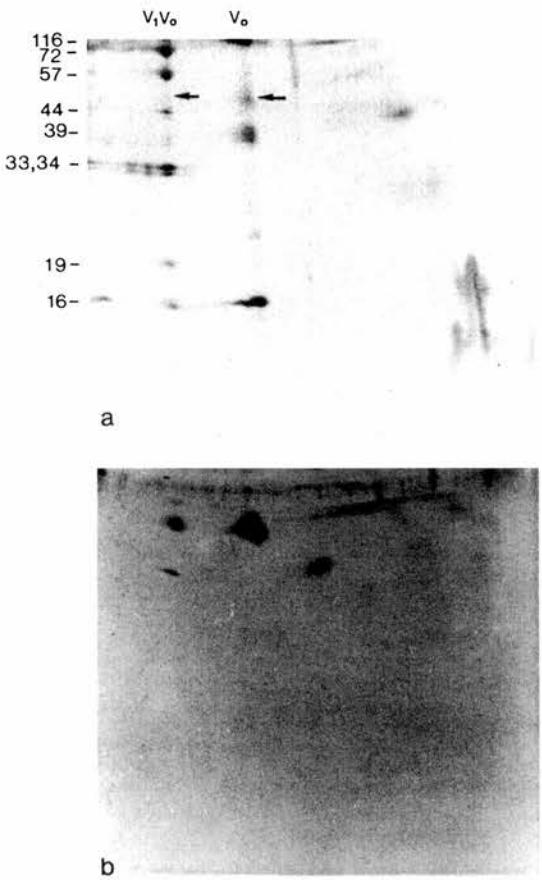


Fig. 2. Ac45/Glycoprotein IV is a component of the membrane sector of vacuolar ATPase. (a) Partially-purified chromaffin granule V-ATPase complexes were separated by Blue native gel electrophoresis (left to right) followed by SDS-tricine gel electrophoresis (top to bottom), then stained with Coomassie blue G to visualize the ATPase subunits. The positions of the V-ATPase subunits are shown on the left. Ac45 stains poorly, and its position is shown by arrows. (b) Immune blot of a similar gel, using the anti-glycoprotein IV serum.

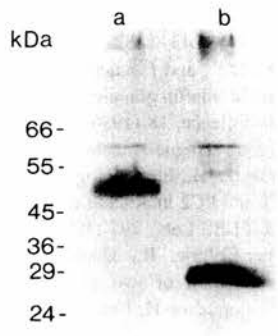


Fig. 3. Ac45/Glycoprotein IV has an apparent peptide molecular mass of 29 kDa. Partially-purified chromaffin granule V-ATPase was deglycosylated with peptide *N*-glycosidase F. then proteins separated by SDS-PAGE and an immune blot made using the anti-glycoprotein IV serum. (a) Control, incubated without the enzyme; (b) deglycosylated enzyme. The scale on the left shows the positions of molecular mass markers.

sequence was obtained, while in a control experiment with cytochrome b from bovine heart, similarly prepared, cleavage was observed.

Enzymatic deglycosylation yields a protein of apparent molecular mass 29 kDa (Fig. 3). The processed form of Ac45, containing the C-terminal 222 amino acids of the sequence deduced from cDNA, would have an unglycosylated molecular mass of 25 390 Da; the difference is within the error expected in estimating the molecular mass of membrane proteins. Expression of the cDNA encoding Ac45 gave a 50 kDa product, consistent with translation of the entire sequence [9]; furthermore this could be glycosylated to an even larger form in pancreatic microsomes. The sequence of post-translational events in the adrenal medulla remains to be elucidated.

Since glycoprotein IV and Ac45 are identical the designation glycoprotein IV could now be discontinued, and the protein numbered with the H^+-ATPase subunits. Its function in the ATPase complex is not yet known, nor is it clear whether it is peculiar to the chromaffin granule H^+-ATPase, or whether Ac45 homologues are present in other vacuolar-type ATPases. The heterogeneity of Ac45 and its weak staining with Coomassie blue tend to hinder its detection even in highly-purified ATPase preparations. In this context is also noteworthy that glycoprotein IV/Ac45 was not detected by periodic acid-Schiff staining of chromaffin granule membranes from pig and horse [1].

This work was supported by a Libyan Government Studentship to F.G., and funds from the Austrian Science Foundation (A.L.), Deutsche Forschungsgemeinschaft (H.S.) and the Wellcome Trust (D.K.A.)

