MOLECULAR PROPERTIES OF CARNITINE PALMITOYLTRANSFERASES I AND II FROM RAT LIVER MITOCHONDRIA

MYLES C B MARSHALL

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DECLARATION

I hereby declare that this thesis was composed by myself and that all the work described herein is my own.

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ABSTRACT

Carnitine palmitoyltransferases (CPT) I and II are the enzymes responsible for enabling activated long chain fatty acids to be translocated across the mitochondrial impermeable barrier in rat liver cells. Carnitine is esterified onto the fatty acid on the cytosolic side of the mitochondrial inner membrane, releasing CoA, and the activated fatty acid is regenerated on the matrix side, releasing free carnitine. The carnitine-fatty acid is translocated across the inner membrane by a carnitine acyl-carnitine translocase. Once in the mitochondrial matrix, the activated fatty acid is available for β-oxidation.

The aim of this thesis was to purify both CPT I and II, with a view to isolating the nuclear genes which encoded them. This would answer questions intrinsic to the CPT system; are the two enzymes identical; do they reside on the same membrane; are they encoded by one gene; and how are the targeted to two different locations in the mitochondrion?

CPT I was localised in the outer mitochondrial membrane and then partially purified. The protein was digested with specific peptidases and the fragments N-terminally sequenced and used to generate oligonucleotides for cDNA library screening and polymerase chain reaction (PCR). The partially purified protein was also used to generate polyclonal antibodies, which were used to screen λgt11 cDNA libraries. A positive clone was isolated from the λgt11 cDNA library using affinity purified serum. When sequenced, the clone was identified as long chain fatty acid CoA synthase.

CPT II was purified from rat liver mitochondrial inner membranes and N-terminally sequenced. The purified protein was used to generate polyclonal antibodies. Oligonucleotides were generated to the cDNA encoding CPT II reported by Woeltje *et al* (1990b) and used to PCR up a 300 bp fragment, which when cloned and sequenced was identified as the C-terminus of CPT II. This fragment was then used unsuccessfully to screen λgt10, λgt11 and λgt11-stretch cDNA libraries, in order to find a full length clone.

The affinity purified sera was used to show that CPT I and II were immunologically distinct and were localised on the rat liver mitochondrial outer and inner membranes respectively. Using the information generated by this thesis along with other recent

publications a possible topology of CPT I in the rat liver mitochondrial outer membrane was elucidated.

ABBREVIATIONS

A - adenine

ATP - adenosine triphosphate

BSA - bovine serum albumin

BrP- CoA-bromopalmitoyl-Coenzyme A

C - cytosine

cAMP - cyclic adenosine phosphate

CAT - carnitine

CHAPS - (acetyltransferase)3L(3-Cholamido propyl)dimethyl -ammonio]-1-propansulphanate

CPT I - carnitine palmitoyltransferase I

CPT II - carnitine palmitoyltransferase II

CoA - coenzyme A

COT - carnitine octanoyltransferase

DDT - 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane

DEPC - diethyl pyrocarbonate

dH_2O - distilled water

DHFR - dihydrofolate reductase

DMSO - dimethyl sulphoxide

DNA - deoxyribonucleic acid

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

FAD^+ - flavin adenine dinucleotide

FADH - reduced flavin adenine dinucleotide

$F_1\beta$-β - subunit of F_1 ATPase

FFA - free fatty acids

G - guanine

GIP - general insertion protein

GPAT - glycerol phosphate acyltransferase

GTP - guanisine triphosphate

HMG-CoA - hydroxy-3-methylglutaryl-coenzyme A

HSP - heat shock protein

IPTG - isopropyl thiogalactoside

kb - kilobase

kDa - kilodaltons

LDL - low density lipoproteins

LSB - Laemmli sample buffer

MAO - monoamine oxidase

MEGA 8 - Octanoyl-N-methyl glutamide

MEGA 10 - decanoyl-N-methyl glutamide

MPP - mitochondrial processing peptidase

NAD^+ - nicotinamide adenine dinucleotide

NADH - reduced nicotinamide adenine dinucleotide

NTP - adenosine triphosphate and guanosine triphosphate

OD - optical density

OG - octyl glucoside

PEG - polyethylene glycol

PEP - processing enhancing protein

PCR - polymerase chain reaction

pfu - plaque forming unit

PMSF - phenyl methyl sulphonyl fluoride

PPi - pyrophosphate

Pi - inorganic phosphate

R - purine

RNA - ribonucleic acid

SDS - sodium dodecyl sulphate

T - thymine

TEMED N,N,N',N'-tetramethylethylene diamine

TCA - trichloroacetic acid

TDG-CoA - 2-tetradecylglycidyl-coenzyme A

VLDL - very low density lipoproteins

Y - pyrimidine

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CHAPTER 1

INTRODUCTION

CHAPTER 1

Introduction

1 General introduction

The uptake of activated long chain fatty acids into the mitochondrion prior to β-oxidation, is facilitated by a transport system involving the membrane bound proteins; carnitine palmitoyltransferases (CPT) I & II, and a carnitine-acylcarnitine translocase protein. There has been growing interest in the CPT step as a potential target for pharmacological intervention in diabetes therapy (Tutwiler *et al*, 1985; Wolf and Engel, 1985). CPT II has also been identified as a site of inherited mutations, some with severe consequences (Declerq *et al*, 1985). Such developments have promoted renewed efforts to explore the CPT system at a molecular level.

1.1 Energy storage within the eukaryotic cell

Within higher eukaryotes there is a considerable diversity of function at the cellular level. These observed physiological differences are reflected in biochemical differences such as the choice of fuel substrate in different cell types. Adipocytes (fat cells) have, as their name suggests, the purpose of storing energy in the form of fat, mainly triacylglycerols, as a reserve for the body in times of starvation. Up to 90% of the body's energy reserve is stored in the form of triacylglycerols. The hydrophobic nature of triacylglycerols enables them to be stored almost completely dehydrated hence devoid of excess weight due to water.

The other main store of energy within the body is glycogen which is stored in the liver and the skeletal muscle. The mobilisation of glycogen reserves releases glucose-6-phosphate which is either converted into glucose, which is released into the blood stream by tissues that contain glucose-6-phosphatase (e.g. liver) or intracellularly converted into pyruvate. However, glycogen storage is limited due to the hydrated nature of the glycogen. The mass of glycogen trebles when hydrated and therefore stores less potential energy than an equal weight of triacylglyceride. Nevertheless, a degree of glycogen storage is necessary for the maintenance of blood

glucose levels and the provision of energy under anaerobic conditions (e.g. for fast muscle contraction).

1.2 Role of fatty acids in energy metabolism

With a few exceptions (brain and red blood cells) most tissues have the ability to oxidise fatty acids. Usually, these tissues will use a mixture of glucose and fatty acids, the ratio of which is determined by the physiological state.

Apart from their physiological importance as a fuel substrate, fatty acids are also used in the synthesis of complex lipids, such as phospholipids and triacylglycerols. They are also responsible for the storage and transport of the fat soluble vitamins A, D, E and K.

The oxidation of fatty acids is aerobic since the reducing equivalents formed during fatty acid oxidation can only be oxidised through the electron transport chain. Therefore fatty acid oxidation requires a constant delivery of fatty acids and oxygen. The products of complete oxidation of fatty acids are ATP, H_2O and CO_2, which can never be used as a carbon source for the synthesis of either carbohydrate or protein. Incomplete oxidation of fatty acids gives rise to ketone bodies. This process occurs primarily in the liver which metabolises fatty acids not only for energy, but also for the production of ketone bodies. These substitute for glucose in most tissues including some tissues that are unable to metabolise fatty acids (e.g. brain). The liver uses fatty acids for triacylglycerol synthesis. These are secreted within very low density lipoproteins (VLDL) which are responsible for the transport of triacylglycerols to peripheral tissues such as heart, skeletal muscle and adipose tissue. The fatty acids released through VLDL triacylglycerols, are either oxidised or used for synthesis of triacylglycerols at the periphery.

1.3 Conditions determining substrate selection by tissues

The supply of glucose, fatty acids (lipoproteins) and ketones are controlled by the physiological state of the animal, which is governed by a variety of factors. In a normal state the diet of the animal supplies both glucose and fatty acids which are metabolised in the liver to triacylglycerols, and are transported by VLDL to both adipose tissue for storage and other tissues such as heart and muscle

for metabolism. The liver does not produce ketone bodies in this state. In this normal state the level of the hormones insulin and glucagon are high and low respectively, (high insulin/glucagon ratio).

In a glucose deficient animal, e.g. starved, the insulin/glucagon ratio is low. Tissues such as heart, diaphragm and red skeletal muscle, must therefore metabolise fatty acids. These can either come directly from their own stores, from fatty acids delivered by VLDL secreted by the liver, or from fatty acids complexed to albumin delivered directly by the circulation after release from adipose tissue. They also metabolise ketone bodies which are produced in the liver. In humans the metabolism of ketone bodies is not only the result of a prolonged glucose deficiency, but can also be the result of longer than normal overnight fast.

1.4 The role of insulin and glucagon

Insulin stimulates the uptake of glucose by binding to specific receptors on the plasma membrane of cells. This activates a protein phosphorylation cascade system. If the level of blood glucose becomes too low, then glucagon is released from the α-cells of the pancreatic islets. Glucagon receptors on the cell membrane are coupled to the activation of sensitive adenylate cyclase. So receptor occupation results in increased levels of intracellular cAMP. This second messenger mainly acts by its activating cAMP dependent protein kinases. One of these cAMP-dependent kinases hydrolyses glycogen reserves to form glucose-6-phosphate. In the liver glucose-6-phosphate is hydrolysed to glucose which is released into the blood stream. cAMP also stimulates gluconeogenesis in the liver such that glucose can be synthesised from precursors such as lactate and alanine.

The uptake of glucose by the brain is not insulin dependent and therefore is proportional to the blood glucose level. This is one of the main reasons why blood glucose levels must be strictly controlled at all times, regardless of the physiological state of the animal. This control is referred to as glucose homeostasis.

Insulin and glucagon also play an important role in the control of fatty acid metabolism. They generally have opposite effects on lipolysis. In adipose tissue insulin decreases lipolysis, and glucagon increases lipolysis. They also have opposite effects on

liver ketogenesis, again with insulin decreasing ketogenesis and glucagon increasing ketogenesis.

Although glucagon supports lipolysis in adipose tissue, it does not support lipolysis in other tissues such as heart or skeletal muscle. This is a vital selective mechanism to ensure that the immediate fatty acid reserves of the organs are not mobilised during starvation, and are therefore left for use by the organ. Therefore, during glucose deficiency an animal will utilise not only the lipolysed stored fatty acids from the adipose tissue, but also the non-lipolysed fatty acids stores in the other tissues (heart, diaphragm red skeletal muscle etc.).

1.5 Types of absolute or relative glucose deficiency

There are two main categories of deficiency. Firstly true glucose deficiency, which is caused either by starvation or by insufficient food intake to satisfy the glucose demand of bodily functions. The second is a relative deficiency, such as in uncontrolled diabetes. In this condition there are very high blood glucose levels but no glucose uptake by the cells, due to either insulin deficiency or insulin resistance.

Diabetes is the general name for a group of glucose metabolism disorders that prevents glucose uptake by tissues. The result of such a disorder is a lack of adequate glycogen in the muscle cells and, unless controlled, a high glucose level in the blood stream. The main symptoms of diabetes, associated with hyperglycaemia, are thirst, weakness, weight loss and polyuria. In extreme cases, the rate of production of ketone bodies in the liver is markedly increased resulting in ketoacidosis. If the blood glucose level is left unattended it would result in coma and ultimately give rise to brain damage and death. There are two types of diabetes mellitus which require different methods of control. Type I is referred to as an absolute insulin deficiency due to non-functional β-cells, whereas type II is due to a relative insulin deficiency. In this state, insulin secretion and/or insulin action may be deficient.

1.5.1 Starvation

During prolonged physical exercise or a period of starvation the animal's glycogen reserves are depleted before its triacylglyceride reserves. During starvation this readily available energy source will last for only a short time in the liver, after which the animal is totally reliant on fatty acid catabolism. Fatty acid reserves can last for a much longer period depending on the physiological state of the animal before starvation. After the depletion of the fat reserves, the animal will start to degrade its protein to generate amino acids which can be used, via glucogenesis, to generate glucose in the liver. In the final stages of starvation protein catabolism will serve as the only source of energy.

1.5.2 Diabetes mellitus type I

Diabetes mellitus type I is caused by an inability of an animal to produce insulin. In humans the defect usually becomes symptomatic during the first 25 years of life. It is caused by either damage to and loss of the β-cells of the pancreatic islets of Langerhans with resulting loss of insulin production. The agent that damages the islets is not known. Two main factors which have been implicated are virus infection and autoimmunity. The latter has been implicated because complement fixing islet cell antibodies can be found in genetically predisposed individuals. It should be noted, however, that not all of these genetically predisposed individuals develop diabetes.

The treatment for the majority of cases involves the administration of exogenous insulin. The intravenous method is usually used during stabilisation of an individual or when the blood contains life-threateningly high glucose levels. Intramuscular insulin injections are the normal method of control for insulin dependent diabetics. There are many modified forms of insulin available (isophane, protamine, zinc and lente etc.) each having a characteristic onset time, peak effect and duration time. These forms of insulin fall into three categories of fast, medium and slow acting insulin. A normal scenario would be a diabetic who would inject twice a day (morning and evening) with a mixture of fast and slow acting insulin. This would be injected usually 30 minutes before meals and would cover two meals from each injection. This method is very

effective after the diabetic has been stabilised. However care must always be taken with the calorific value of the meals. The key element however is the stabilisation of individuals. Stabilisation, on an individual basis, can be very easily obtained or almost impossible for reasons which are not yet understood. In cases where stabilisation is difficult the individuals are referred to as 'brittle' diabetics. In order to avoid either hyper- (section 1.4) or hypo-glycaemia, brittle diabetics must pay great attention to their blood and urine glucose levels at all times. Hypoglycaemia is a frequent and serious problem, which is caused by the blood sugar falling below 5 mM. In many cases the symptoms are undetectable to the sufferer and can manifest themselves as severe character alterations. These include confusion, blurred vision, drowsiness and aggression. The patient will then slip into unconsciousness and convulsions, leading ultimately to coma, brain damage and death. Most type I deaths occur at night due presumably to hypoglycaemia, although post mortem blood glucose levels are not accurate enough to provide proof.

Even in stabilised individuals problems arise when the treatment has been continued over a number of years as is the case in most sufferers. Eye deterioration, although preventable, is common in long term juvenile onset diabetic individuals. This is thought perhaps to be caused by fluctuating glucose levels in the blood stream which cause alterations in the refraction of the lens. This can cause cataracts and in extreme cases haemorrhaging. Ulcerations of the foot are also a common problem along with limb nerve damage which prevents sensations such as touch and pain being experienced from the hands and feet.

Recently molecular biological techniques have been developed to produce human recombinant insulin. This has proved very effective in 90% of sufferers and hopefully should not cause side effects to the same extent as its porcine counterpart. In the remaining cases a more serious problem has been encountered. In these cases stabilisation is unobtainable with the recombinant human insulin, and violent side effects resulting in frequent comas are common. Signs of oncoming hypoglycaemia, which were detectable in people treated with the porcine insulin, are no longer detectable. The cause of such effects is not known, and the reason why they should happen to a greater

extent with human recombinant insulin and not porcine insulin is confusing.

1.5.3 Diabetes mellitus type II

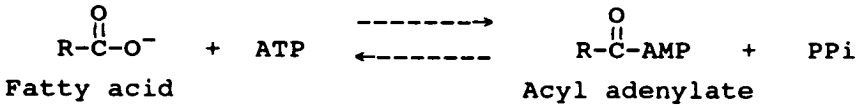
Maturity onset diabetes (mellitus type II) is a rather different scenario, and is sometimes referred to as non-insulin dependent diabetes. Individuals must be genetically predisposed to the disorder but need not necessarily develop the condition. The individual is usually in the latter part of life (50 plus) and has a history of obesity. Non-insulin dependent diabetic patients can secrete insulin, but their serum insulin concentrations may be diminished, normal, or even elevated. The problem lies with the receptors on the cells to which the insulin binds. The insulin receptors in the cell membrane are either blocked or desensitised. The cell therefore can not respond to insulin, and hence glucose cannot be transported into the cells. This is referred to as insulin resistance. The usual treatment for insulin resistant diabetics is dietary control. The patients must eat only enough to maintain hour by hour functions, and must strictly avoid simple carbohydrates of any kind as this would almost certainly result in hyperglycaemia. If dietary control is not sufficient to keep the blood sugar normal, then there is a wide spectrum of oral drugs available. Most of the drugs available fall into two categories. The sulphonylurea group act chiefly by stimulating insulin release from the β-cells of the pancreatic islets, and the biguanides act by decreasing hepatic glucose reduction. The drugs can be used individually or in combinations with each other. Problems arise if the drugs are taken in conjunction with other drugs such as alcohol, beta-blockers and aspirin, which cause hypoglycaemia, or corticosteroids which cause hyperglycaemia. In the type II disorder a more attractive cure is one of prevention. Even if the individuals are genetically predisposed, they will never develop the condition if they eat a normal diet, avoiding simple carbohydrates and obesity.

1.6 Fatty acid metabolism

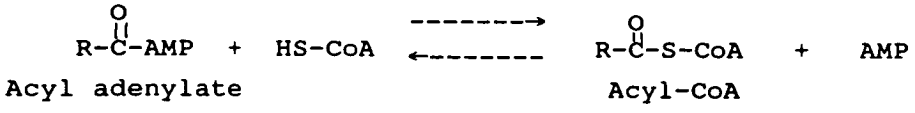
Fatty acids have an important role in the metabolism of the cell (section 1.1), and are oxidised in a manner unaffected by either insulin deficiency or insulin resistance. Therefore, theoretically, the energy produced from fatty acid oxidation could be used to alleviate the effects of diabetes.

1.6.1 Activation of fatty acids

Once in the cell the fatty acids are activated, before being transported into the mitochondria or the peroxisomes prior to β-oxidation. This activation of the fatty acids is catalysed by carbon chain length dependent fatty acyl-CoA synthases (or fatty acid thiokinases). These enzymes are widespread through the cell and are usually associated with the membranes of several organelles. The reaction occurs in two stages. Firstly the carboxyl group of the fatty acid binds to the α-phosphoryl group of adenosine triphosphate (ATP) to form an acyl adenylate and releasing pyrophosphate (PPi) as a by-product. The PPi is hydrolysed by pyrophosphatase.



Secondly the sulphydryl group of CoA attacks the acyl adenylate, which is tightly bound to the enzyme, to form acyl-CoA and AMP.



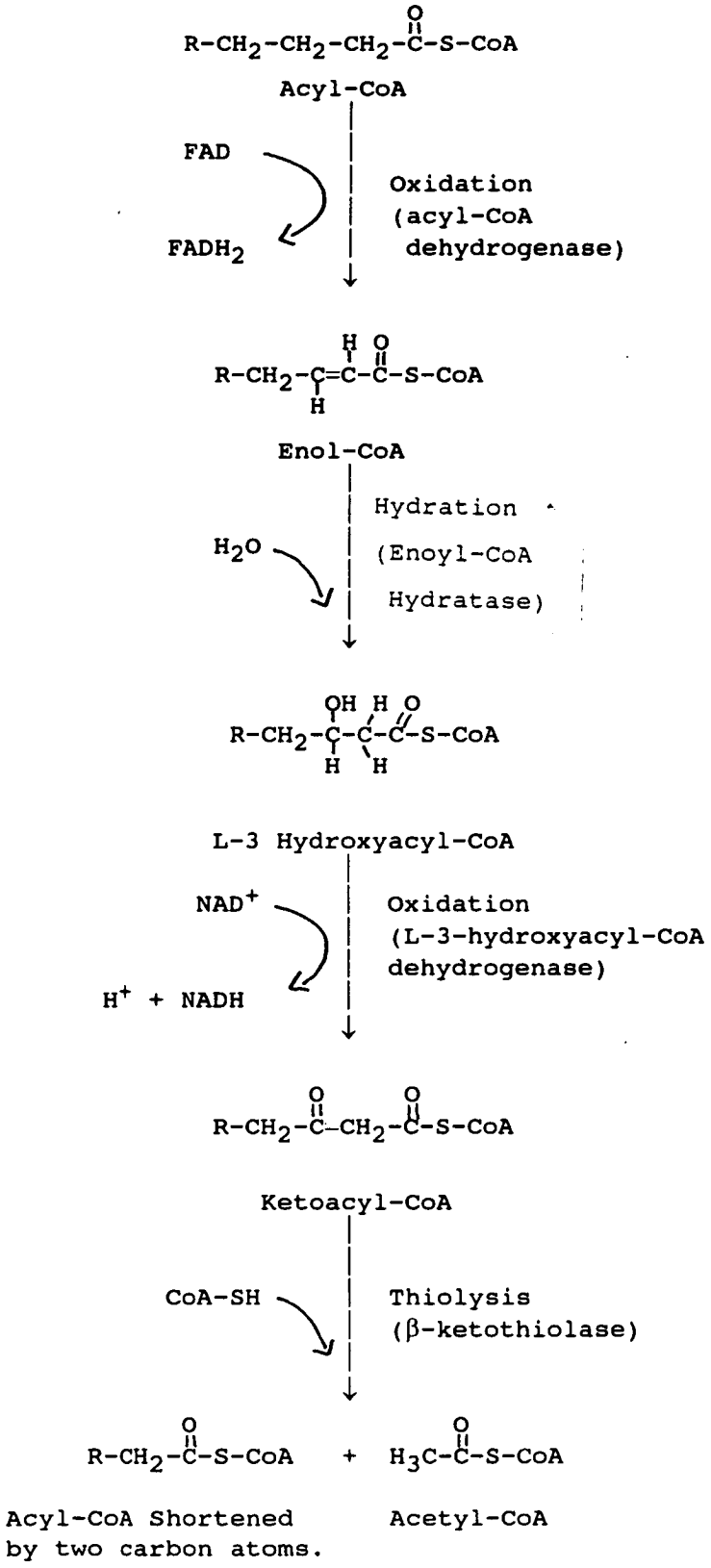
It is interesting to note that the partial reactions are freely reversible and the equilibrium constant is close to one. The reaction is actually driven forward by hydrolysis of pyrophosphate by

pyrophosphatase. This makes the overall reaction irreversible since two high energy bonds are broken, but only one is formed. Long chain acyl-CoA synthase from rat is a very interesting protein because it has no typical targeting sequence as deduced from its cDNA sequence (Suzuki *et al*, 1990). Also there is no difference in the size of the mature protein and the precursor which is synthesised free in the cytoplasm. This feature is common in mitochondrial outer membrane proteins. The authors also reported that the enzyme could be purified not only from mitochondrial outer membrane, but also from microsomes and peroxisomal membrane. In each case the proteins have an identical molecular weight and an immunogenic similarity. If these results were to be believed then this would be the only known enzyme to be distributed in three different organelles. The absence of a classical targeting sequence to either mitochondria, peroxisomes or endoplasmic reticulum is unusual, especially as the enzyme is so widely targeted. Doubts must therefore be cast as to the reliability of the subcellular fractionations used during the isolation of the enzyme from the different organelles. In such procedures contamination from fraction to fraction is a common problem (Parsons *et al*, 1966).

1.6.2 β-oxidation of long chain saturated fatty acids

β-Oxidation, in eukaryotes, takes place both in the peroxisomes and in the mitochondrial matrix. The process is carried out by four reactions which sequentially remove two carbon units from the activated fatty acid in a cyclic manner. The reactions are (i) oxidation linked to flavin adenine dinucleotide (FAD), (ii) hydration, (iii) oxidation linked to nicotinamide adenine dinucleotide (NAD^+), and (iv) thiolysis by CoA (Fig 1.1). The principal reactions involved in the degradation of saturated fatty acyl-CoA produce reduced flavin adenine dinucleotide ($FADH_2$), reduced nicotinamide adenine dinucleotide (NADH), and acetyl-CoA. The first reaction in each cycle of the β-oxidation is the oxidation of acyl-CoA by an acyl-CoA dehydrogenase to give an enol-CoA with a *trans* double bond between C-2 and C-3. The next step is the hydration of this double bond by enol-CoA hydratase. This reaction is stereospecific and only the L-isomer of 3-hydroxyacyl-CoA is formed when the *trans*-Δ^2 double bond is hydrated. The second oxidation then takes place converting the hydroxyl group at C-3 into a keto group,

Fig 1.1 β-oxidation of medium to long chain saturated fatty acids



generating NADH. This oxidation is catalysed by the enzyme L-3-hydroxyacyl-CoA dehydrogenase, which is absolutely specific for the L-isomer of the hydroxyacyl substrate. The final step is the cleavage of 3-ketoacyl-CoA by the thiol group of a second molecule of CoA, which yields acetyl-CoA and an acyl-CoA shortened by two carbon atoms. This thiolytic cleavage is catalysed by β-ketothiolase. The shortened acyl-CoA then goes through further cycles of degradation using the same enzymes (since they are not specific to chain length) until, on the last cleavage the acyl-CoA remaining is either also acetyl-CoA (in the case of even numbered fatty acids) or propionyl-CoA (in the case of the less common odd numbered fatty acids). In such cases the propionyl-CoA is converted into succinyl-CoA, firstly by carboxylation to yield D-methylmalonyl-CoA at the expense of one ATP, (catalysed by propionyl-CoA carboxylase). The D-isomer is then racemised to the L-isomer of methylmalonyl-CoA, which is the substrate for the methylmalonyl-CoA mutase enzyme which converts it into succinyl-CoA. The succinyl-CoA is then ready to enter the citric acid cycle.

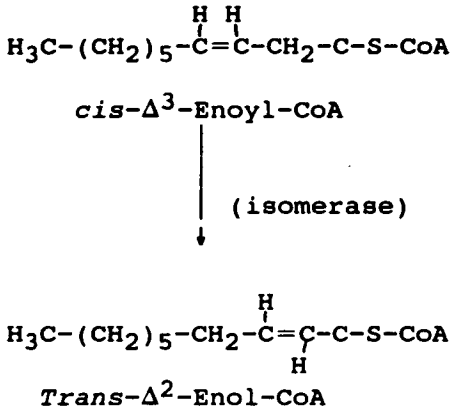
The number of ATP molecules obtained from the complete oxidation of a typical long chain fatty acid such as palmitate (a C-16 saturated fatty acid) can be calculated. The complete degradation of palmitate requires seven reaction cycles yielding 8 molecules of acetyl-CoA. Three molecules of ATP are generated for each NADH that is oxidised, two for each $FADH_2$ and 12 for each acetyl-CoA being completely oxidised by the citric acid cycle. Hence the total yield of ATP, calculated from the stoichiometric equation of oxidation, is 131 molecules of ATP per molecule of palmitate.

$$\text{Palmitoyl-CoA} + 7\ \text{FAD} + 7\ \text{NAD}^+ + 7\ \text{CoA} + 7\ \text{H}_2\text{O} \rightarrow 8\ \text{acetyl-CoA} + 7\ \text{FADH}_2 + 7\ \text{NADH} + 7\ \text{H}^+$$

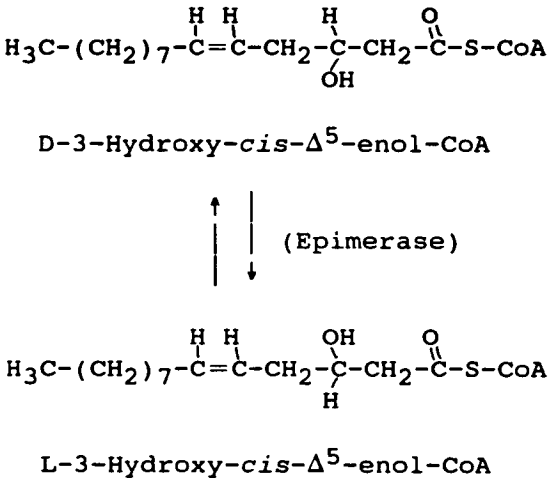
However during the activation of palmitate two high energy bonds are used up so the net gain of ATP from the complete oxidation of palmitate is 129.

1.6.3 β-oxidation of long chain unsaturated fatty acids

The oxidation of unsaturated fatty acids requires two additional enzymes. The first is an isomerase which converts a double bond in the *cis* configuration to a *trans* configuration by substituting the double bond between C3-C4 for the single bond between C2-C3.



This reaction occurs prior to hydration by enoyl-CoA hydratase (section 1.6.2). This means that after hydration both L- and D-isomers will exist since the enzyme also hydrates a *cis*-Δ^2 double bond, but the product then is the D-isomer which is not a substrate for the next enzyme. An epimerase must therefore be used to change the D-isomer to the L-isomer which, as indicated above is the only substrate of the L-3-hydroxyacyl-CoA dehydrogenase.



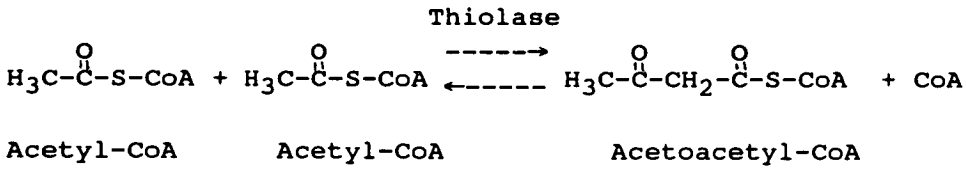
1.6.4 By-products of β-oxidation of fatty acids

Acetyl-CoA, formed by the oxidation of the fatty acids, is oxidised by entering the citric acid cycle. It can only do this when carbohydrates are also being metabolised. This is because its entry into the citric acid cycle depends on the availability of oxaloacetate for the formation of citrate.

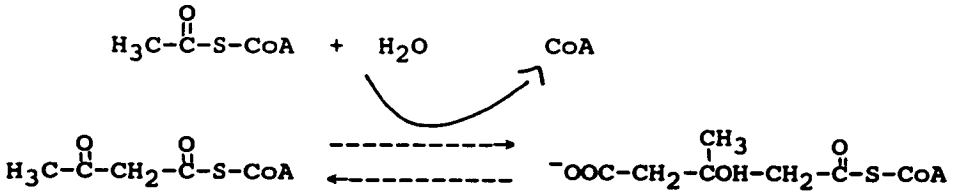
In states of fasting, diabetes or extreme exertion, oxaloacetate is also used for gluconeogenesis and is therefore unavailable for condensation with acetyl-CoA.

Acetyl-CoA is indirectly responsible for the activation of the enzyme pyruvate carboxylase. This enzyme catalyses the first reaction in the glucogenic pathway ie the formation of oxaloacetate from pyruvate. Pyruvate carboxylase has biotin as a prosthetic group, which must first be carboxylated before it can catalyse the reaction. Biotin can only be carboxylated when acetyl-CoA is associated with the enzyme, although acetyl-CoA plays no further part in the formation of oxaloacetate. Therefore when acetyl-CoA is abundant in the liver the glucogenesis will be activated.

Under such conditions acetyl-CoA is diverted to the formation of ketone bodies (acetoacetate, D-3-hydroxybutyrate). Acetoacetate is formed from acetyl-CoA in three steps. Firstly two molecules of acetyl-CoA condense to form acetoacetyl-CoA.



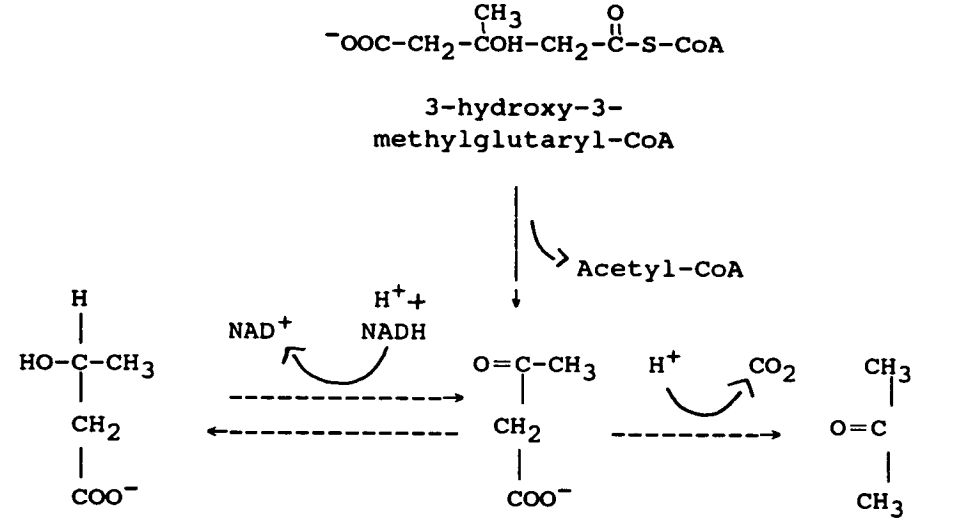
This reaction, which is catalysed by thiolase, is a reversal of the thiolysis step in the oxidation of fatty acids (Fig 1.1). Acetoacetyl-CoA then reacts with acetyl-CoA and water to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This is catalysed by HMG-CoA synthase.



Acetoacetyl-CoA

3-hydroxy-3-methylglutaryl-CoA

The unfavourable equilibrium in the formation of acetoacetyl-CoA is overcome by the favourable equilibrium of this reaction, which is due to the hydrolysis of a thioester linkage. 3-Hydroxy-3-methylglutaryl-CoA is then cleaved to acetyl-CoA and acetoacetate. 3-Hydroxybutyrate is formed by the reduction of acetoacetate in the mitochondrial matrix and is dependent on the NADH/NAD^+ ratio inside the mitochondrion. The acetoacetate also undergoes slow but spontaneous decarboxylation to acetone.



3-hydroxy-3-methylglutaryl-CoA

D-3-Hydroxybutyrate

Acetoacetate

Acetone

1.6.5 Control of fatty acid β-oxidation

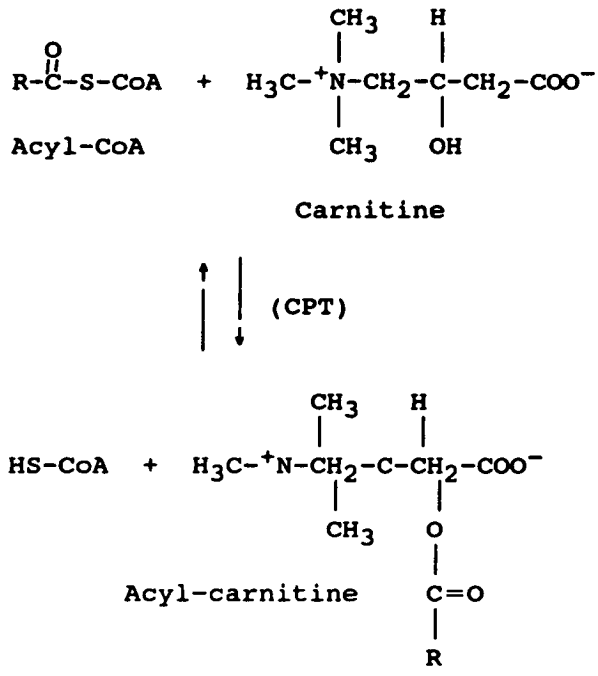
The simplified pathway for the oxidation of fatty acids in the liver has three expected control points.

Fatty acid → acyl-CoA --||→ β-oxidation → ketone bodies

The first point of control is at the partitioning of acyl-CoA between esterification to glycerol and esterification to carnitine outside the mitochondrial permeability barrier. This step is controlled by the activity of overt carnitine palmitoyltransferase I (CPT I). The second branchpoint is at the level of acetyl-CoA. Acetyl-CoA can either form citrate or acetoacetyl-CoA. Citrate can either be used in the Krebs cycle or efflux to the cytosol. Under conditions of high flux, CO_2 production in the citric acid cycle from fatty acid oxidation accounts for only about 5% of total acetyl-CoA. The third point of control is at the intermediate between acetoacetyl-CoA and acetoacetate, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA synthase can undergo reversible inhibition by succinylation, but this is likely to have a small influence on flux (≅ 20%).

The main control point of fatty acid β-oxidation is at the CPT step. This step is catalysed by two enzymes, carnitine palmitoyltransferases (CPT) I and II. It is CPT I which is responsible for the control of the transport of medium to long chain fatty acids into the mitochondrial matrix.

As discussed earlier, the oxidation of medium to long chain fatty acids takes place within the mitochondrial matrix (section 1.6.2). Free fatty acids in the cytosol are readily activated to their CoA esters on the mitochondrial outer membrane surface by long chain fatty acid CoA synthase (section 1.6.1). The mitochondrial outer membrane is permeable to molecules of molecular weight less than or equal to 10000 (section 1.11.1), and is therefore permeable to activated fatty acids. The inner membrane however, is not permeable to activated fatty acids and a method of transport is required. This is facilitated via an acyl-carnitine/carnitine translocase. The acyl group is cleaved from the CoA and transesterified onto carnitine by carnitine palmitoyltransferase I (CPT I).



The acyl-carnitine is then translocated into the mitochondrial matrix via the carnitine-acylcarnitine translocase. The reaction is then reversed by carnitine palmitoyltransferase II (CPT II), resulting in reformation of activated fatty acid and in so doing the releasing of free carnitine.

Factors governing the activity of CPT I include the availabilities of carnitine, glycerol-phosphate and the relative affinities of the two pathways for carnitine.

Acyl-glycerol-phosphate production is catalysed by the enzyme glycerol phosphate acyltransferase (GPAT). It occurs, in different isoforms, as mitochondrial and microsomal enzymes, that are reliant on the level of glycerol phosphate present. This can either be supplied from glycerol in the blood or synthesised from glucose-6-phosphate. The affinity of GPAT for acyl-CoA is not as high as the affinity of carnitine for acyl-CoA. Therefore when acyl-CoA is abundant, the carnitine system (if not inhibited) has preferential choice of the acyl-CoA over the GPAT system.

1.6.6 Control of CPT I

The activity of CPT I is governed by four factors. The amount of CPT I present; the amount of carnitine present; the Vmax of CPT I; and whether or not there are activators or inhibitors affecting its activity.

McGarry and Foster (1977) reported the potent inhibitory affect of malonyl-CoA on CPT I as a key component in the regulation of hepatic fatty acid oxidation and ketogenesis. The characteristics of this inhibition are of a competitive nature with respect to palmitoyl-CoA. This competitive inhibition is however not straight forward since malonyl-CoA and palmitoyl-CoA appear to bind to different active sites. Rather than sterically inhibiting the binding of each other, they seem to change the kinetics of binding via a conformational change in the protein.

The physiological importance of such inhibition can be related to changes in CPT I activity during different physiological states (section 1.3). During carbohydrate feeding (high insulin; low glucagon/insulin ratio) adipose tissue lipolysis is suppressed and plasma free fatty acids (FFA) are low. Within the liver carbon flow through acetyl-CoA carboxylase is brisk, resulting in an elevated concentration of malonyl-CoA and a high rate of lipogenesis. Because of the inhibitory effect of malonyl-CoA on CPT I, newly formed long chain acyl-CoAs are prevented from entering the mitochondria for β-oxidation, and are instead directed into triacylglycerol synthesis and VLDL formation. A futile cycle involving the simultaneous oxidation and synthesis of fatty acids is thus avoided.

Conversely, in ketotic states (low insulin; high glucagon/insulin ratio) lipolysis in adipose tissue is stimulated and plasma FFA levels rise. Simultaneously, because of cAMP mediated interruption of carbon traffic through glycolysis and acetyl-CoA carboxylase in liver, the tissue concentration of malonyl-CoA falls dramatically and fatty acid synthesis is much diminished. Such conditions allow for the de-repression of CPT I, accelerated fatty acid oxidation and a marked increase in the production of acetoacetate and β-hydroxybutyric acid. Once the ketogenic machinery of the liver is activated the output of ketone bodies is governed principally by delivery of fatty acids to the liver.

1.7 Ketone bodies are energy sources within peripheral tissues

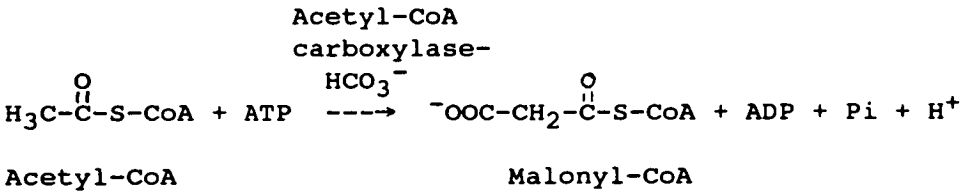
Acetoacetate and 3-hydroxybutyrate are major fuels in the body for respiration and are quantitatively important as a source of energy (section 1.2). Indeed heart muscle and the renal cortex prefer acetoacetate to glucose, and during starvation the brain (which in the normal, fed state utilises glucose) can adapt to using acetoacetate for 75% of its energy requirements. During starvation, extreme exertion or diabetes the ketone bodies are released into the blood stream to reach the major tissues that require energy.