[1] Huber, E., König, P., Schuler, G., Aberer, W., Plattner, H. and Winkler, H., Characterization and topography of the glycoproteins of adrenal chromaffin granules, J. Neurochem., 32 (1979) 35–47.
[2] Gavine, F.S., Pryde, J.G., Deane, D.L. and Apps, D.K., Glycoproteins of the chromaffin granule membrane: separation by two-

dimensional electrophoresis and identification by lectin blotting, J. Neurochem., 43 (1984) 1243–1252.
[3] Winkler, H., Apps, D.K. and Fischer-Colbrie, R., The molecular function of adrenal chromaffin granules: established facts and unresolved topics, Neuroscience, 18 (1986) 261–290.
[4] Kirchmair, R., Gee, P., Hogue-Angeletti, R., Laslop, A., Fischer-Colbrie, R. and Winkler, H., Immunological characterization of the endoproteases PC1 and PC2 in adrenal chromaffin granules and in the pituitary gland, FEBS Lett., 297 (1992) 302–305.
[5] Laslop, A., Fischer-Colbrie, R., Hook, V., Obendorf, D. and Winkler, H., Identification of two glycoproteins of chromaffin granules as carboxypeptidase H, Neurosci. Lett., 72 (1986) 300–304.
[6] Hieber, D.A. and Christie, D.L., Characterization of glycoprotein II from bovine adrenal medullary chromaffin granules, J Biol. Chem., 268 (1993) 11073–11078.
[7] Palmer, D.J. and Christie, D.L., The primary structure of glycoprotein III from bovine adrenal medullary chromaffin granules. Sequence similarity with human serum protein 40,40 and rat Sertoli cell glycoprotein 2, J. Biol. Chem., 265 (1990) 6617–6623.
[8] Apps, D.K., Percy, J.M. and Pérez-Castiñeira, J.R., Topography of a vacuolar-type H^+-translocating ATPase: chromaffin-granule ATPase I, Biochem. J., 263 (1989) 81–88.
[9] Supek, F., Supekova, L., Mandiyan, S., Pan, Y.-C.E., Nelson, H. and Nelson, N., A novel accessory subunit for vacuolar H^+-ATPase from chromaffin granules, J. Biol. Chem., 269 (1994) 24102–24106.
[10] Laslop, A., Steiner, H.J., Egger, C., Wolkersdorfer, M., Kapelari, S., Hogue-Angeretti, R., Erickson, J.D., Fischer-Colbrie, R. and Winkler, H., Glycoprotein III (clusterin, sulphated glycoprotein 2) in endocrine, nervous and other tissues: immunochemical characterization, subcellular localization and regulation of biosynthesis, J. Neurochem., 61 (1993) 1498–1505.
[11] Clegg, J.C.S., Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to arenavirus and flavevirus proteins, Anal. Biochem., 127 (1982) 389–394.
[12] Pérez-Castiñeira, J.R. and Apps, D.K., Vacuolar H^+-ATPase of adrenal secretory granules. Rapid partial purification and reconstitution into proteoliposomes, Biochem. J., 271 (1990) 127–131.
[13] Schägger, H. and von Jagow, G., Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, Anal. Biochem., 199 (1991) 223–231.
[14] Schägger, H. and von Jagow, G., Tricine-sodium dodecyl sulphatepolyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem., 166 (1987) 368–379.
[15] Schägger, H., Denaturing electrophoretic techniques. In G. von Jagow and H. Schägger (Eds.), A Practical Guide to Membrane Protein Purification, Academic Press, San Diego, 1994.
[16] von Jagow, G., Schägger, H., Engel, W.D., Machleidt, W., Machleidt, I. and Kolb, H.J., Bovine heart complex III: isolation and characterization of cytochrome b, FEBS Lett., 91 (1978) 121–125.
[17] Anderson, S., De Brujin, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G., Complete sequence of bovine mitochondrial DNA, J. Mol. Biol., 156 (1982) 683–717.
[18] Webster, L.C. and Apps, D.K., Analysis of nulcleotide binding by a vacuolar proton-translocating ATPase, Eur. J. Biochem., 240 (1996) 156–164.