Interestingly the intermediates 3-hydroxy-3-methylglutaryl-CoA (in cholesterol synthesis) and malonyl-CoA (in fatty acid synthesis) are products formed from acetyl-CoA on its reconstitution once outside of the mitochondrion. Obviously in times of starvation neither fats or cholesterol would be synthesised to any great extent so ketone bodies would be produced.

1.8 Synthesis of fatty acids, cholesterol and isoprenol from acetyl-CoA

When the cell requires acetyl-CoA for biosynthetic purposes, it combines with oxaloacetate in the mitochondrial matrix to form citrate which can efflux from the mitochondrion on the tricarboxylate carrier.

In the cytosol, acetyl-CoA is again formed by the action of citrate lyase. Both fatty acids and cholesterol can then be synthesised. The carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase is an irreversible reaction and a committed step to long chain fatty acid synthesis.



The synthesis of long chain fatty acids is carried out by sequential additions of malonyl-CoA. The enzyme responsible for the elongation of the fatty acid is fatty acid synthase. In mammals this consists of two polypeptide chains each of which has several enzymic

activities (multifunctional proteins). One subunit has covalently attached pantothenic acid as a prosthetic group. This compound is a constituent of CoA, and contains a sulphydryl group to which the fatty acids combine as thioesters. This is unlike the enzymes responsible for the oxidation of the fatty acids which show no enzyme complex formation. The first committed step towards cholesterol and isoprenol synthesis is the formation of mevalonate from 3-hydroxy-3-methylglutaryl-CoA.

The enzyme responsible for this reaction is 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). It is a 97 kDa integral membrane protein, that contains seven hydrophobic membrane spanning regions and a binding site for cholesterol. The accomodation of cholesterol in the binding site causes end product inhibition by making the enzyme more susceptible to degradation rather than directly inhibiting the activity of the enzyme. Another level of control of HMG-CoA reductase is effected by insulin and glucagon (insulin being the more dominant). This is thought to be via phosphorylation-dephosphorylation of the enzyme causing deactivation and activation respectively.

1.9 Protein targeting

The structure within the eukaryotic cell is highly organised, and contains many highly specialised organelles, each of which is separated from the cytosol by at least one phospholipid membrane bilayer. The vast majority of polypeptides are synthesised on polysomes in the cytosol. This creates a two fold problem for protein targeting within the cell. Firstly, newly synthesised proteins must be localised to their specific destination and not elsewhere within the cell, creating the need for a highly specific and efficient address system on the protein. Secondly, organelle proteins have to

be translocated into or across biological membranes by processes which are poorly understood. Several different targeting pathways coexist within eukaryotic cells. The biogenesis of many organelles, such as vacuoles, Golgi bodies and the plasma membrane, depends upon delivery of proteins via branches of the secretory pathway and in these cases the initial targeting steps occur at the endoplasmic reticulum (von Heijne, 1986). Transport of proteins into other organelles including mitochondria, peroxisomes and the nucleus, occurs directly from the cytoplasm and therefore requires a vast range of different primary targeting sequences incorporated within the precursor protein.

1.9.1 Cytosolic protein targeting to the nuclear envelope

Targeting sequences that direct proteins to the nucleus have been reported by Silver (1991) to consist of short sequences rich in basic amino acids. The nuclear targeting sequence is present in the mature protein, which depicts a fundamental difference in its targeting pathway compared to other targeting pathways. This property can be rationalised by observing that, during mitosis, the nuclear membrane of many organisms breaks down into vesicles with the result that the soluble components of the nucleus are released into the cytosol. These components are reabsorbed via their targeting pathways after reformation of the nuclear envelope.

1.9.2 Cytosolic protein targeting to the peroxisomes

Peroxisomes are vesicular organelles which belong to the microbody family, and contain a varying array of oxidative enzymes. Peroxisomes are found universally in eukaryotes although in many cases their biological role is unclear. They are particularly abundant in the methylotrophic yeasts such as *Hansenula polymorpha*. When this yeast is grown with methanol as a sole carbon source, it undergoes a massive induction of methanol oxidase which forms crystalline arrays in enlarged peroxisomes. Proteins are imported into peroxisomes directly from the cytosol. Many of these proteins in higher eukaryotes have been found to contain a C-terminal tripeptide targeting signal, serine-lysine-leucine (Ser-Lys-Leu), or a closely related sequence (Gould *et al*, 1989). It has also been shown by Gould

et al (1990), that firefly luciferase, an insect peroxisomal protein, is efficiently targeted to peroxisomes when expressed in the yeasts *H. polymorpha* and *Saccharomyces cerevisiae*, indicating that targeting to peroxisomes has been well conserved throughout eukaryotic evolution.

1.9.3 Cytosolic protein targeting to the mitochondria

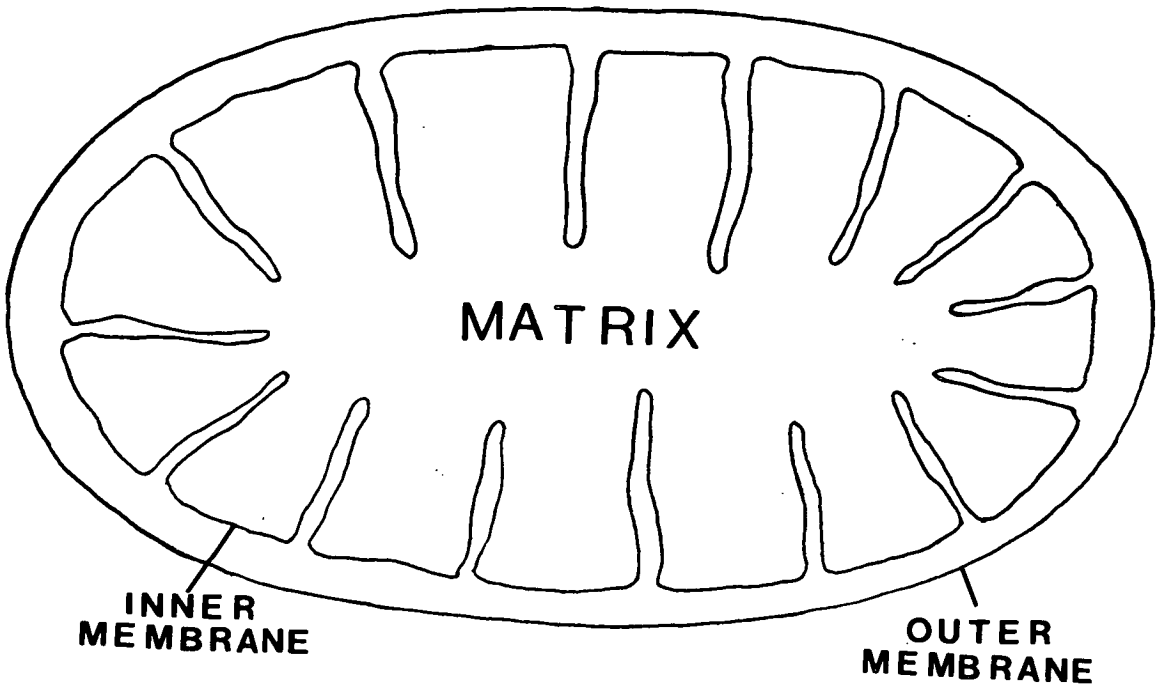
(For review Glover and Lindsay, 1992)

Within the mammalian cell targeting of proteins to mitochondria is made complicated by the complexity of the organelle. The mitochondria are comprised of two membranes an inter-membrane space and a matrix, all of which require specific proteins to be targeted accurately (Fig. 1.2). The mitochondrial targeting sequence is located at the N-terminus of a protein and is usually 20 - 25 amino acids long. Mitochondrial targeting sequences are characterised by a lack of acidic amino acids and a predominance of basic amino acids, and are predicted under biological conditions to form alpha helices. The targeting sequence is usually removed by a protease in the mitochondrial matrix. By following the behaviour of fusion proteins the targeting sequences have in several cases been shown to target proteins which are normally extramitochondrial. For example it was shown by Walker *et al*, (1990) that the mouse cytosolic protein dihydrofolate reductase (DHFR) could be targeted to mitochondria by a fusion with the first 15 amino acid residues of the β-subunit of mitochondrial ATP synthase. The authors also reported that when a larger leader peptide was fused from the ATP synthase (35 amino acids) to DHFR, proteolytic processing was also observed.

1.10 Mitochondrial biogenesis

The mitochondria are generally thought to have originated from a symbiotic prokaryote. This prokaryote allowed the earliest eukaryotes, otherwise anaerobes, to utilise oxygen. The mitochondrial genome is still replicated independently of nuclear chromosomes and is inherited maternally in all mammals. Over 90% of DNA contained within the mitochondrion has, during the passage of time, either been translocated to, or copied into the nucleus of the cell, making the mitochondrial version redundant.

Fig. 1.2 Diagrammatic representation of the mitochondrion



The nuclear encoded mitochondrial proteins are synthesised on cytosolic polysomes as precursor proteins, and are then translocated to their functional destination in one of the four mitochondrial compartments.

1.11 Translocation of precursor proteins into mitochondria

Translocation of precursor proteins into mitochondria is a multi-step process. This has been shown by accumulating translocation intermediates at different stages of the import pathway into mitochondria. This approach has allowed the identification of distinct functional steps in protein import.

1.11.1 ATP dependent unfolding of precursor proteins

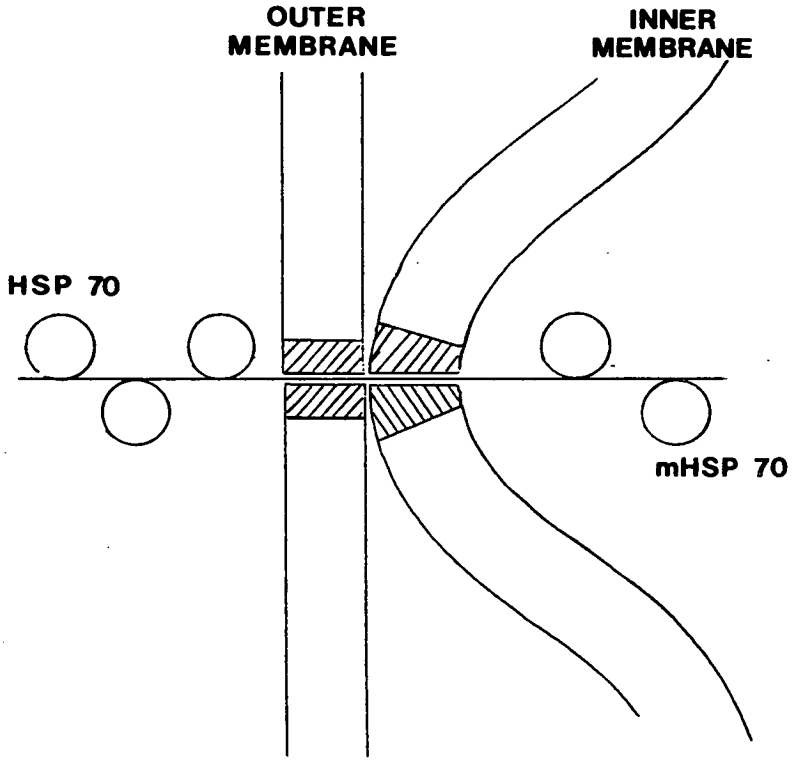
Precursor protein was accumulated in contact sites (section 1.11.3) in a two membrane spanning manner by Schleyer and Neupert (1985). They reported that the amino terminus of the precursor protein had entered the mitochondrial matrix where it was processed by a processing peptidase. They also demonstrated that the carboxyl terminus was still outside the mitochondrion by its susceptibility to proteolytic cleavage. The protein they used in their experiments was F_1-ATPase β-subunit which in a mature form has a diameter far smaller that the distance between the two membranes. This suggests that the precursor protein must have been incompletely folded, to enable it to span both membranes. It was also shown that depletion *in vitro* of NTPs (ATP and GTP) prevents protein import and that re-addition of NTPs restores import (Pfanner and Neupert, 1986; Pfanner *et al*, 1987a; Chen and Douglas, 1987; Eilers *et al*, 1987). The requirement for NTPs was shown to be independent of membrane potential by the import of the mitochondrial protein porin which requires NTPs but does not require a membrane potential (Kleene *et al*, 1987). Porin does not require a membrane potential because it does not cross the inner mitochondrial membrane during its translocation. It has also been shown that precursor protein treated with small quantities of protease, in the absence of mitochondria, was more sensitive to digestion in the presence of NTPs. This is possibly because they are more loosely folded than their non-protease treated counterparts and do not need to be further unfolded (Pfanner *et al*, 1987a). Different

lengths of precursor protein, with the same targeting sequence, have been shown to require differing quantities of NTPs. However once the precursor protein has been inserted into the mitochondrial outer membrane it no longer requires NTPs. The authors suggested that NTPs are required in the unfolding of precursor proteins possibly via a NTP dependent unfoldase resident in the cytoplasm or associated with the mitochondria. Precursor proteins once in an unfolded state must remain so until translocation has been completed. The unfolded or loose conformation is thought to be maintained by a group of proteins called molecular chaperones which are contained in the cytosol, [including heat shock protein 70 (HSP70)] which bind to the precursor polypeptide. When the precursor proteins appear in the mitochondrial matrix they become associated with mitochondrial Hsp70 proteins (Kang *et al*, 1990; Scherer *et al*, 1990). It is suggested that this interaction provides the main driving force for transport subsequent to the translocation initiation event mediated by the targeting sequence (Fig 1.3). The precursor protein is then passed on to HSP60 proteins in the mitochondrial matrix which are thought to assist in the correct folding of the mature protein. ATP hydrolysis is associated with the release of polypeptides from each of these heat-shock proteins. It has also been suggested that this model may have relevance in the transport of polypeptides across other eukaryotic membranes. Substantiating this claim are reports that heat-shock proteins have also been implicated in other transport pathways, although their exact function has not been elucidated.

1.11.2 Specific recognition of precursor proteins

The mitochondrial outer membrane contains receptor proteins which are thought to bind mitochondrial targeting sequences. It has been known for some time that mild pre-treatment of mitochondria with protease prevented the import of precursor, (for review see Pfanner and Neupert, 1987a). Recently, the binding of ADP/ATP carrier to mitochondria has been resolved into two sequential steps (Pfanner and Neupert, 1987b; Pfanner *et al*, 1987a; Söllner *et al*, 1988). The precursor first reacts with protease sensitive receptors on the mitochondrial surface where it remains accessible to externally added proteases or antibodies.

Fig. 1.3 Translocation of precursor protein across the mitochondrial membranes at contact sites in conjunction with HSP 70 proteins



Precursor proteins initially bind to a receptor in the outer membrane. Translocation is then initiated by $\Delta\varphi$-dependent insertion of the N-terminus through contact sites between the outer and inner membrane. The precursor proteins are maintained in a 'loose' unfolded state by interaction of HSP 70 proteins. Mitochondria HSP 70 (mHSP 70) bind the precursor protein as it appears in the mitochondrion matrix. Neupert *et al* (1990) suggested that the binding of mHSP 70 proteins provides the driving force for translocation after the initial $\Delta\varphi$-dependent insertion.

The precursor then inserts into a protease protected site in the outer membrane which are sometimes referred to as general insertion proteins (GIP). Pfanner *et al*, (1988), calculated the number of receptor sites to be approximately 0.25 pmol/mg mitochondrial protein and the number of GIP sites to be around 2.5 pmol/mg mitochondrial protein. They also reported that although other proteins such as porin, ADP/ATP carrier, the bc_1 and iron sulphur (Fe-S) complex of the respiratory chain had different receptor proteins, they were all inserted by the same GIP protein. The only protein that did not require GIP for insertion was cytochrome c which seemed to use a completely separate insertion pathway. More recently a number of proteins have been identified in the mitochondrial protein translocation pathway both in *S. cerevisiae* and *Neurospora crassa* (Baker and Schatz, 1991; Pfanner *et al*, 1991) These proteins seem to function as a complex of 400-600 kDa (Kiebler *et al*, 1990). This complex includes the receptor proteins MOM72 and MOM19 (Söllner *et al* 1989), an integral transport protein MOM38 (ISP 42) and an outer membrane polypeptide MOM22. A possible mechanism for translocation was suggested by Neupert *et al*, (1990), whereby precursor polypeptides synthesised in the cytosol are recognised by receptors on the mitochondrial surface. Translocation is initiated by insertion of the N-terminal targeting sequence of these polypeptides into the outer membrane of the mitochondrion. In response to the transmembrane potential difference, generated by the respiratory chain, the N-terminus crosses the inner membrane, resulting in the unfolding of the membrane spanning domain.

1.11.3 Import of precursor proteins via translocation contact sites

The accumulation of precursor proteins in contact sites between both mitochondrial membranes was reported by Schleyer and Neupert (1985). Their experimental evidence showed sites of close contact between both mitochondrial membranes. A role for contact sites had first been proposed by Kellems *et al*, (1975). Three distinct methods were used to produce translocation intermediates spanning both membranes; import of precursor proteins at low temperature; pre binding of antibodies to the carboxyl terminus of precursor proteins; and import of precursor proteins at low levels of NTPs. The arrested

translocation intermediates were then examined for their accessibility to the matrix processing protease, to externally added proteases and to antibodies in order to show that the precursor protein had in fact partly traversed both mitochondrial membranes. Thus even when precursor unfolding was taken into consideration, the two membranes had to be in very close proximity to each other. These points of close proximity are referred to as contact sites.

1.11.4 The insertion of precursor protein into translocation sites requires a membrane potential

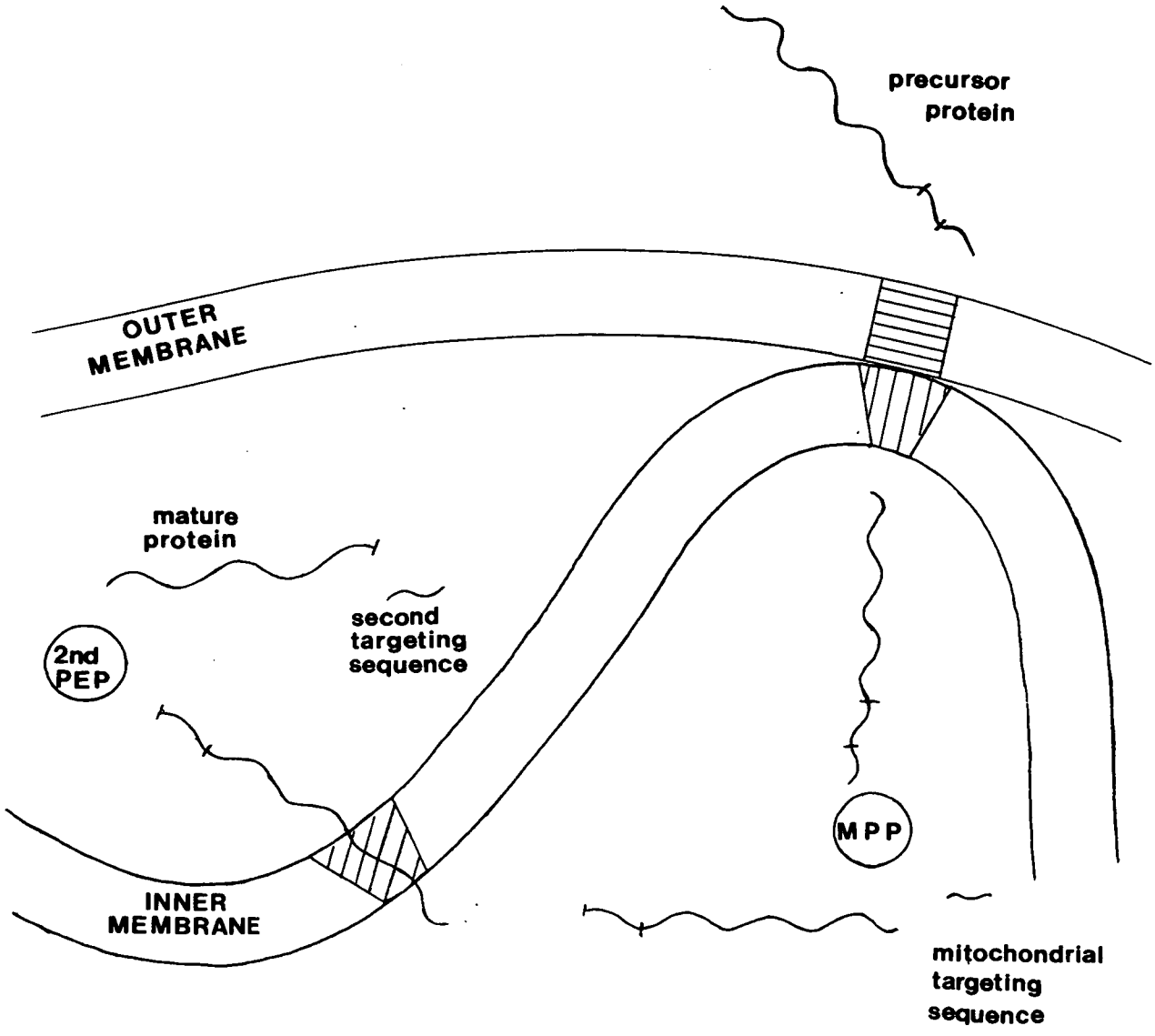
The completion of precursor translocation into the inner membrane or matrix is independent of membrane potential (Schleyer and Neupert, 1985; Pfanner *et al*, 1987b). As described in section 1.11.3, binding of precursor protein and insertion are independent of membrane potential, suggesting that the only step of translocation which requires a membrane potential is the initial insertion of the precursor protein into the inner membrane. This step usually requires a positively charged pre-sequence which is a targeting leader peptide. However it has been shown by Pfanner and Neupert (1985) that only the electrical component of the total protonmotive force ($\Delta\psi$) and not the chemical component (ΔpH), is required for protein import. The authors concluded from this that the membrane potential across the inner membrane (negative inside) exerts an electrophoretic effect on positively charged regions of the precursor proteins thereby triggering the initial entrance of precursor into the inner membrane.

1.11.5 Conservative sorting of precursor proteins

In the mitochondrial matrix the amino-terminal presequence of the precursor protein is proteolytically removed by the matrix-processing peptidase (MPP), a soluble 57 kDa protein. A 52 kDa inner mitochondrial membrane associated protein, the processing enhancing protein (PEP), greatly enhances processing of the precursors. It is thought that PEP binds the precursor and presents it for processing in the correct orientation for the peptidase. Several proteins are processed in two steps. The first step has been described as a cleavage of presequence by the MPP. The second cleavage may also be performed by this peptidase (Schmidt *et al*, 1984) or by the processing activities located in the intermembrane space (Hartl *et*

al, 1987). For example cytochrome c_1, cytochrome c peroxidase and cytochrome b_2 undergo a second cleavage in the intermembrane space. These precursor proteins contain an uncharged stretch of around 20 amino acids in the second part of their presequences, which is involved in the intramitochondrial sorting of the precursor. Two models have been proposed for the function of these intermembrane space targeting sequences. Firstly they could act as stop transfer sequences that allow only the N-terminus of the precursor to cross the inner membrane, leaving the majority of the protein ie. the mature protein, in its functional location outside the inner membrane (Reid *et al* 1982). Alternatively it may act, as suggested by Hartl *et al*,(1987) in a manner depicted in Fig 1.4. The precursor proteins are imported through a hydrophilic membrane environment via translocation sites into the mitochondrial matrix. The positively charged first half of the presequence is then removed by the MPP. The protein together with the hydrophobic second part of the presequence is then redirected back across the inner membrane. On the outer surface of the inner membrane the second proteolytic cleavage is performed. Cytochrome b_2 is then released as a soluble protein into the intermembrane space, whereas cytochrome c_1 is anchored to the inner membrane via a hydrophobic sequence at the extreme C-terminus of the mature protein. These results can be rationalised in terms of the endosymbiotic origin of the mitochondria. In the prokaryotic ancestor, periplasmic proteins were synthesised in the cytoplasm, which corresponded to the mitochondrial matrix. These proteins had a signal sequence similar to the hydrophobic second half of the mitochondrial presequence. When the genes of the endosymbiont were transferred to the nucleus of the host after the endosymbiotic event, proteins requiring to be translocated into the endosymbiotic organelle were endowed with a hydrophilic targeting signal. This enabled import via contact sites into the organelle matrix. Precursors that were therefore translocated into the organelle matrix could then follow their ancestral sorting and assembly pathways. It is for this reason that this type of import and sorting has been named conservative sorting (Hartl and Neupert, 1990).

Fig. 1.4 Conservative sorting of precursor protein in the mitochondrion



After translocation (Section 1.11.4) of the precursor protein into the mitochondrial matrix, the N-terminal mitochondrial targeting sequence is cleaved by a matrix processing peptidase (MPP). This leaves a second sequence that targets the precursor protein to the intermembrane space. A second peptidase then proteolytically removes the second sequence leaving the mature protein either free in the intermembrane space or anchored to the inner membrane.

1.12 Protein topology within membranes

Membrane-spanning domains are usually considered to consist of an uninterrupted series of 22-26 hydrophobic amino acids (Gierasch, 1989). The yeast mitochondrial outer membrane protein OMM 70 (MAS70) was shown by Gierasch (1989) to contain a uninterrupted stretch of 29 hydrophobic amino acids in the first 38 amino acids of its N-terminal sequence.

Li and Shore (1992) however reported that only the first 19 amino acids of this hydrophobic stretch were required for transmembrane orientation of the protein. The orientation of OMM 70 in the mitochondrial outer membrane has its N-terminus exposed to the intermembrane space and its C-terminus exposed to the cytoplasm (N_{in}-C_{cyt}).

A hybrid protein was constructed by Li and Shore (1992) where the N-terminal amino acids (1 through 29) of OMM 70 were fused, in-frame, to a cytosolic reporter protein DHFR. The fusion protein was targeted to the mitochondrion and was translocated into the outer membrane. However, the 19 amino acid membrane spanning domain was reversed in its topology (N_{cyt}-C_{in}).

Typically, transmembrane sequences are flanked by highly charged stretches of amino acids (von Heijne, 1985; von Heijne, 1986). These stretches are usually 10 to 15 residues long, and generally the more positive of the two flanking portions faces the cytosol. Where there is more than one transmembrane sequence, the orientation of them all is determined by the most N-terminal one (Hartmann *et al*, 1989).

Parks and Lamb (1991) reported that the removal of positive charges from the N-terminal side of the membrane spanning domain of a N_{cyt}-C_{in} protein changes the topology to N_{in}-C_{cyt}. Addition of positive charges at the C-terminal side however had little effect. The authors concluded from this that eukaryotic membrane protein topology is governed by the presence or absence of a N-terminal signal for retention in the cytoplasm that is composed in part with positive charges.

1.13 Common genes for proteins located in different compartments within the cell

The existence of single genes that encode two (or more) polypeptide products has been documented in yeast (Perlman and Halverson, 1981), mammals (Suzuki *et al*, 1989) and viruses (Kozak, 1986). For example the SUC2 gene, described by Perlman and Halverson (1981), encodes distinct yeast invertase mRNAs for secreted and cytoplasmic invertase. Until recently similarly functioning proteins in different compartments were thought to be encoded by different genes. This conclusion was based on the biochemical properties or electrophoretic mobility of the proteins. It should be noted however that proteins which are translocated across organelle membranes often undergo modifications which could account for differences in the mature proteins. In addition, further evidence for the existence of more than one message being encoded for by a single gene is accumulating. The existence of genes containing more than one transcription initiation site, multiple translation starts or single mRNAs that under go RNA processing such as splicing have now been documented. Thus one gene can produce two proteins that differ in location, molecular weight, electrophoretic mobility and other properties.

1.13.1 RNA processing (splicing)

Alt *et al*, (1980) reported that B lymphocyte tumour cells directed the synthesis of two forms of μ heavy chain, one with a molecular weight of 67 kDa and one of 64 kDa. When the B cells were converted into IgM secreting cells it was shown that membrane bound IgM contained the 67 kDa heavy chain (μ-m) and secreted IgM contained the 64 kDa heavy chain (μ-s). The authors reported that cells which produced both proteins did so via two mRNAs with different 3' ends. Rodgers *et al*, (1980) reported that during differentiation B lymphocytes underwent a shift from membrane bound IgM synthesis to secretory IgM synthesis. They also reported the presence of splicing sites at the 3' end of the fourth constant region of the μ gene (Cμ4) where the μ-m and μ-s cDNAs diverge, suggesting that the two species of mRNA were manufactured from the same transcript by differential RNA splicing. Early *et al*, (1980) reported that the only splicing that occurs in the Cμ4 boundary is in the μ-m mRNA. The authors

presumed that the role of this splicing reaction was to introduce a membrane binding sequence which would anchor the μ chain to the B lymphocyte membrane. The system would presumably operate by an inducible positive (or negative) regulator molecule that would facilitate the shift from μ-m to μ-s. This control of antibody secretion is probably not unique to IgM, and is possibly conserved throughout a wide spectrum of lymphocyte secretion of antibody. Indeed a similar method of control was reported for IgE by Yaoita *et al*, (1982) involving RNA splicing and possibly a DNA rearrangement.

1.13.2 Double start sites at the transcriptional level

Wu and Tzagoloff, (1987) reported that disruption of the chromosomal copy of the FUM1 gene in *S. cerevisiae*, caused a respiratory defect, due to an absence of fumarase in the mitochondria, and an absence of cytoplasmic fumarase. The authors concluded that the FUM1 gene encoded both mitochondrial and cytoplasmic fumarase. Sequencing of the FUM1 gene revealed multiple start codons (ATGs), some of which were in the coding region of the gene. Two mRNAs were also found which differed in length by several hundred nucleotides. However, only the more abundant, longer transcript was detected by a probe from the 5' end of the gene. Given this evidence the authors suggested that the shorter transcript was transcribed further downstream from the initial ATG and translation initiated from other ATG sites. This was in accord with the translation product from the longer transcript being targeted to the mitochondria, since the sequence of amino acids encoded by the 5' end of the gene were generally basic (3 arginine, 3 lysine), hydroxylated (3 serine, 2 threonine) and lacked any acidic residues. This is in accordance with classical mitochondrial targeting sequences.

Another example of double transcriptional start sites, was described by Natsoulis *et al*, (1986). They reported that both cytoplasmic and mitochondrial histidine-tRNA synthetases were encoded by a single gene, HTS1. In this case, as with the fumarase from *S. cerevisiae*, the two proteins are encoded by different length mRNAs. The shorter mRNA encodes the cytoplasmic protein and the longer mRNA encodes the mitochondrial counterpart. The translation initiation sites of the two transcripts are located 60 base pairs (bp) apart

resulting in a 20 amino acid leader peptide on the mitochondrial protein which is basic in nature.

1.13.3 Multiple translational initiation sites

tRNA-modification enzymes in yeast have been shown to be common to mitochondria, cytoplasm and nucleus. This suggestion was based on indirect data obtained by studying the effect of nuclear mutations in the *trm*1 gene in activating the first enzyme in both mitochondria and cytoplasm, and in the *trm*2 gene causing the damage of the second enzyme in both cellular compartments (Hopper *et al*, 1982). The same conclusion may be true for the *mod*5 gene, which controls the isoprenylation of both cytoplasmic and mitochondrial yeast tRNA (Martin *et al*, 1982; Dihanich *et al*, 1987). Recently the genes for the yeast tRNA modifying enzymes have been cloned confirming that both the mitochondrial and cytoplasmic enzymes are encoded by the same genes (Dihanich *et al*, 1987; Najarian *et al*, 1987). Najarian *et al* (1987) also reported that the *mod*5 gene may encode two proteins, one of which is a mitochondrial form with an amino terminal extension of basic charge. Although mRNAs of heterogeneous size are transcribed from this gene, all of them initiate upstream of the first ATG codon of the open reading frame. It was therefore suggested by the authors that *mod*5 transcripts generated two proteins through differental translation. This would mean that initiation at the first ATG would generate a protein with a basic N-terminal extension, whereas initiation at the second in-frame ATG located ten codons downstream would give rise to the cytoplasmic-nuclear form of the same enzyme.

It was reported by Suzuki *et al* (1989) that rat liver mitochondrial and cytosolic fumarate hydratase which are almost equally distributed between the two compartments were encoded by the same gene, similarly to yeast fumarase (Wu and Tzagoloff, 1987). However under northern blot analysis only one species of mRNA could be found. This gave rise to two possibilities. Either the two fumerases were encoded by different mRNAs of the same length, or one mRNA gave rise to both proteins. The second theory is more interesting since there is a second AUG just upstream of GCA, which is the codon of the N-terminal alanine of both mature proteins. If this second AUG codon does initiate translation then both fumarases would be encoded by the same mRNA by differential translation.

1.14 Carnitine palmitoyltransferases I and II

As discussed in section 1.6.5 the CPT system is the pivotal role in fatty acid metabolism. It has been known for some time that both CPT I and II are synthesised in the cytosol and are therefore targeted to the mitochondria, and translocated to either side of the mitochondrial inner membrane (Brady *et al*, 1986).

The exact position of the two enzymes has been the subject of great conjecture for many years. However most evidence now points towards a CPT system which operates as depicted in Fig 1.5.

The reactions of the CPT enzymes are freely reversible, and the direction of the reaction is dictated by the environment of the enzyme. CPT II would appear to be more easily solubilised than CPT I, which is therefore presumed perhaps to be an integral membrane protein.

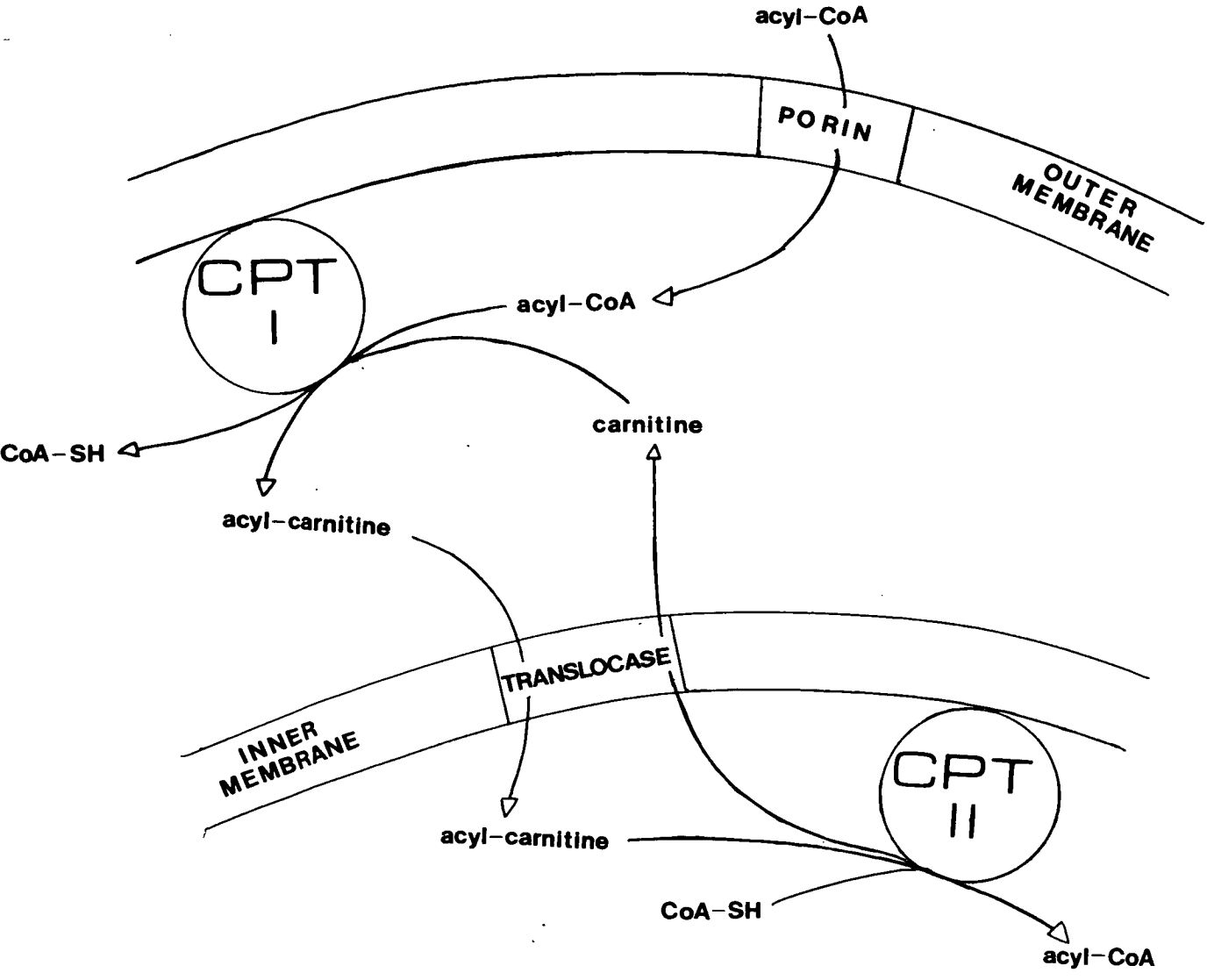
There are many more questions that can be asked about these two proteins. For example, are CPT I and CPT II identical proteins; are they encoded for by the same gene; and if so, how are they targeted to the different locations in the mitochondrion. The answers would be an important asset in the understanding of the complex mitochondrial targeting system.

Elucidation of the CPT system would also be of benefit to diabetes research. It has been known for some time now that CPT holds a pivotal role in the control of fatty acid oxidation (McGarry and Foster, 1980). Both starvation and ketotic diabetes have been shown to cause a two fold increase in CPT I active protein (Brady and Brady, 1987). Along with this increase of CPT I active protein, is a desensitisation of CPT to inhibitors, such as malonyl-CoA (Grantham and Zammit, 1988; Kolodziej and Zammit, 1990a) This is thought to be related to membrane fluidity, which is altered by a decrease of cholesterol incorporation into the membranes.

1.15 Aims of this project

The aim of this study was to examine the CPT system in order to elucidate the connection or distinction between the two rat liver mitochondrial CPT enzymes. The enzymes would have to be extracted from their membrane environments, purified, and then used to clone the gene (or genes) encoding them.

Fig. 1.5 The uptake of long chain length fatty acids by the mitochondrion for β-oxidation



Activated fatty acids are permeable through the outer mitochondrial membrane. Carnitine palmitoyltransferase I (CPT I) then catalyses the esterification of carnitine to the fatty acid with the release of CoASH. The acyl-carnitine is then translocated through the inner membrane by a antiport translocase. Carnitine palmitoyltransferase II (CPT II) then reverses the previous reaction reforming the activated fatty acid and releasing carnitine to be translocated back out of the matrix. The activated fatty acid is then ready to be oxidised in the mitochondrial matrix (Murthy and Pandy 1987a & b).

One of the main objectives of this study to elucidate which of the mitochondrial membranes contained CPT. Comparisons of rat liver mitochondrial CPT I, CPT II and other known acyl-transferases could be made in order to establish relationships, and possible ancestral roles of the system involved in fatty acid oxidation within eukaryotes.

Inhibition studies on the two enzymes could be carried out and when compared to other acyl-transferases would gradually build up a more complete picture of the CPT system in rat liver mitochondria.

Finally a mechanism for the uptake of long chain fatty acids by rat liver mitochondria could be more correctly conceptualised.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

Materials and Methods

2.1 MEDIA

2.1.1 Media for *Escherichia coli*

Luria broth; Difco Bacto tryptone, 10 g; Difco Bacto yeast extract, 5 g; NaCl, 5 g; $MgSO_4$, 6 g; per litre.

Luria agar; As luria broth with 15 g Difco Bacto agar per litre.

Luria top agar; As luria broth with 5 g Difco Bacto agar per litre.

Ampicillin, kanamycin and tetracycline, were added to the media immediately prior to use, to final concentrations of 100, 25, and 25 μg/ml respectively.

2.1.2 Media for *Saccharomyces cerevisiae*

YPD Yeast extract 10 g; peptone 20 g; glucose 20 g per litre. Plates were made by adding Difco Bacto agar (20 g/L) to above media.

2.2 SOLUTIONS

2.2.1 Sucrose solution (300 mM)

300 mM Sucrose	102.60 g
5 mM Tris.Cl	0.605 g
1 mM EGTA	0.38 g
	Per Litre

The solution was adjusted to pH 7.4 at 0°C.