Supek F, Supekova L, Mandiyan S, Pan Y-CE, Nelson H, Nelson N (1994) A novel accessory subunit for vacuolar H^+-ATPase from chromaffin granules. *J. Biol. Chem.* **269:**24102-24106

Supekova L, Sbia M, Supek F, Ma Y, Nelson N (1996) A noval subunit of vacuolr H^+-ATPase related to the b subunit of F-ATPases. *J. Exp. Biol.* **199:**1147-1156

Sutcliffe JG, Shinnick TM, Green N, Liu F-T, Niman HL, Lerner RA (1980) Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. *Nature* **287:**801-805

Sutton R, Apps DK (1981) Isolation of a DCCD-binding protein from bovine chromaffin granule membranes. *FEBS lett.* **130:**103-106

Tagaya M, Toyonaga S, Takahashi M, Yamomoto A, Fujiwara T, Akagawa K, Moriyam Y (1995) Syntaxin 1 (HPC-1) is associated with chromaffin granules. *J. Biol. Chem.* **270:**15930-15933

Takahashi T, Funatsu M (1962) Biochemical studies on caster bean hemagglutinin. *J. Biochem.* **51:**288-292

Tatemoto K, Carlquist M, Mutt V (1982) Neuropeptide Y, a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **296:**659-660

Tatemoto K, Efendic F, Mutt V, Makk G, Feistner GJ, Barchas JD (1986) Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature* **324:**476-478

Taugner G, Wunderlich I (1981) Phosphoryl group transfer by a fraction of the soluble proteins of catecholamine storage vesicles. *J. Neurochem.* **36:**1879-189

Taylor CS, Kent UM, Fleming PJ (1989) The membrane-binding segment of dopamine β-hydroxylase is not an uncleaved signal sequence. *J. Biol. Chem.* **264:**14-166.

Terland O, Flatmark T, Kryvi H (1979) Isolation and characterization of noradrenaline storage granules of bovine adrenal medulla. *Biochim. Biophys. Acta.* **553:**460-463

Terland O, Flatmark T (1980) Subcellular distribution of ascorbate in bovine adrenal chromaffin cells. Evidence for accumulation in chromaffin granules against a concentration gradient. *Biochim. Biophys. Acta.* **628:**182-189

Thomas L, Betz H (1990) Synaptophysin binds to physophilin, a putative synaptic plasma membrane protein. *J. Cell. Biol.* **111:**2041-2052

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350-4354

Treimann M, Weber W, Gratzl M (1983) 3`, 5`-cyclic adenosine monophosphate and Ca^{2+} calmodulin-dependent endogenous protein phosphorylation vesicles: identification of two phosphorylated components as tyrosine hydroxylase and protein kinase regulatory type II. *J. Neurochem.* **40:**661-669

Trifaró M, Fournier S, Novas ML (1989) The p65 protein is a calmodulin-binding protein present in several typse of secretory vesicles. *Neurosci.* **29:**1-8

Tsao J, Lin X, Lackland H, Tous G, Wu Y, Stein S (1991) Internally standardized amino acid analysis for determining peptide carrier protein for coupling ratio. *Anal. Biochem.* **197:**137-142

Tugal HB, van Leeuwen F, Apps DK, Haywood J, Phillips JH (1991) Glycosylation and transmembrane topography of bovine chromaffin granule p65. *Biochem. J.* **279:**699-703

Vassilacopoulo D, Ripellino J, Tezapsidis N, Hook VYH, Robakis NK (1995) Full-length and truncated Alzheimer amyloid precursors in chromaffin granules: solubilization of membrane amyloid precursor is mediated by an enzymatic mechanism. *J. Neurochem.* **65:**1423-1426

Von Jagow G, Schägger H, Engel WD, Machleidt W, Machleidt I, Kolb HJ (1978) Bovine heart complex III: Isolation and characterization of cytochrome b561. *FEBS Lett.* **91:**121-125

Von Zastrow M, Tritton TR, Castle JD (1986) Exocrine secretion granules contain peptide amidation activity. *Proc. Natl. Acad. Sci. USA* **83:**3297-3301

Voyta JC, Slakey LI, Westhead EW (1978) Accessibility of lysolecithin in catecholamine secretory vesicles to AcetylCoA: Lysolecithin acyl transferase. *Biochem. Biophys. Res. Commun.* **80:**413-417

Walker JE, Runswick MJ, Saraste M (1982a) Subunit equivalence in *Escherichia coli* and bovine heart milochondria F_1F_O ATPases. *FEBS lett.* **146:**393-396

Walker JE, Runswick MJ, Saraste M (1982a) Subunit equivalence in *Escherichia coli* and bovine heart milochondria F_1F_O ATPases. *FEBS lett.* **146:**393-396

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982b) Distantly related sequence in the alpha and beta subunits of ATP synthase, myosin kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1:**945-951

Wallace EF, Krantz MJ, Lovenberg W (1973) Dopamine β-hydroxylase: A tetrameric glycoprotein. *Proc. Natl. Acad. Sci. USA* **70:**2253-2255

Walter G, Scheidtmann K-H, Carbone A, Laudano AP, Doolittle RF (1986) Antibodies specific for the carboxyl- and amino-terminal regions of simian virus 40. Large tumor antigen. *Proc. Natl. Acad. Sci. USA* **77:**5197-5200

Wang S-Y, Moriyama Y, Mandel M, Hulmes JD, Pan Y-CE, Danho W, Nelson H, Nelson N (1988) Cloning of cDNA encoding a 32-kDa protein: An accessory polypeptide of the H^+-ATPase from chromaffin granules. *J. Biol. Chem.* **263:**17638-17642

Wary W, Boulikas T, Wary VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118:**197-203

Weber A, Winkler H (1981) Specificity and mechanism of nucleotide uptake by adrenal chromaffin granules. *Neurosci.* **6:**2269-2276

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:**4406-4412

Webster LC, Apps DK (1996) Analysis of nucleotide binding by a vacuolar proton-tanslocating ATPase. *Eur. J. Biochem.* **240:**156-164

Weiler R, Steiner H-J, Schmid KW, Obendorf D, Winkler H (1990) Glycoprotein II from adrenal chromaffin granules is also present in kidney lysosmes. *Biochem. J.* **272:**87-92

Westhead EW, Winkler H (1982) The topography of ganglioside in the membrane of chromaffin granule of bovin adrenal medulla. Neurosci. **7:**1611-1614

Wiedemann C, Schäfer T, Burger MM (1996) Chromaffin granule-associated phosphtidylinositol 4-kinase is required for stimulated secretion *EMBO J.* **15:**2094-2101

Winkler H (1976) The composition of adrenal chromaffin granules: an assessment of controversial results. *Neurosci.* **1:**65-80

Winkler H, Apps DK, Fischer-Colbrie R (1986) The molecular function of adrenal chromaffin granules: established facts and unresolved topics. *Neurosci.* **18:**261-290

Winkler H, Carmichael SW (1982) The chromaffin granule. In the secretory granule (eds Poisner A. M. and Trifaro J. M.), pp.3-79. Elsevier Biomedical press. Amsterdam.

Winkler H, Westhead E (1980) The molecular organization of adrenal chromaffin granules. *Neurosci.* **5:**1803-1823

Wood SL, Apps DK, Phillips JH (1985) Purification of chromaffin granule membrane glycoproteins by affinity chromatography on lectin columns. *Biochem. Soc. Trans.* **13:**710-112

Wu HJ, Rozansky D, Parmer RJ, Gill B, O'Conner DT (1991) Structure and function of the chromogranin A gene. *J. Biol. Chem.* **266:**13130-13134

Xie XS, Crider BP, Stone DK (1989) Isolation and reconstitution of the chloride transporter of clathrin-coated vesicles. *J. Biol. Chem.* **264:**18870-18873

Zachowski A (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J.* **294:**1-14

Zachowski A, Henry J-P, Devaux PF (1989) Control of transmembrane lipid asymmetry in chromaffin granules by an ATP- dependent protein. *Nature* **340:**75-76

Zhang JZ, Davletov BA, Südhof TC, Anderson RGW (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* **78:**751-760

Zhang J, Myers M, Forgac M (1992) Characterization of the V_o domains of the coated vesicles (H^+)-ATPase. *J. Biol. Chem.* **267:**9773-977

Zimniak L, Dittrich P, Gogarten JP, Kibak H, Taiz L (1988) The cDNA sequence of the 69-kDa subunit of the carrot vacuolar H-ATPase. Homology to the β-chain of the F_1F_o-ATPases. *J. Biol. Chem.* **263:**9102-9112

Yariv J, Kalb AJ, Katachalzki A (1967) Isolation of an L-fucose binding protein from *Lotus tetragonolobus seed. Nature* **215:**890-896

Yokoi H, Hadano S, Kogi M, Kang X, Wakask K, Eikeda J (1994) Isolation of expressed dequences encoded by the human Xq terminal portion using microclone probes generated by laser microdissection *Genomics* **20:** 404-411