2.2.2 SM-Phage buffer

NaCl	5.8 g
$MgSO_4.7H_2O$	2-4 g
1 M Tris.HCl(pH 7.5)	50 ml
2% gelatin	5 ml
	per litre

2.2.3 TE buffer

Tris.HCl	10 mM
EDTA	1 mM

2.2.4 10 x TBE

Tris base	108 g
Boric acid	55 g
0.5M EDTA pH8.0	40 ml
	per litre

2.2.5 10 x Loading buffer

0.25% Bromophenol blue

15% Ficoll 400

2.2.6 Prehybridisation solution

20x SSC	7.5 ml
100 x Denhardts	1.25 ml
10% SDS	1.25 ml

2.2.7 Denhardts (100X)

2% BSA

2% ficoll

2% polyvinylpyrollidone

2.2.8 2 X Laemmli sample buffer (LSB)

1 M Tris.HCl pH 6.8	3.13 ml
SDS	2 g
Glycerol	9 ml
2- mercaptoethanol	5 ml
1% bromophenol blue	1 ml
Distilled water	80 ml

2.2.9 Solutions for polyacrylamide gel electrophoresis (PAGE)

4 X resolving buffer	per litre	4 x stacking buffer	per 500 ml
Tris.base	181.6 g	Tris.base	30.28 g
SDS	4.0 g	SDS	2.0
dH_2O	600 ml	dH_2O	450.0 ml
conc.HCl	10 ml	conc.HCl	15.0 ml
pH to 8.8		pH to 6.8	

10 X running buffer	per litre
Tris.base	60.56 g
glycine	288.5 g
SDS	20.0 g
dH_2O to 1 litre	

2.2.10 10X Transfer buffer

250mM Tris.HCl pH 8.3; 1.5M Glycine

2.2.11 Tris-buffered saline (TBS)

10ml 1M Tris.HCl pH 7.5, 37.5ml 4M NaCl, 952.5ml water

2.2.12 90% Percoll

Percoll	900 ml
Sucrose 2.5 M	100 ml
	Per Litre

The solution was adjusted to pH 7.4 at 0°C.

2.2.13 Potassium phosphate (KPi)

KPi 20 mM	400 ml
Bovine Serum Albumin (BSA) 0.2%	0.8 g
EGTA 1 mM	0.16 g

The solution was adjusted to pH7.4 at 0°C.

2.2.14 Magnesium ATP (MgATP)

ATP	1.79 g
$MgCl_2$	0.161 g

Made up to a final volume of 15 ml with KCl and adjusted to pH7.4 with solid Tris.base. The solution was then devided into 2 ml aliquots and stored at -20°C.

2.2.15 Potassium chloride (KCl)

KCl 150 mM	11.18 g
Tris.HCl 5 mM	0.605 g
EGTA 1mM	0.38 g
	Per Litre

Adjusted to pH 7.4 at 0°C.

2.3 DETERGENTS

Octyl Glucoside (OG)

3L(3-Cholamido propyl)dimethyl
-ammonio]-1-propansulphanate.(CHAPS)

Octanoyl-N-methyl glutamide(MEGA 8)

Decanoyl-N-methyl glutamide(MEGA 10)

(Detergents were obtained from Sigma)

2.4 SUPPLIERS

2.4.1 Enzymes

T_4 DNA ligase, restriction endonucleases and Klenow fragment of DNA polymerase were obtained from Gibco-BRL, Paisley, UK. Pyruvate kinase was obtained from Boehringer Mannheim (BCL), Lewes, Sussex. Pancreatic ribonuclease A was obtained from Sigma, and was boiled for 10 minutes to remove any DNase activity before use. Sequenase® was obtained from United States Biochemical Corporation, Cleveland, Ohio.

2.4.2 Antisera

Antibodies against CPT I and CPT II were raised in sheep at Hannah Research Institute, Ayr. HRP-conjugated anti-rabbit IgG was obtained from Bio-Rad Laboratories, Richmond, California, USA. HRP-conjugated anti-sheep/goat IgG was obtained from Scottish Antibody Production Unit.

2.4.3 Isotopes - Amersham International

2.4.4 General laboratory chemicals - Sigma chemical company, Poole, Dorset or, BDH, Poole, Dorset.

2.4.5 Proteinase inhibitors

Antipain, Leupeptin & Pepstatin were obtained from Sigma

2.5 BACTERIAL STOCKS

Name	Genotype	Reference
E.coli Y1090	Δ*lac*U169, *pro*A+, Δ*lon*, *ara*D139, *str*A, *sup*F [trpC22::Tn10] (pMC9 = pBR322)	Young and Davis, 1983b
E.coli Y1088	Δ*lac*U169, *sup*E, *sup*F, *hsd*R$^-$, *hsd*M$^+$, *met*B, *trp*R, *ton*A21, [proC::Tn5] (pmC9 = pBR322)	Young and Davis, 1983b
E.coli TG1	*sup*E, *hsd*Δ5, *thi*, Δ(*lac*-*pro*AB), F′, *traD*36, *pro*AB$^+$, *lacI*^{q}, *lacZ*ΔM15	Gibson, 1984

2.6 PHAGE

Name	Descrption and use	Reference
λgt11	bacteriophage expression vector	Young and Davis, 1983a
λgt10	bacteriophage cloning vector	Huynh *et al*, 1985
λgt11 stretch	bacteriophage expression vector	Young and Davis, 1983a
λC126	bacteriophage constitutive lysis	Meselson, 1964
M13KO7	Helper phage	Vieira and Messing, 1987

2.7 PLASMIDS

Name	Description and use	Reference
pTZ18R and pTZ19R	phagemid cloning vectors	Rokeach *et al*, 1988

2.8 OLIGONUCLEOTIDES

N = (A,G,C or T); R = A or G; Y = C or T.

Sequence 5'-3'	Name
GTNGCNTTYCARTTYACNGT	a CPT I sense
ACNGTRAAYTGRAANGCNAC	a CPT I antisense
CARCARATGCARCARAT	b CPT I sense
ATYTGYTGCATYTGYTG	b CPT I antisense
CAGCAGGCTGTGGCTTTTCAGTTTACCGTGACCCCA	c CPT I sense
GCCATCTGGGGTCACGGTAAACTGAAAAGCCACAGCCTGCTG	c CPT I antisense
CAGCAGATGCAGCAGATCCTGGATGATCCA	d CPT I sense
TGGATCATCCAGGATCTGCTGCATCTGCTG	d CPT I antisense
GARAARGGNGTNCARAC	e1 CPT II - Brady
GARAARGGNGTNCARACNGGNCAR	e2 CPT II - Brady
GATGAATTCATGATGCCGCGCCTGCTGTTTC	f CPT II
GTCAAGCTTCTAAGTTTTGATGGCTTTGCCT	g CPT II
CAGCACATGATGGCTGAGTGTTCCA	h CPT II
TACAGCTGCGACCTCACACTACCT	i CPT II
GAAGCAGCGATGGGCCAG	j CPT II
TCGAAAATGTCTTCCAAGCA	k CPT II

2.9 SUCROSE STEP GRADIENTS

The layers were added to the tubes in reverse order:

Top layer : Sucrose 0.76 M (26% sucrose), Tris.HCl 5 mM, EGTA 1 mM.

Middle layer: Sucrose 1.0 M (34% sucrose), Tris.HCl 5 mM, EGTA 1 mM,

Bottom layer: Sucrose 1.17 M (40% sucrose), Tris.HCl 5 mM, EGTA 1 mM

The pH of all three layers was adjusted to 7.4 at 0°C.

Depending on availabilty, two centrifuges were used. The volumes of each layer were as follows:

	Combi swing out tubes (10 ml)	Prepspin swing out tubes (20 ml)
Top layer:	3 ml	7 ml
Middle layer:	3 ml	6 ml
Bottom layer:	1.5 ml	6 ml

2.10 PREPARATION OF CYANOGEN-BROMIDE-SEPHAROSE CYTOCHROME C

15g of cyanogen-bromide-sepharose (CNBr-sepharose) was swollen in 500ml of dH_2O for 2 hours then stored overnight at 4°C. The suspension was then washed sequentially with the following:

1 litre dH_2O

1 litre 1 mM HCl (15 minutes)

1 litre dH_2O

The CNBr-sepharose was then suspended in 30 ml of $NaHCO_3$ (pH 8.0). 360 mg of cytochrome c (equine SIGMA C2506) were added along with 30 ml of $NaHCO_3$. This was then incubated at room temperature (RT) for 1.5 - 2 hours with occasional stirring. The "slurry" was then washed with the following solutions:

0.1M Na acetate	pH 5.0	200ml
1.0M KCl + 1% Lubrol		200ml
0.1M $NaHCO_3$	pH 8.0	200ml

This cycle of washes was repeated three times before the "slurry" was washed with 1 litre of dH_2O. In order to fully oxidise the cytochrome c, 1 litre of 1mM $K_3Fe(CN)_6$ was added and left for 15 minutes. The "slurry" was then washed with 1 litre of dH_2O before being washed (equilibrated) with sucrose 0.25M ; Tris.HCl 10mM ; EDTA 1mM (all at pH 7.4). It was then divided into three aliquots and stored in sucrose buffer (as above) at 0°C.

2.11 PREPARATION OF RAT LIVER MITOCHONDRIAL OUTER MEMBRANE FRAGMENTS

(Parsons *et al*, 1966)

Six rats were starved in wire bottomed cages for 48 hours and killed by cervical fracture. The livers were removed from the rats and immediately placed in ice cold sucrose solution. The livers were then finely minced (still on ice), transferred to a teflon-glass potter homogeniser and homogenised carefully by hand. The homogenate was then centrifuged at 500 X g (Sorvall SS-34 rotor) for 10 minutes and decelerated with the brake on. The supernatant was decanted and centrifuged at 9000 X g (Sorvall SS-34 rotor) for 10 minutes (brake on). The fat at the top of the tube was removed with a tissue, and the supernatant decanted. After the pellets had been resuspended in ice cold sucrose solution by gentle homogenisation, the volume was made up to 135 ml with ice cold sucrose solution. 75 ml of 90% Percoll solution (4°C) was added and the mixture was split evenly into 8 x 35 ml Oakridge tubes. The tubes were then centrifuged at 35000 X g (OTD-Combi) for 20 minutes at 4°C. At this point the KPi solution was prepared (section 2.2.13).

The tubes were decanted almost down to the mitochondrial layer, visible as a pale fibrous layer almost at the bottom of the tube. The mitochondria were carefully pipetted into a homogeniser. Care was taken not to collect any of the red blood cells that were still present at the very bottom of the tube.

The mitochondria were gently resuspended in 300 mM sucrose solution (section 2.2.1) and then centrifuged at 18000 X g (Servall SS-34 rotor) for 15 minutes (brake off). The pellets were then resuspended in 160 ml of KPi medium (section 2.2.13), transferred to a 200ml beaker and left on ice for 20 minutes. This had the effect of swelling the mitochondria by osmosis.

2 ml of 200 mM MgATP solution were then added. This resulted in the sudden shrinking of the mitochondrial inner membranes. After 5 minutes the suspension was centrifuged at 35000 X g (OTD-Combi) for 20 minutes (4°C).

These pellets were resuspended in KPi and homogenised vigorously creating large shearing forces to disrupt the mitochondria. This suspension was then centrifuged at 2000 X g (Sorvall SS-34 rotor) for 20 minutes.

The supernatant was then harvested and put in a 100 ml measuring cylinder. 0.1 x the volume of the supernatant of 1.5 M KCl was then added and mixed thoroughly in order that the 150 mM concentration of KCl was obtained. The resultant solution was then centrifuged at 45000 X g (OTD-Combi) for 20 minutes (4°C) to pellet the outer membrane fragments. The sucrose step gradients were prepared at this point (section 2.9).

The pellets were resuspended by washing with 150 mM KCl solution and homogenising vigorously. The total volume of the suspension was kept to less than 4ml, which was layered on top of the sucrose step gradients. These gradients were centrifuged at 40000 X g (OTD-Combi) for 2.5 hours at 4°C in swing out rotor. The mitochondrial layer was collected with a bent Pasteur pipette and either; extracted with Tween 20, before being frozen in liquid nitrogen and stored at -70°C; or put in a 50 ml measuring cylinder and made up to 25 ml with dH_2O.

An aliquot of activated-Sepharose (section 2.10) was then added to the measuring cylinder, and incubated for 20 minutes at 35°C while being constantly mixed. The Sepharose was centrifuged out at 200 X g

(Sorvall SS-34 rotor) for 10 minutes (4°C). The supernatant was then filtered through Pasteur pipettes filled with glass wool in order to remove the reacted Sepharose. The Sepharose was resuspended in sucrose solution and the process was repeated.

Both filtered supernatants were then centrifuged at 45000 X g (OTD-Combi) for 45 minutes (4°C). The pellets were resuspended in dH_2O (volume variable) and frozen in liquid nitrogen before being stored at -70°C.

2.12 RAT LIVER MITOCHONDRIAL ENZYME ASSAYS

2.12.1 Cytochrome c oxidase assay

(Smith , 1955)

The assay buffer was prepared as follows:

K_2HPO_4/KH_2PO_4 (pH 7.2 at 22°C)	70 mM
EDTA	5 mM

1.8 ml cytochrome c oxidase assay buffer was added to a cuvette along with 50 µl of cytochrome C type II (20 mg/ml) (Sigma C2506). A few crystals of sodium dithionite were added to give 90% reduction.

The samples to be assayed were diluted with 5 volumes sample and 1 volume sample buffer (EDTA 10 mM; $NaHCO_3$ 10 mM; 1% Triton X-100) and incubated for 5 minutes before being assayed.

The optical density (OD) at 550 nm of the assay buffer (30°C) was adjusted to an OD of 1.000. The recorder speed was set to an appropriate speed (dependent on sample) and the sample was added very quickly on a stirring rod. The decrease in OD_{550nm} was recorded continuously from 0 - 30 seconds, during which time the change in OD was linear. The gradient (right to left) of the slope obtained and the molar extinction coefficient, Σ = 19200 $lmol^{-1}cm^{-1}$, were used to calculate the amount of cytochrome oxidase that was present in the sample.

2.12.2 Monoamine oxidase (MAO) assay

(Schnaitman *et al*, 1967)

The assay buffer was prepared as follows:

K_2HPO_4/KH_2PO_4 (pH 7.2 at 22°C)	70 mM
EDTA	5 mM

1.9 ml assay buffer was added to a cuvette along with Triton X-100 (20 μl) and fresh 1 M benzylamine-HCl (10μl). The assay buffer was then left for 20 minutes before the sample was added.

2 ml of the assay buffer (30°C) was added to 10 μl of fresh benzylamine (1 M) and 10 μl of 10% (w/v) Triton X-100. The spectrophotometer was then set to an OD around 1.000 and 20 μl of sample was added. The decrease in absorbance at 250 nm was recorded continuously for about 3 minutes during which time the change in absorbance was linear. The amount of monoamine oxidase present was then calculated using the gradient of the slope and the molar extinction coeficient of benzaldehyde (Σ = 13000 $1mol^{-1}cm^{-1}$).

2.12.3 Colorimetric CPT assay

CPT was assayed at 25°C in a final volume of 1ml. Reactions were initiated by the addition Of 0.4 mM L-carnitine to a reaction mix of 150 mM sucrose; 60mM KCl, 25 mM Tris.HCl (pH 6.8), 1mM EDTA, 0.1 mM 4,4'-dithiodipyridine, BSA (1.3mg/ml), and 40 μM palmitoyl CoA. The reaction was monitored at 324 nm. (Ghadiminejad and Saggerson, 1991).

2.12.4 Radiochemical CPT assay

Carnitine palmitoyltransferase activity was assayed radiochemically by measuring the incorporation of [^{3}H] or [^{14}C]-carnitine into palmitoylcarnitine in the presence of palmitoyl CoA. The acylcarnitine formed was extracted into butanol which was washed three times with butanol-saturated water.

The reaction mixture was as follows:

KCl	150 mM
EGTA	1 mM
Tris.HCl	5 mM
DDT	1 mM
BSA	10 mg/ml

The pH of this mixture was then adjusted to 7.4 at 37°C and 2 μ g/ml of rotenone/actinomycin A in ethanol were added. Each assay was 2 ml in final volume. The substrates used were 50μl of palmitoyl-CoA (5.3 mM) and 50 μl of [^{3}H] or [^{14}C] Carnitine (20.8 mM), which were added to start the reaction. The reaction was then run for 2 minutes before being stopped by the addition of 300 μl of concentrated HCl. Water saturated butanol (4.5 ml) was then added to the reaction mix which was then shaken well and centrifuged at 3000 X g on a MSE bench top centrifuge. The butanol layer was removed to a clean tube, to which 3 ml of butanol saturated water was added. The tubes were mixed well and centrifuged as before. The water was then removed and the previous step repeated twice more. After the three butanol saturated water washes 3 ml of the butanol layer were transferred into 10 ml of Opti-fluor 12 and counted against palmitoyl-CoA blanks in a scintillation counter.

2.13 PROTEIN TECHNIQUES

2.13.1 Estimation of proteins

All protein standards for the protein calibration curves were obtained by dissolving BSA (obtained from Sigma) in the buffer used to store the samples. Care was taken during all estimations that standards were measured in identical conditions as the samples, especially where detergents were used.

2.13.1.1 Lowry method

Protein was estimated using the method described by Lowry *et al*, (1951).

Solution A- 2% Na_2CO_3, 1.6% Na tartrate, 0.4% NaOH, 1% SDS
Solution B- 1 ml 4% $CuSO_4$ and 100 ml solution A
Folin reagent- diluted 1:1 with dH_2O

The sample to be assayed was diluted in dH_2O to a volume of 1 ml, as were standards of BSA (0-100 μg/ml). To these, 3 ml of solution B was added. After 30 minutes, 1 ml of diluted Folin reagent was added, and the absorbance at 660 nm was read after a further 30 minutes. The concentration of the sample was then calculated from a standard curve.

2.13.1.2 Bradford method

(Bradford, 1976)

Bradford reagent was obtained from BDH. Reagent (2.5 ml) was added per 0.5 ml of sample, and the absorbance at 595 nm was measured against a reagent blank within one hour. The concentration of the sample was then calculated from a standard curve.

2.13.2 Enzymatic digestion of proteins

2.13.2.1 Digestion of proteins by endoproteinase Asp-N

Protein samples (40 mg/ml) were diluted in the same volume of 1 M sodium phosphate (pH8). The sample was concentrated for one hour in a speed vacuum. 25 μl of endoproteinase Asp-N was then added and the mixture incubated for varying amounts of time at 22-37°C.

2.13.2.2 Succinylation and clostripain digestion of proteins

In order to limit clostripain to the digestion of arginine residues, succinylation of the lysine residues was first performed. This was carried out by mixing together in a microfuge tube 0.1 ml of 2 M Tris.base and 0.1 ml of concentrated protein solution (1-10 mg/ml). A couple of flakes of succinic anhydride were the added every

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30 minutes for 2-4 hours. The succinylated protein was then dialysed against 1% ammonium bicarbonate for 2 hours.

Clostripain was activated by pre-incubating the enzyme at a concentration of 2 mg/ml in 1 mM calcium acetate and 2mM dithiothreitol (DTT) over night at 4°C.

0.5-0.05 mg of succinylated protein (100 μl of dialysed sample) were mixed in an Eppendorf tube with 10 μl of 75 mM DTT solution in 1% ammonium bicarbonate. Activated clostripain was then added (protein/clostripain ratio of 50:1), and incubated at 37°C for 2-4 hours. The sample was then dried in a speed vacuum.

2.13.3 Chemical cleavage of protein by cyanogen bromide

All glassware to be used in this procedure was first washed thoroughly with concentrated nitric acid and rinsed with dH_2O before use. The sample of protein to be cleaved was treated with 5% 2-mercaptoethanol (pH8) for 24 hours. The protein was separated from the thiol by dialysis for a minimum of 4 hours at 4°C, before freeze drying.

The sample was dissolved in 70% (v/v) aqueous formic acid to a concentration of 5 mg/ml at room temperature. A spatula tip of cyanogen bromide was added with constant stirring. The mixture was then incubated in the dark under oxygen-free nitrogen at 20-25°C for 16-24 hours.

The mixture was twice diluted with 15 volumes of water and freeze dried.

2.13.4 Separation of peptides in preparation for N-terminal sequencing

Peptide samples were separated in two ways. They were either separated by polyacrylamide gel electrophoresis, as described in section 2.28, or by reverse phase chromatography using the RP-300 Aquipore 250 X 1 mm column (Amersham). Such samples were first dissolved in 0.1% trifluoroacetic acid.

2.14 DETERGENT TREATMENT OF RAT LIVER MITOCHONDRIAL OUTER MEMBRANES

2.14.1 Effect of detergents on CPT 1 activity

All detergents were dissolved in 500 mM KCl. In order to examine the effect on the activity of CPT I by the detergents that might be used for solubilisation, 200 µl of outer membrane preparation were centrifuged at 100000 X g for 10 minutes (Beckman Air-fuge), before the resultant pellet was resuspended in an appropriate volume of 10 mM; 20 mM; and 40 mM detergents. The membrane concentrations were calculated to give protein concentrations of 2.5 - 3.0 mg/ml. Protease inhibitors antipain, leupeptin and pepstatin were added to a final concentration of 100 µg/ml, 4 µg/ml, and 100 µg/ml respectively. Care was taken to avoid frothing of the detergent as this can damage proteins. The suspensions were then left on ice for 30 minutes before being assayed for CPT activity.

2.14.2 Solubilisation of CPT active protein in outer membranes by detergents

Solubilisation of CPT I was carried out in a similar fashion to that described by Murthy & Pande (1987). Mitochondrial outer membranes (800 µl) were centrifuged at 100000 X g (Beckman Air-fuge), for 10 minutes The pellet was then resuspended in an appropriate volume of detergent and proteinase inhibitors were added as before. The suspension was stirred periodically on ice for 30 minutes and centrifuged at 100000 X g (Beckman Air-fuge), for 1 hour. The supernatant was removed and either assayed for activity or counted (when radioactive markers had been used). The pellet was then resuspended in the same volume of detergent containing protease inhibitors as before and measured for CPT activity in the same way as the supernatant. Double solubilisation experiments were carried out by resolubilising the pellet obtained after the first solubilisation. The second supernatant was either measured separately or combined with the first. Any pellet obtained was measured as before. All work was carried out at 4°C.

2.15 ACTION OF INHIBITORS ON CPT ACTIVITY

2.15.1 Inhibition of CPT I by bromopalmitoyl-CoA

Bromopalmitoyl-CoA (BrPCoA), is an inhibitory substrate analogue of CPT where a bromine atom is subsituted onto the α-carbon of palmitoyl-CoA. BrP-CoA was tested for inhibition of CPT using the following method. A pellet obtained by centrifugation at 100000 X g (Beckman Air-fuge) of 200 µl of outer membranes, was dissolved in an appropriate volume of 300 mM KCl to give a protein concentration of 2.5 - 3.0 mg/ml. 1.25 mM "Cold" carnitine (10 µl) was then added along with 5 µl of 10 µM BrPCoA. This mixture was then incubated at 25°C for 45 minutes before being spun at 100000 X g (Beckman Air-fuge) for 10 minutes. A control was run where the BrP-CoA was replaced with 100 mM KCl. The pellets were then assayed for CPT activity as described in section 2.12.4.

2.15.2 Inhibition of CPT I by malonyl-CoA

Assay Conditions

Malonyl-CoA	100 µM
BSA	1%
Palmitoyl-CoA	132 µM
Carnitine	0.5 mM
KCl	150 mM
EGTA	1 mM
Tris.HCl	3 mM
DTT	1 mg

Malonyl-CoA inhibition of CPT I was used to determine the effect of Tween 20 on the membranes. This was done by solubilising 200 µl of a rat liver mitochondrial outer membrane preparation, as described before. After solubilisation the pellet and supernatant were assayed for CPT activity in the presence and absence of 20 µg/ml malonyl-CoA. The effect of 1% Tween 20 (w/v) on the outer membrane preparation (3 mg protein/ml) was also examined against a control where dH_2O was used instead of Tween 20.

2.15.3 Binding of radiolabelled carnitine by rat liver outer membranes in the presense of BrP-CoA

The effect of BrP-CoA on the binding of carnitine to the rat liver mitochondrial outer membrane preparation was examined by the following method (Declerq *et al*, 1987). [^{3}H]-carnitine (0.15 nmol with specific activity 75 Ci/mmol) was dissolved in the appropriate volume of 500 mM KCl to give an outer membrane protein concentration of 2.5 - 3.0 mg/ml when added to the pellet obtained from spinning down 200 μl of outer membrane preparation. 1.25 mM "Cold" carnitine (10 μl) was added along with 3 μl of 10 μM BrP-CoA. This mixture was incubated at 25°C for 45 minutes before being spun at 100000 X g (Beckman Air-fuge) for 10 minutes. A control was prepared whereby the BrP-CoA was replaced with 500 mM KCl. Both control and reaction were washed 4 times in 10% BSA in 100 mM KCl and then counted in 10 ml Opti-fluor 12 in a scintillation counter.

2.16 DESIGN OF MICRO-DIALYSER

In order to dialyse small volumes (10-100 μl) a new method had to be developed. This was accomplished by altering Eppendorf tubes. An Eppendorf tube, with its cap removed, had its coned bottom sliced off with a sharp scalpel, leaving a cylindrical tube. The centre of the removed cap was then hollowed out carefully and the lip, used to open the Eppendorf cap, carved to a circular shape.

Boiled dialysis tubing was then sliced into a sheet and stretched over the cap. The sheet was then pulled tight, and threaded through the cylindrical Eppendorf body. This procedure pulled the visking sheet tightly over the cap at the same time as securing the cap onto the Eppendorf body.

Excess visking tubing was removed from the inside of the Eppendorf with a sharp scalpel. A paper clip was shaped into a handle and attached to the top of the inverted Eppendorf tube.

Shallow containers, filled with buffer, were used during the dialysis. The solution to be dialysed was added carefully into the Eppendorf onto the viscing material. The Eppendorf was then placed into the buffer and dialysed by gentle agitation, ensuring that the Eppendorf remained vertical and inverted at all times.

2.17 PURIFICATION OF CPT II

2.17.1 Solubilisation of CPT II

Total heavy mitochondria were prepared as in section 2.11 up to and including the Percoll gradients. This ensured that only heavy mitochondria were present since light mitochondria are known to contain peroxisomal contamination. The mitochondria used were free from peroxisomes to 5% as measured by catalase activity. The mitochondria were solubilised in 4% (v/v) Tween-20 in the presence of 1 M KCl, 0.1mM DMSO and 0.5 mM dithiothreitol. Solubilisation was carried out by gentle stirring for 15 minutes at 0°C, with sonication every 2 minutes (5 bursts of 15 seconds). The mixture was centrifuged at 30000 X g (OTD-Combi) for 20 minutes. Both supernatant and pellet were assayed for CPT activity (described in section 2.12.3) to determine the percentage solubilisation.

2.17.2 Polyethylene glycol (PEG) precipitation

PEG 6000 was added 'very slowly' with constant stirring to the crude protein preparation suspended in 500mM potassium phosphate buffer (pH7.2) containing PMSF 0.1 mg/ml and 0.5% (v/v) Tween-20. Once added to the required percentage of PEG, the preparation was centrifuged at 30000 X g (OTD-Combi) for 30 min. Both supernatant and pellet were then assayed for CPT activity (section 2.12.3) and the percentage of PEG altered in order to further eliminate the precipitation of unwanted proteins on subsequent cuts.

2.17.3 Ammonium sulphate $(NH_4)_2SO_4$ precipitation

$(NH_4)_2SO_4$ was added 'very slowly' to a 40% saturation and then left on ice for 1 hour, before centrifugation at 30000 X g (OTD-Combi) for 30 minutes. The supernatant was decanted and filtered through butter muslin gauze (three layers) and increased to 50% saturation with $(NH_4)_2SO_4$. After standing in ice for 1 hour the precipitate of CPT II was collected by centrifugation as above, dissolved in 500mM potassium phosphate buffer (pH7.2) containing PMSF (0.1 mg/ml) and 0.5% (v/v) Tween-20 then desalted and equilibrated with 20mM *bis*-Tris-propane buffer (pH7.0) containing 0.5% (v/v) Tween-20 by applying the sample to a Sephadex G-25 column (Pharmacia). The mixture was then concentrated by Amicon

Ultrafiltration with a low molecular weight cut-off point of 30000 Daltons.

2.17.4 Mono Q ion exchange

A Mono Q 10/10 column (Pharmacia), pre-equilibrated with 20 mM *bis*-Tris-propane buffer (pH7.0) containing 0.5% (v/v) Tween-20 was used to purify CPT II after the second $(NH_4)_2SO_4$ cut. Once applied to the column, the enzyme was eluted with a shallow linear gradient of 0.01-0.15 M NaCl dissolved in the same buffer. Most of the CPT activity eluted between 0.05-0.1 M NaCl. All fractions containing activity were pooled, desalted and concentrated by ultrafiltration.

2.17.5 Mono P chromatofocusing

Chromatofocusing produces a pH gradient on an ion exchanger by taking advantage of the buffering action of the charged group of the ion exchanger. Polybuffer 96 (Pharmacia) was adjusted to pH8 and run through a Mono P chromatofocusing column (Pharmacia) as described by Brady and Brady (1986) except that 0.5% (v/v) Tween-20 was incorporated into the buffers instead of octyl glucoside. The column was pre-equilibrated with polybuffer 94 adjusted to pH5.5 before the proteins were loaded and the gradient applied. CPT II activity was eluted from the column between pH6.5-6.0.

2.18 GROWTH AND MAINTENANCE OF *E.coli*

2.18.1 Growth of *E.coli* bacterial cultures

Liquid cultures of *E.coli* were grown in Luria broth by inoculating a given volume of broth with a single colony using a sterile inoculating loop. Cells were shaken at 37°C for an appropriate length of time.

2.18.2 Storage of *E.coli* bacterial cultures

For long term storage 1 ml of fresh overnight culture of bacteria, grown in Luria broth supplemented with antibiotics as appropriate, was mixed with 1 ml of 100% sterile glycerol, and stored in a sterile vial at -80°C. Upon recovery, vials were thawed quickly at 37°C and the culture streaked out on Luria broth agar plates, with antibiotics if required. TG1 was streaked out onto minimal agar

plates to maintain the F' plasmid. After overnight incubation at 37° C, a single colony was picked to propagate a fresh bacterial culture. For short term storage (4 - 6 weeks), bacteria were stored on agar plates at 4°C.

2.19 TRANSFORMATION PROCEDURES

2.19.1 Preparation of competent cells

1 litre of Luria broth was inoculated with 10ml of an overnight culture and shaken at 37°C until an OD_{600} of 0.3 was reached. Cells were placed on ice for 10 minutes to arrest growth, then pelleted by centrifugation in 4 x 250 ml sterile Sorvall pots at 5000 X g for 15 (Sorvall SS-34 rotor) minutes at 4°C. The pellets were resuspended in 125ml of fresh 100 mM calcium chloride. After 30 min on ice, the cells were pelleted at 9000 X g (Sorvall SS-34 rotor) for 10 minutes at 4°C. Each pellet was resuspended in 25 ml of 100 mM calcium chloride, and combined in one of the pots. Glycerol (20 ml) was added (final concentration of 17%) then the cells were devided into 1ml aliquotes, frozen in liquid nitrogen and stored at -80°C.

2.19.2 Transformation of *E.coli*

Supercoiled plasmid DNA (5 to 10 ng), or half of a ligation reaction, were added to 100 µl of competent TG1 cells in a 1.5 ml microcentrifuge tube and left on ice for 40 minutes. The cells were then heat shocked at 42°C for 3 min. Luria broth (1 ml), containing ampicillin, was added to the cells, which were then incubated at 37°C for 1 hour. The cells were then pelleted in a microfuge and resuspended in 100 µl of luria broth containing ampicillin. Aliquots (25 µl and 75 µl) were spread onto luria agar petri dishes containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C.

2.20 ISOLATION OF NUCLEIC ACIDS

2.20.1 Isolation of plasmid DNA from *E.coli*

Plasmid DNA was isolated from *E. coli* using the alkaline lysis method essentially as described by Birnboim and Doly (1979).

2.20.1.1 Small scale isolation of plasmid DNA from *E.coli*

Luria broth (3 ml), supplemented with ampicillin to a final concentration of 100 μg/ml, was inoculated with a single colony of the plasmid carrying strain and incubated at 37°C overnight with shaking. Half of the culture (1.5 ml) was pelleted by centrifugation in a microfuge and resuspended in 100 μl of 25 mM Tris.HCl (pH8), 10 mM EDTA, 50 mM glucose (TEG). Lysis buffer (200 μl of 0.2 M NaOH, 1% SDS) was added and after gentle mixing, placed on ice for 5 min. Following the lysis 150 μl of 3 M sodium acetate (pH5) was added to precipitate chromosomal DNA, SDS and proteins. After a further incubation on ice for 5 minutes the precipitate was removed by centrifugation in a microfuge for 10 minutes. Absolute ethanol (1 ml) was added to the supernatant, mixed, and immediately centrifuged in a microfuge to pellet the plasmid DNA. The DNA was washed with 70% ethanol, dried under vacuum, and resuspended in 50 μl of TE buffer. Plasmid 'miniprep' DNA was stored at 4°C.

2.20.1.2 Large scale isolation of plasmid DNA from *E.coli*

A 250 ml culture of bacteria carrying the desired plasmid was incubated overnight at 37°C with vigorous shaking in Luria broth supplemented with 100 μg/ml of ampicillin. The cells were pelleted by centrifugation at 9000 X g (Sorvall GSA rotor) and resuspended in 4 ml of 25 mM Tris.HCl (pH8), 10 mM EDTA, 50 mM glucose (TEG). Cells were lysed on ice by adding 8ml of 0.2 M NaOH, 1% SDS and left on ice for 20 min. Addition of 6 ml of 3 M sodium acetate (pH5) precipitated chromosomal DNA, SDS and proteins which were spun down at 17000 X g (Sorvall SS-34 rotor) for 15 minutes at 4°C. Isopropanol (11 ml) was added to the supernatant and left at room temperature for 30 min to precipitate plasmid DNA. The DNA was pelleted by centrifugation at 20000 X g (Servall SS-34 rotor) for 20 minutes at 4°C. The pellet was washed in 70% ethanol, dried under vacuum, resuspended in 10 ml TE buffer containing 10 μg/ml RNase and incubated at 37°C for 30 minutes. Plasmid DNA (9.4 ml) was transferred to a fresh tube to which 100 μl of 10mg/ml ethidium bromide and 9.02g of CsCl were added, giving a density of 1.55 g/ml. The DNA was banded by centrifugation at 40000 X g (Beckman Tl-100; TLA-100.3 rotor) for 12 hours at 20°C. DNA was visualised by side illumination with a UV light. The lower band containing supercoiled plasmid DNA was removed

by puncturing the tube with a 19 gauge needle and syringe. A second 19 gauge needle was inserted at the top of the tube to allow pressure release. The ethidium bromide was removed by extraction several times with butanol, and the CsCl was removed by dialysis against 2 litres of TE buffer for 8 to 15 hours at room temperature. The TE buffer was changed 3 to 4 times during dialysis. The DNA was then stored at 4°C for up to 12 months or under ethanol at -70°C for long term storage.

2.20.2 Isolation of single stranded DNA from *E.coli*

Single stranded DNA from plasmids with the F1 origin of replication (phagemids) were prepared from *E. coli* using M13KO7 helper phage. This method yielded approximately 1 μg single stranded DNA which was suitable for use in sequencing reactions.

A single colony of *E. coli* host containing the phagemid was grown in 2 ml selective medium to mid-log phase (OD_{600} of 0.5). M13KO7 helper phage was added to this, giving a multiplicity of infection of 10, and the culture was shaken vigorously. After 1 hour, 400 μl of infected cells were mixed with 10 ml of selective medium, to which kanamycin was added at a final concentration of 70 μg/ml to select for the phage. The culture was grown over night, with vigorous shaking to give good aeration.

Cells were removed from the culture supernatant by centrifuging 1.5 ml of the overnight culture at 12000 x g for 5 minutes. To precipitate the phage, 1.2 ml of the supernatant was added to 0.3 ml of NaCl/PEG solution (2.5M NaCl, 20% polyethylene glycol 6000), and left at room temperature for 15 minutes. The phage were pelleted at 12000 x g for 5 minutes. After removing all NaCl/PEG solution, the phage were resuspended in 100 μl TE. Phenol (50 μl) was added to the phage. The suspension was vortexed and centrifuged for 1 minute. DNA, which was located in the aqueous layer, was removed and cleaned by Geneclean™.

2.20.3 Preparation of rat liver nucleic acid

All equipment and solutions were pre-treated with diethyl pyrocarbonate (DEPC) when RNA was being prepared.

Preparation of CsCl Density Gradients;

Guanidinium isothiocyanate (GIT).

GIT	100.0 g
dH_2O	100.0 ml
Tris.HCl 1 M (pH 7.6)	10.6 ml
EDTA 0.2 M	10.6 ml

This solution was stirred for 30 min at 4°C. Then 21.2 ml of 20% sarkosyl (sodium lauryl sarcosinate) were added and the resultant solution was filtered through a Nalgene (0.45 μm) filter. Finally 2.1 ml of β-mercaptoethanol was added before the GIT was aliquoted and stored at -20°C.

Caesium Chloride (CsCl).

Per litre

CsCl	5.7 M
EDTA	0.1 M

The CsCl was autoclaved and filtered before use.

A liver was removed from a rat and immediately frozen in liquid nitrogen (N_2). It was then broken up into 1 g quantities and stored at -70°C until required. Rat liver (1 g) was ground to a fine powder in liquid N_2 using a mortar and pestle. The powder was transferred (as a slurry in N_2) to thaw. The thawed homogenate was passed through a 19 gauge needle and then through a 23 gauge needle (5 to 6 times each), in order to disperse small clumps of tissue and shear genomic DNA. Solid CsCl (1g per 2.5ml) was added to the homogenate which was then layered on top of a "cushion" of 1.2ml of 5.7M CsCl in a 5ml swing out centrifuge tube. The tubes were filled up with GIT, balanced and centrifuged at 30000 X g (OTD Combi) for 30 hours at 20° C. After centrifugation the tube contained three visible bands. A top band of fat, a middle band of protein and a bottom band of DNA. There was also a transparent gelatinous pellet of RNA.

2.20.3.1 Preparation of rat liver DNA

The gradient was removed from the Corex tubes (section 2.20.3) to just above the DNA band which in turn was collected down to the interface with the cushion and transferred to a 30 ml Corex tube. The DNA sample was then diluted to 15 ml with TE buffer, extracted with phenol-chloroform and precipitated with ethanol as in section 2.20.5.

2.20.3.2 Preparation of rat liver RNA

After removal of most of the gradient by a pasteur pipette, the corex tubes (section 2.20.3) were drained by inversion leaving only the gelatinous RNA pellet. The tubes were stored on ice after the sides had been dried carefully with clean tissue. The pellet was resuspended in 0.5 ml of sterile H_2O and transferred to a 15 ml falcon tube. An equal volume of a mixture of chloroform and butanol (4:1) was added. After vigorous shaking, the mixture was centifuged in a MSE bench top centrifuge at 5500 X g for 5 minutes at 0°C. The aqueous layer was then trasfered to a Corex tube and the organic layer was re-extracted. The aqueous layers were pooled and then precipitated as described in section 2.20.5. The RNA was recovered by centifuging at 15000 X g (Sorvall SS-34 rotor), for 20 minutes at 4° C. The RNA was dissolved in TE buffer and stored at -80°C.

2.20.4 Extraction of proteins from nucleic acid by phenol and chloroform

Distilled phenol was equilibrated with buffer at pH8 for extraction of proteins from DNA, by mixing with an equal volume of 1M Tris.HCl (pH8). The phases were allowed to separate and the upper aqueous phase was discarded. This process was repeated until the pH of the phenol reached 8. The Tris.HCl layer was then replaced with TE buffer. Extraction of proteins from RNA required phenol to remain acidic (pH5-6). Phenol was therefore equilibrated as above except Tris was replaced with distilled water. The nucleic acid to be extracted was mixed thoroughly with 0.4 to 1 X the volume of phenol then centrifuged at 2000 X g (Sorvall SS-34 rotor) for 2 - 5 minutes. The upper aqueous phase was removed into a fresh tube, taking care not to collect any proteins located at the interface, and re-extracted with phenol if necessary. Instead of, or following phenol

extraction, DNA or RNA was extracted with phenol-chloroform. The aqueous phase was then extracted with an equal volume of chloroform to remove any remaining phenol. After centrifugation as above, the upper aqueous phase was transferred to a fresh tube.

2.20.5 Precipitation of nucleic acid with ethanol

Nucleic acid was precipitated from solution by addition of 0.1 volumes of 3 M sodium acetate (pH5) and 3 volumes of absolute ethanol at room temperature. The solution was mixed thoroughly and allowed to stand at room temperature for 10 minutes, -20°C for 20 minutes, or -70°C for 15 minutes depending on the DNA concentration. DNA was pelleted by centrifugation at 5000 X g (Servall SS-34 rotor) for 15 to 30 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged as above for 2 minutes. The supernatant was discarded and the pellet dried under vacuum until no visible traces of ethanol remained (2 to 5 min). The DNA was dissolved in sterile distilled water or TE buffer. Precipitation of RNA from solution with ethanol was identical except that 0.033 volumes of 3M potassium acetate (pH5) and 0.5 volumes of ethanol were added. Alternatively, RNA was precipitated by adding 2 volumes of 3 M LiCl and left over night at 4°C before centrifugation, washing and drying.

2.21 GEL ELECTROPHORESIS OF DNA

2.21.1 Agarose gel electrophoresis of DNA

DNA was separated in 0.7 - 2% (w/v) agarose BRL electrophoresis grade with 0.5 μg/ml ethidium bromide in 1 X TBE (for Southern blotting), or 1 X TAE (for routine gels). Prior to loading, DNA samples were mixed with 0.1 X volume of loading buffer (20% glycerol; EDTA; 0.1% bromophenol blue). Electrophoresis was carried out horizontally across a potential difference of 1-10 V/cm. Bacteriophage λcI857 DNA cut with HindIII was used as size markers. DNA was visualised by UV illumination and photographed.

2.21.2 Recovery of DNA from agarose gels

DNA was electrophoresed through 1% agarose BRL in 1 X TAE, containing 0.5 μg/ml ethidium bromide. The desired fragment was visualised by UV illumination, cut out, and extracted from the agarose using Geneclean™. The agarose was weighed and 2 - 3 volumes of 6M NaI were added per mg agarose. The agarose was dissolved by heating to 55°C for 5 minutes with occasional mixing, and then cooled on ice for 5 minutes. The molten agarose was treated with 5μl of 'glass milk' (a silica matrix suspended in water), and left for 5 minutes on ice with occasional mixing to allow the DNA to bind to the silica matrix. The 'glass milk' was pelleted by centrifugation at 15000 X g (microfuge) for 1 minute. The supernatant was discarded and the pellet washed three times with 500 μl of 'NEW wash' (NaCl/ethanol/water mix). After a final spin all the NEW wash was discarded and the DNA was eluted from the 'glass milk' in 5-20 μl of TE buffer or water at 55°C. The mixture was spun in a microfuge, and the supernatant containing the DNA, transferred to a fresh microfuge tube and stored at -20°C. Recovery of DNA fragments of 500 base pairs or greater was usually around 80%.

2.21.3 Recovery of DNA fragments of 300 bp or less from agarose gels

The DNA was electrophoresed through a 1-2% low melting point agarose gel (LMP Agarose; BDH) in 1 X TAE containing ethidium bromide. The required DNA fragment was visualised with UV, as described before, and excised from the gel into an microfuge tube. To every 0.1g of agarose, 200 μl water, 1 μg glycogen, and SDS to final concentration of 0.1% were added. The agarose was dissolved at 55°C for 20 minutes until all the agarose had dissolved. The DNA was then separated from the agarose by phenol/chloroform extraction followed by ethanol precipitation.

2.21.4 Agarose gel electrophoresis of RNA

RNA isolated from rat livers was separated on agarose gels prior to Northern blotting using the method of McMaster and Carmichael (1977). A 1.5% agarose gel was prepared with DEPC treated water and 10 mM sodium phosphate (to a final concentration of 10 μM. The RNA sample was prepared by mixing 5 μl of RNA with 20 μl of glyoxal mix, [100 μl glyoxal (30%) pH6; 250 μl formamide pH7; 10 μl

0.5 M sodium phosphate; 40 μl water], and incubated at 50°C for 30 minutes. The running buffer used for the gel was 500 mM sodium phosphate buffer (pH7). The RNA was electrophoresed into the gel before adding more buffer to submerge the gel. Electrophoresis was carried out at 200 mA for 2 hours, recirculating the buffer every 10 min.

2.21.5 Polyacrylamide gel electrophoresis of DNA

To separate DNA fragments less than 400 base pairs long, electrophoresis was carried out with a vertical gel of 6 - 10% polyacrylamide (40:1 acrylamide : *bis*-acrylamide) in 1 X TBE buffer at 10 - 70 v/cm.

2.22 DNA MANIPULATION TECHNIQUES

2.22.1 Cleavage of DNA with restriction enzymes

All restriction enzyme digests were performed using BRL enzymes and buffers. DNA (0.1 to 20 μg) was cut in 10 - 200 μl of 1 X appropriate 'React' buffer for 2 - 8 hours at the appropriate temperature. For double digests involving enzymes with different recommended buffers, the buffers were checked individually in double digests to determine which gave the most efficient digestion.

2.22.2 Filling in of protruding 5' ends of fragments

Protruding 5' ends were filled in using the DNA polymerase activity of the Klenow fragment of DNA polymerase 1 from *E.coli*. The fragment of DNA (1 μg) in 21.5 μl of distilled water was added to 2.5 μl of 10 X Nick Translation buffer (0.5 M Tris.HCl pH7.5; 0.1 M $MgSO_4$; 10 mM DTT; 500 μg/ml BSA) and 1μl of a 2mM solution of all 4 dNTPs. Klenow fragment (2 units) was added and the reaction mixture was incubated at 22°C for 30 minutes. The Klenow fragment was inactivated by heating to 70°C for 5 minutes.

2.22.3 Ligation of DNA ends

50-100 ng of vector (cut with the appropriate restriction enzyme) was incubated with a four-fold molar excess of fragment in 1 X ligation buffer (10 mM Tris.HCl pH 7.2; 1 mM EDTA; 10 mM $MgCl_2$; 10 mM DTT; 1 mM ATP) with 10 units of T4 ligase. The reaction was made

up to a final volume of 10 μl and incubated for 12 to 48 hours at 16° C.

2.22.4 Polymerase chain reaction

All reaction were carried out on a Techne Programmable Dryblock (PHC-2). The amplification strategy was based on that of Scharf *et al* (1986), reviewed by Mullis and Faloona (1987). Primers were designed such that they shared 15-25 nucleotide homology to the template and where appropriate a 6 base restriction enzyme site at their 5' end. Where amino acid sequence had been used to elucidate primer sequence, either primers containing total heterogeneity or 'guessmers' using selective heterogeneity were made. In the latter case care was taken to ensure that the 3' end was always complementary. Template DNA (100 pg - 1 μg) was mixed with 10 μl of 10 X amplification buffer [100 mM Tris.HCl pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.1% gelatin(w/v); 0.1% Tween 20 (v/v); 0.1% NP40 (v/v)], 2 μl of 10 mM dNTPs, 1-2 μg of each primer, and 2.5 units of Taq polymerase (Cetus, Perkin Elmer), and made up to a volume of 100 μl with DEPC treated water then submerged under 100 μl of mineral oil (Sigma). One initial 95°C 5 minutes denaturation cycle preceded all amplifications from either single or double stranded DNA. Where RNA/DNA duplex was used as a template the initial denaturation step was at 99°C for 10 minutes. After this initial step all cycling was identical where the annealing of the primers to the template was carried out at 37-55°C for 1 minute, polymerisation at 72°C for 2 - 5 minutes and further denaturation steps at 94°C for 1 minute. The number of cycles used varied according to the abundance of the target DNA, but was usually between 25 and 55 cycles. Following the final cycle a further period of 10 minutes at 72°C was carried out to ensure that all products were full length. PCR products were checked by polyacrylamide gel electrophoresis and excess primers were removed by first removing the oil by addition of 0.5 ml of chloroform, and then phenol and chloroform extraction of the DNA, followed by ethanol precipitation.

2.23 SEQUENCING OF SINGLE STRANDED DNA

Sequencing of DNA was carried out using the Sequenase™ Version 2.0 kit (United States Biochemicals). The dideoxy chain termination method was used.

Appropriate sequencing primer (1 μl of 3 ng/μl) was annealed to 7 μl of template DNA (approximately 1 μg DNA) in 2 μl of 5 X reaction buffer (200 mM Tris.HCl pH7.5; 100 mM $MgCl_2$; 250 mM NaCl) by heating to 65°C for 2 minutes then cooled slowly to below 37°C. Extension from the annealed primer was done by adding 1 μl 0.1 M DTT, 2 μl dGTP label mix (a 1 in 4 dilution of 7.5 μM dGTP, dCTP, dATP and dTTP), 0.5 μl α-[^{35}S]-dCTP (400 Ci/mmole) and 2 μl diluted sequenase™(a 1 in 8 dilution of sequenase at 13 U/μl in 10 mM Tris.HCl pH7.5; 5 mM DTT; 0.5 mg/ml BSA). The extension mixture was left at room temperature for 2 - 5 min. Further extension and termination was done by dispensing 3.5 μl of extension mix into four tubes preheated to 37 °C containing 2.5 μl of one of the four termination mixes:

ddGTP mix - 80 μM dNTPs; 8 μM ddGTP; 50 mM NaCl
ddATP mix - 80 μM dNTPs; 8 μM ddATP; 50 mM NaCl
ddCTP mix - 80 μM dNTPs; 8 μM ddCTP; 50 mM NaCl
ddTTP mix - 80 μM dNTPs; 8 μM ddTTP; 50 mM NaCl

The termination reaction was allowed to proceed at 37°C for 5 minutes and the reaction was stopped by the addition of 4 μl of stop solution (95% formamide; 20 mM EDTA; 0.05% bromophenol blue). Extension products were separated by electrophoresis through a 6% denaturing polyacrylamide gel made up by mixing 25.2 g of urea, 6 ml 10 X TBE, 12.5 ml Protogel (30% acrylamide (30:1 acrylamide: *bis*-acrylamide)) made up to a final volume of 60ml with water. Polymerisation of the acrylamide was achieved by adding 140 μl of 10% ammonium persulphate and 140 ml of TEMED immediately prior to pouring the gel. Sequencing reaction were heat denatured at 75°C for 3 minutes, loaded onto the gel and electrophoresed in 1 X TBE buffer at 65 watts for 1 hour 45 minutes to 6 hours. The gel was fixed in 10% acetic acid (v/v), washed with water and then dried under vacuum at 80°C for 45 minutes. The gel was then autoradiographed at room temperature overnight.

2.24 NUCLEIC ACID HYBRIDISATION TECHNIQUES

In order to detect specific sequences in either DNA or RNA, the nucleic acid was separated in an agarose gel and transferred to a nylon membrane before hybridisation with a DNA probe.

2.24.1 Transfer of nucleic acids to filters

The capillary blotting method used for transfer was essentially that described by Southern (1975), and as detailed below for the transfer of DNA (Southern transfer) or RNA (Northern transfer).

2.24.1.1 Southern transfer

Discs and sheets of both nitrocellulose (Hybond-C) and nylon (Hybond-N) were obtained from Amersham International. Chromosomal DNA was digested with the restriction endonucleases *Hind*III, *Eco*R1, *Bam*H1, and a mixture of all three. The digests were then separated on a 0.8% agarose gel by electrophoresing in 1 X TBE. The gel was placed in denaturing solution (1.5 M NaCl; 0.5 M NaOH) for 30 min, and then in neutralising solution [1.5 M NaCl; 0.5 M Tris.HCl (pH 7.2), 0.001 M Na_2EDTA] for 30 minutes. The gel was placed upside down on a piece of 3 MM filter paper, the ends of which were resting in a reservoir of 20 X SSC (3 M NaCl; 0.3 M sodium citrate). A sheet of nylon membrane (Hybond-N) was cut to the size of the gel and placed on top. Care was taken to avoid any air bubbles forming between the gel and the membrane as they would prevent migration of the DNA. Three layers of presoaked filter papers were placed on top of the membrane followed by a 10 cm stack of absorbent paper towels, and a glass plate with a weight placed on top of it to ensure even transfer. Transfer was allowed to proceed overnight, after which the filter was removed and washed in 2 X SSC. The filter was then allowed almost to dry on a piece of filter paper before being wrapped in Saran wrap and placed upside down on a UV transilluminator for 5 min. This procedure covalently bonded the DNA to the nylon membrane.

2.24.1.2 Northern transfer

The RNA sample was electrophoresed in a denaturing agarose gel. Transfer of RNA to the nylon membrane was essentially as described for Southern Transfer above, using the method described by Thomas (1980) except that the membrane was baked for 2 hours at 80°C after

transfer to reverse glyoxylation and to therefore allow the subsequent hybridisation steps to take place efficiently.

2.24.2 Detection using radioactive probes

2.24.2.1 5' end labelling

Probing mixture:

2 μl(20μCi)γ^{32}P-ATP(3000Ci/mmol;Amersham PB10168)

3 μl 1 M Tris.HCl, pH8.0

1.5 μl 0.1 M DTT

1 μl 0.3 M $MgCl_2$

20 pmol oligonucleotide

H_2O to 30 μl

0.5 μl (5 units) polynucleotide kinase

This reaction mixture was incubated at 37°C for 1 hour before the enzyme was inactivated by incubation at 68°C for 10 minutes. Incorporation of the [^{32}P] into the oligonucleotides was checked by electrophoresing 2μl of the completed digests on a 10% polyacrylamide gel. An autoradiograph was then taken of the gel. A 20 minute exposure of the gel was sufficient to see incorporation. The probes made were 20 and 17 oligomers. They were obtained by interpretation of the amino acid sequence of the amino terminus of CPT II, and constructed in the Orwell DNA synthesis unit, Department of Chemistry, University of Edinburgh.

2.24.2.2 Random-primed labelling of DNA

(Feinberg and Vogelstein, 1983)

Linear DNA (50-100 ng) in a total volume of 35 μl was heat denatured by boiling. After snap cooling on ice the DNA was mixed with 30 μCi α[^{32}P]-dCTP (3000 mCi/mmol), 10 μg BSA, 10 μl 5 X OLB (0.05 M β-mercaptoethanol; 1 M HEPES pH 6.6; 0.03 U $pd(N)_6$; 0.26 M Tris.HCl pH8; 0.26M $MgCl_2$; 0.1mM dATP, dTTP and dGTP) and 1 μl of Klenow fragment (5 U/μl) from *E.coli* DNA polymerase 1. The reaction took place at room temperature over a 5-12 hour period. The radiolabelled DNA was separated from unincorporated nucleotides by firstly making the total volume of the reaction mixture to 200 μl with 4 X SSC (final concentration of 3 X SSC), then passing the

mixture through a 1 ml Sephadex G-50 column (made up in 3 X SSC), (modified from Maniatis *et al*, 1989).

2.24.2.3 Incorporation of [^{32}P] by PCR techniques

Linear DNA (50-100 ng) was used in a PCR reaction (normal conditions, see section 2.22.4) along with 50 μCi α[^{32}P]-dCTP (3000 mCi/mmol). The radiolabelled DNA was then separated from unincorporated nucleotides (section 2.24.2.2).

2.24.3 Hybridisation conditions

The nylon membrane to which the DNA was bound was placed in a heat sealable bag with 0.2 ml of prehybridisation solution per cm^2 of membrane. The bag was sealed and then incubated at 50°C for >3 hours with constant shaking. Hybridisation was then carried out by adding 50 ng of denatured labelled probe to the bag, resealing it and incubating it at 37°C for > 6 hours with shaking. Care was taken during both prehybridisation and hybridisation to avoid air bubbles in the heat sealed bags. After hybridisation the membranes were washed with increasing stringency of temperature until all the background radiation had been washed off. The membranes were then allowed to dry before being autoradiographed at -70°C.

2.25 EXTRACTION OF TOTAL CELL PROTEIN FROM *E.coli*

A single colony of *E.coli*. was grown in LB broth, supplemented with ampicillin, to stationary phase before being harvested by centrifugation at 9000 X g (Sorvall SS-34 rotor). The pelleted cells were resuspended in 1 ml of ice cold 10% TCA and transferred to an Eppendorf tube. Approximately 2 ml of glass beads (0.45 - 0.5, 40 mesh) were added in order to fill the tube to just below the meniscus of the cell suspension. The tubes were then vortexed at full speed for 2 minutes. 10% TCA (1 ml) was added and the tubes which were vortexed again. The liquid was then removed to a fresh tube and combined with 2 X 1 ml 10% TCA washes of the beads. The precipitated protein was pelleted by centrifugation at 20000 X g (Sorvall SS-34 rotor) for 5 minutes and was washed twice in cold ethanol to remove residual TCA. The protein pellet was dried and resuspended in 200 μl of 2 X LSB. Protein concentrations of these samples were estimated by spotting 2 μl dilutions of the cell extract onto a piece of filter paper, alongside 2 μl standards of

bovine serum albumin (1 μg/ml) and staining with Page Blue 83 'Electran' (BDH) solution made up in 20% methanol and 5% acetic acid. The amount of protein present could then be estimated by the comparative intensity of the blue spots.

2.26 EXTRACTION OF TOTAL CELL PROTEIN FROM THE YEAST *Saccharomyces cervisiae*

A single colony of *S.cervisiae* was grown in yeast extract broth to an OD_{600} of 0.7. Cells were then harvested by centrifugation, washed in distilled water and resuspended in 1ml of ice-cold 10% TCA. The preparation of the protein was then carried out identically to the procedure for *E.coli* protein preparation.

2.27 EXTRACTION OF TOTAL CELL PROTEIN FROM RAT LIVER

Rat liver (1 g) was finely minced and homogenised in 3 ml of sucrose solution to form a thin emulsion. A sample was taken (0.5 g) and the rest was centrifuged at 1000 rpm (Servall SS-34 rotor) to remove cell debris. The supernatant was then centrifuged at 7000 rpm (Sorvall SS-34 rotor) to spin out mitochondria. This pellet was resuspended in sucrose solution as in section 2.11. This pellet of crude mitochondria was taken as the second sample. To each of the two samples an equal volume of 2% (v/v) Triton X 100 was added. The samples were then left on ice (with occasional stirring) for 30 minutes. The samples were then centrifuged at 9000 X g (Sorvall SS-34 rotor) for 20 minutes at 4°C. An equal volume of 20% PEG was added to the supernatant and the samples were then left on ice for a further 30 minutes. The protein-PEG precipitate was then pelleted at 10000g for 20 minutes (4°C). Care was taken to remove all of the PEG before resuspending the protein pellet in SDS sample buffer. The protein suspensions were boiled for 3 minutes and then centrifuged at 9000 X g (Sorvall SS-34 rotor) for 3 minutes. The supernatant was transferred to Eppendorfs and frozen at -20°C until required. The approximate concentration of the protein solution was estimated (section 2.13.1.1).

2.28 ONE DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

This technique was used to separate proteins according to their molecular weight, (Laemmli, 1970). The polyacrylamide gel was prepared in two phases, a resolving gel for the separation of the protein samples and a stacking gel for the concentration of the protein samples before separation. The resolving gel was prepared by mixing the following solutions in the following order:

30% Acrylamide : 0.8% *bis*-acrylamide	10.0 ml
4 X Resolving Buffer	7.5 ml
dH_2O	12.3 ml
TEMED	30.0 μl
10% Ammonium Persulphate (APS)	190.0 μl

The gel was mixed well, poured between two glass plates and overlaid with water saturated butanol. It was then left to polymerise for 1 hour. Once the resolving gel had polymerised the water saturated butanol was rinsed off with distilled water and a stacking gel was prepared by mixing the following solutions in the following order:

30% Acrylamide : 0.8% *bis*-acrylamide	1.6 ml
4 X Stacking Buffer	2.5 ml
dH_2O	5.86 ml
TEMED	10.0 μl
10% Ammonium Persulphate (APS)	30.0μl

The stacking gel was mixed well and then poured on top of the resolving gel. A comb was then inserted into the top of the stacking gel and the gel was allowed to polymerise for 30 minutes. Once polymerised the gel was clamped into a vertical electrophoresis tank filled with 1 x running buffer. The comb was then carefully removed and the protein samples (in 2 X LSB) were loaded into the wells. The gel was then run at 10 volts per cm for 4 - 5 hours. The gels were stained with 1% PAGE Blue Electran in 20% (v/v) methanol, 5% (v/v) acetic acid.

2.29 ELECTOPHORETIC TRANSFER OF PROTEIN TO MEMBRANES BY WESTERN BLOTTING

In order that specific proteins could be detected from a cell extract, the proteins were firstly electrophoresed and then transferred to a nylon membrane (Hybond-N) or a nitrocellulose membrane (Hybond-C). Once run the gel was soaked in 1 X transfer buffer for two minutes. It was then assembled into a "sandwich" with the gel adjacent to a piece of nylon membrane (Hybond-N) placed between 2 x 2 layers of 3 MM filter paper and foam sponge, (all pre-soaked in 1 X transfer buffer). The proteins were then transferred onto the membrane, by immersing the sandwich in a tank containing 1 X transfer buffer and passing a current of 1 Amp through it for 2 hours. The membrane was on the positive electrode side of the gel, since proteins migrate towards the positive electrode. The membrane was then dried or used immediately for immunodetection.

2.30 METHODS OF BLOCKING THE UNFILLED SITES ON THE MEMBRANE FILTERS

Agents used to block the unbound sites of the membrane were BSA and skimmed milk. These blocking agents were tried at various concentrations for both blocking and antibody incubation (both in the presense and absense of Tween 20) in order to find the most sensitive detection system. All blocking agents were made up in TBS, and once antibody was introduced, were stored at -20°C and used up to 5-8 times. Both nylon (Hybond-N) and nitrocellulose (Hybond-C) were used in the comparisons.

2.30.1 Filters blocked with BSA

The filters were blocked for 6 hours in 2% BSA made in TBS. First antibody incubations were carried out (individually) on each filter over night at R.T. in 2% BSA, 0.05% Tween 20 made in TBS. The filters were then washed extensively in 2% BSA, 0.05% Tween 20 in TBS before the second antibody or antibody affinity protein, was added to the filters. Incubations were again carried out in 2% BSA, 0.05% Tween 20, TBS, for 4 hours. The filters were then washed extensively before developing.

2.30.2 Filters blocked with skimmed milk proteins

The filters were blocked for 6 hours in 2%,5%, and 20% (w/v) skimmed milk powder in TBS, at 25°C overnight. The solution was then replaced with 20 ml of 0.5%, 2% and 5% respectively, skimmed milk powder in TBS. Antibody (30 μl) was added and the membranes/filters incubated at 25°C for 5 hours. The antibody solution was stored at -20°C and was reused many times. After incubation with the antibody the membrane was washed in 100 ml of TBS, four times for 5 minutes. The membrane was then placed in fresh skimmed milk powder in TBS (at the same relative concentration) and second antibody or antibody affinity protein, was added. After incubation for 4 - 5 hours the membrane was washed as before and then developed.

2.31 PREPARATION OF SERUM

2.31.1 Removal of *E. coli* proteins from antibody serum

A culture of a λgt11 lysogen of Y1090 was grown at 30°C until an OD_{650} of 0.5 was obtained. The culture was induced into lysis by incubation at 42°C (shaking at 300 rpm) for 20 minutes. The culture was then incubated at 37°C for 3 hours until total lysis had occurred. Chloroform was added to the culture if total lysis had not occurred at the end of 3 hours, and incubation continued for a further 15 minutes. After this the culture became clear. Membranes (82 mm Hybond-C) were then added to the culture and left to absorb *E.coli* proteins. These membranes were then incubated with the antibody serum at RT for 4 hours. Approximately 10 of these membranes were required to mop up all the antibodies against *E. coli* proteins present in the serum.

2.31.2 Affinity purification of antibody

Two thin strips were cut from each side of a (pre blocked) Western blot of partially purified proteins containg the main protein to which the antibodies were raised. These strips were then incubated with the serum and then with second antibody and developed (section 2.32.1). The position of the desired protein was then be accurately excised from the remaining undeveloped blot. The excised horizontal strips were then incubated with the impure IgG serum. Bound antibody was eluted with 0.2 M glycine (pH2.8), at 15°C for 2 hours. The

eluted antibody was neutralised with sodium hydroxide, containing 1 mg/ml soyabean trypsin inhibitor and dialysed against three changes of TBS containing 10% (v/v) glycerol, for three hours at 4°C. BSA (2% w/v) was added and the purified antibody was stored at -70°C.

2.32 DETECTION SYSTEMS

2.32.1 Horse radish peroxidase

Horse radish peroxidase-conjugated anti-rabbit IgG (Biorad) and anti-sheep/goat IgG (Scottish Antibody Production Unit) were used as second antibodies. Antibody (10-20 μl) was added to a small volume of the diluted blocking agent and incubated with the washed membranes at 25°C for 2 hours. The membranes were then washed thoroughly and developed by shaking the membrane in 10 ml of developing solution (0.5 ml of 5 mg/ml dianisidine, 1 ml of 0.1 M imidazole (pH 7.4), 0.1 ml of 30% H_2O_2 and 8.4 ml of water) until a pink band appeared. Where larger membranes were used the volumes were scaled up accordingly. The reaction was stopped by rinsing the membrane in distilled water and the membrane left to dry. The developed membrane was photographed since the colour tended to fade with time.

3.32.2 [^{125}I]-Protein G

[^{125}I]-Protein G was also used as a detection system for sheep/goat antibodies. The method was as above but after incubation with protein G (5 μCi/100 ml) the membranes were autoradiographed at -80°C for 40 - 70 hours.

2.33 TECHNIQUES INVOLVING BACTERIOPHAGE λ

2.33.1 Preparation of λ libraries for screening

Mid log phase bacteria were prepared making a 1:10 dilution of an overnight culture of *E.coli*. This was grown to mid-exponential phase (3 hours) and was then spun down at 9000 X g (Sorvall SS-34 rotor) for 10 minutes. The pelleted cells were suspended in 1 mM $MgSO_4$ and aliquoted into 100 μl quantities directly before use. The $MgSO_4$ suspension could be stored at 4°C for up to 3 days. Confluent plaques were obtained by incubating 100 μl of plating cells with 100 μl of the appropriate dilution of the bacteriophage λ at 37°C for 20

- 45 minutes. The mixture was then poured onto fresh ampicillin supplemented LB plates along with 3 ml of LB ampicillin supplemented top agar. The plates were then incubated at 37°C for 8 to 12 hours.

2.33.2 λ DNA small scale preparation

Individual plaques were picked and placed in 1 ml of SM buffer with 10 μl of chloroform. The phage were allowed to elute into the buffer (kept at 4°C) for 5 hours before being titred by drop inoculation on a lawn of *E.coli* Y1088, From this titre the volume of SM/Phage stock required to cause 10 X confluent lysis was plated out as in section 2.33.1. After incubation, 5 ml of SM buffer was added to the plates of confluent lysis and left shaking at 25°C for 5 hours. The SM-phage was harvested and centrifuged at 9000 X g (Sorvall SS-34 rotor) for 10 minutes. The supernatant was then incubated with DNase and RNase (50 μg/ml) for 30 minutes at 37°C. An equal volume of polyethylene glycol (20% PEG 6000;2 M NaCl; 10 mM $MgSO_4$) was added to the buffer which was then kept in a slush of ice and water for 1 hour. The buffer was then centrifuged at 17000 X g (Sorvall SS-34 rotor) for 20 minutes at 4°C to precipitate the phage. The phage pellet was then resuspended in 10mM $MgSO_4$ (0.25-0.5ml) with vortexing. The phage were then centrifuged (Sorvall SS-34 rotor) at 9000 x g to clarify the solution. To the supernatant 5μl of 10% SDS and 5μl of 0.5M EDTA were added per 0.5ml of resuspension volume. This solution was then incubated at 68°C for 15 minutes. The phage DNA was then extracted twice with phenol : chloroform and then ethanol precipitated. The phage DNA was then precipitated by centrifugation at 20000 X g (Sorvall SS-34 rotor) for 20 minutes (4° C). The DNA pellet was then resuspended in TE buffer.

2.33.3 Screening cDNA library contained in λgt11 and λgt10

The percentage of recombinants in the libraries was checked in two ways. Firstly by adding 50 μl of X-gal (20 mg/ml) and 50 μl of IPTG (20 mg/ml) to 3 ml of top agar and plating out 1000 pfu. The plaques which were blue in colour were non recombinants, whereas recombinant plaques would be colourless.

Secondly, prepared λ DNA from confluent plates was digested (in T.E. buffer) with the restriction enzyme *Eco*R1 (1-2 U), and

electrophoresed in a 0.8% agarose gel. Both the insert size and abundance were then visualised under UV light.

2.33.4 Plaque lifts using nylon and nitrocellulose membranes (Hybond-N and Hybond-C respectively) for DNA detection systems

In order to increase the efficiency of transfer of the DNA to the membrane, the discs were pre-wet by placing on a blank agar plate. This also stopped the discs from slipping on the agar surface during lifting. Hybond-N discs however did not require to be pre-wet since they are hydrophilic. The discs were then placed on plates containing lawns of plaques to be screened and orientated with three pins. The discs were removed from the plates, washed in 2 X SSC and placed on a filter paper which had been pre-soaked in denaturing solution (1.5 M NaCl; 0.5 M NaOH). The discs were left for 7 min, before being placed, face up, on a filter paper which had been pre-soaked in neutralising solution (1.5 M NaCl; 3.0 M sodium citrate) for 6 minutes. The soaked filter paper being renewed after 3 minutes. The transferred DNA was then covalently bound to the discs by UV illumination for the Hybond-N and baking in a vacuum oven (80°C for 2 hours) for the Hybond-C. The filters were then ready for prehybridisation (section 2.24.2).

2.33.5 λgt11 plaque lifts for screening with antibodies
(Young and Davis, 1983a&b)

Lawns of plaques (10,000 pfu per 82 mm plate) were grown on plates of *E.coli* Y1090 at 42°C for 4 hours. The plates were then overlaid with dry membranes (Hybond-C) that had been presoaked in 50mM IPTG. The plates complete with membranes were then incubated at 37°C for 12 hours. The position and orientation of each membrane was marked by pushing 3 pins through the membrane into the agar. The membranes were blocked (section 2.30.1), incubated with antibody and developed as in section 3.32.1. Positive plaques were isolated using the sterile blunt end of a Pasteur pipette and the plug eluted in 1ml of SM plus 10 μl of chloroform. After 4 to 5 hours elution the putative positive plaques were replated and screened at a density of 500 pfu per 82mm plates. The positives were rescreened 4 to 5 times until pure positive plaques were obtained. Phage DNA of the positive plaques was prepared as described in section 2.33.2 and the insert

excised using the restriction enzyme EcoR1. The insert was then separated from the λ arms by agarose gel electrophoresis on a 1% gel in TBE buffer. The insert was excised from the gel and purified by Gene Cleaning. The fragment was then ligated into the EcoR1 site in the plasmid pTZ from which large quantities of double stranded and single stranded DNA could be prepared for restriction analysis, probing and sequencing respectively.

CHAPTER 3

THE PURIFICATION OF CARNITINE PALMITOYLTRANSFERASE I

CHAPTER 3

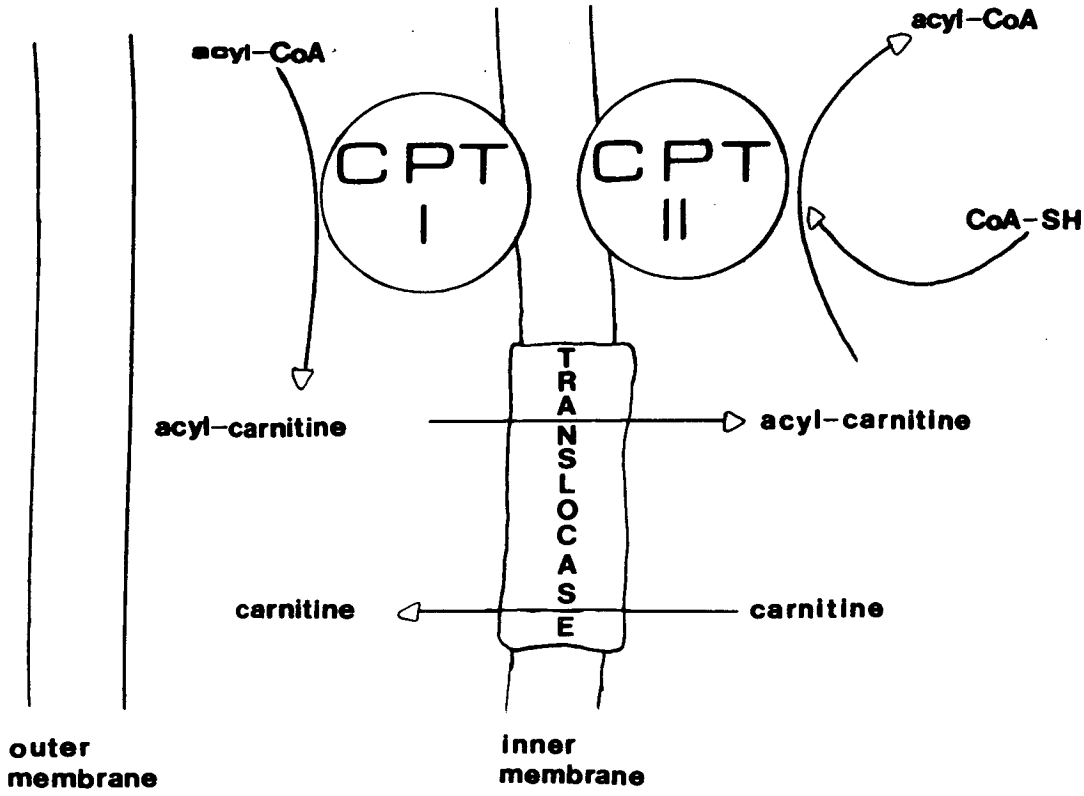
The Purification of Carnitine Palmitoyltransferase (CPT) I

3.1 INTRODUCTION TO THE PURIFICATION OF CPT I

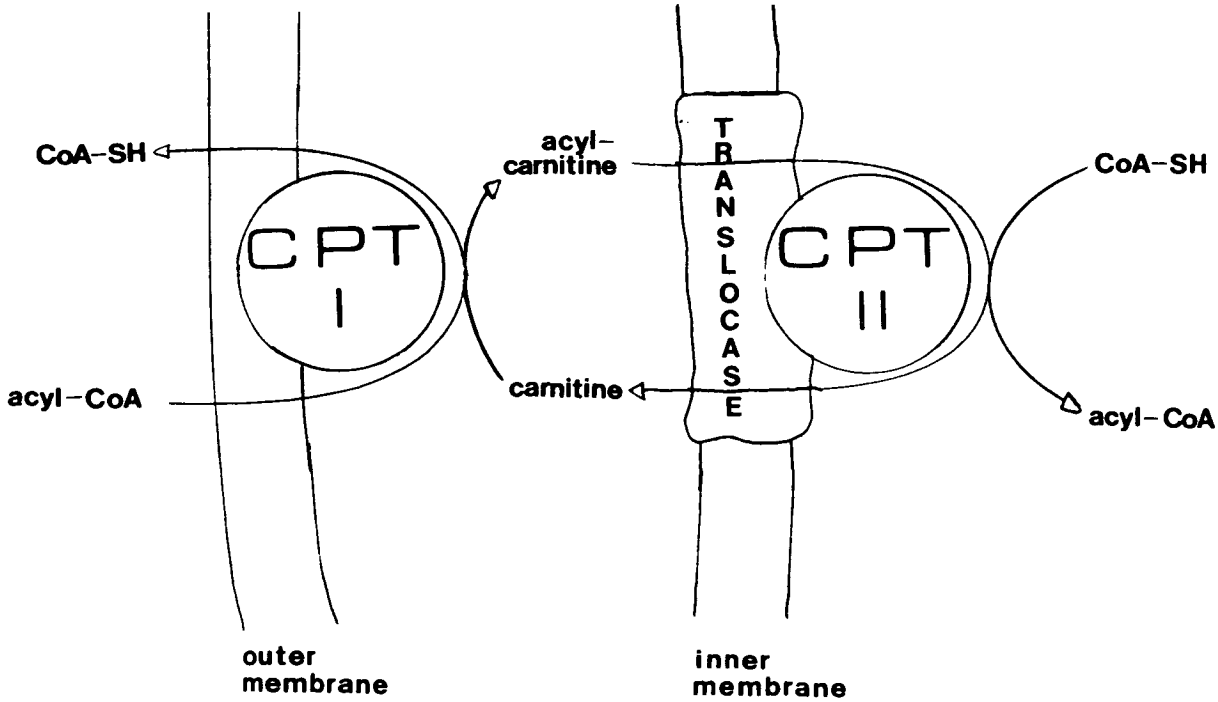
There has been much speculation regarding the system whereby long chain fatty acids enter the mitochondria prior to β oxidation. There are three components involved, carnitine palmitoyltransferases I and II, (CPT I and II), and a carnitine-acylcarnitine translocase. The system was originally conceptualised by the pioneering work of Fritz and Yue (1963), and Bremer (1963). Since the initial work, the system has been refined (Pande, 1975; McGarry and Foster, 1980; Murthy and Pande, 1987a), and is currently thought to function as shown schematically in Fig. 3.1. Since the late 1970s it has become evident that CPT plays a pivotal role in regulation of fatty acid oxidation in liver (and probably other tissues) due to the marked sensitivity of CPT I to malonyl-CoA (McGarry and Foster, 1980). The inhibition of CPT I was seen with intact mitochondria, but not with solubilised membranes. After treatment of the mitochondria with detergents such as Tween 20, the amount of CPT activity in the supernatant was almost 50% more than the total CPT activity of intact mitochondria. This solubilised CPT activity was no longer malonyl-CoA sensitive. The residual membrane however, still showed significant CPT activity, and this remaining activity was as sensitive to malonyl-CoA as the activity of the intact mitochondria. Since these discoveries, two main theories have been suggested. One is that CPT I and CPT II are in fact identical proteins residing on either side of the mitochondrial inner membrane and that the malonyl-CoA sensitivity is due to a regulatory subunit associated with CPT I, which is dissociated by solubilisation of the membranes (McGarry and Foster, 1980; Bremer, 1983; Hoppel and Brady, 1985). The second theory is that CPT I resides in the outer mitochondrial membrane, (Murthy and Pande, 1987b) and is distinct from CPT II (Woeltje *et al*, 1987; Declerq *et al*, 1987; Zammit *et al*, 1989; Murthy and Pande, 1990). In either case many of the questions regarding the relationship between the two enzymes could only be answered by purification of either or both of the proteins, followed by cloning of the gene or genes concerned.

Fig. 3.1 Two theories as to the location of carnitine palmitoyltransferase I in rat liver mitochondrial membranes

a; CPT I and CPT II reside on either side of the mitochondrial inner membrane



b; CPT II resides in the mitochondrial inner membrane but CPT I resides in the mitochondrial outer membrane



3.2 PURIFICATION OF RAT LIVER MITOCHONDRIAL OUTER MEMBRANES

Rat liver mitochondrial outer membranes were purified as detailed in section 2.11. Since the two CPT activities cannot readily be differentiated from each other, assays of marker proteins unique to either outer or inner mitochondrial membrane were used to determine the degree of purification of the enzymes. Monoamine oxidase activity (MAO; section 2.12.2) and cytochrome c oxidase activity (section 2.12.1), were used as markers for outer and inner membranes respectively. The ratios of these activities before, during and after purification along with the CPT activities (radiolabelled assay, section 2.12.4) were used to determine the degree of purification of the outer membrane preparation. The purification of the outer membranes from mitochondria resulted in a 96.9% loss of MAO activity (the outer membrane marker); a 99.9% loss of cytochrome c oxidase activity (the inner membrane marker); and a 75.2% loss of CPT activity (Table 3.1). However after purification there was a 13 fold increase and a 20 fold decrease in the specific activities of MAO and cytochrome c oxidase, respectively (Table 3.2) as would be expected when enriching for outer membranes. The increase in the specific activity of MAO was accompanied by a 20 fold enrichment of CPT activity (Table 3.2). The purification profile was altered when the last activated-sepharose step was replaced by solubilisation of the membranes with Tween 20 (Tables 3.3 & 3.4). Although the specific activity of CPT purified by Tween 20 solubilisation was slightly less than that of a sepharose treated membrane preparation, the reproducibility of purification was greatly improved, and the amount of malonyl-CoA insensitive enzyme activity decreased. This was demonstrated by measuring malonyl-CoA sensitive CPT activity before and after solubilisation of both fractions. Table 3.4 shows that 7-12% of the CPT activity present in the outer membrane fragments was in fact malonyl-CoA insensitive CPT II, therefore the CPT activity within the outer membranes is predominantly due to malonyl-CoA sensitive CPT I. Preparations of mitochondrial outer membranes were used to study the effect of inhibitors on the activity of CPT I and also served as a good starting point in the purification of CPT I.

Table 3.1. Measurement of the total activity of the enzymes in a preparation of purified outer membranes

	KPi	Final	% loss of Activity
CPT (nmol/min)	4982	1233	75.2
MAO (nmol/min)	5760	178	96.9
Cyt c oxidase (units)	142200	44	99.9

Table 3.2. The specific activity of the enzymes in a preparation of purified outer membranes

	KPi	Final	Number of Fold Enrichment
CPT (nmol/min/mg)	9.4	272	29
MAO (nmol/min/mg)	9.4	119	13
Cyt c oxidase (units /mg)	593	29	0.05

Table 3.3. The specific activity of the enzymes after final stage purification by Tween 20 extraction

	KPi	Final	Number of Fold Enrichment
CPT (nmol/min/mg)	7.7	170	22
MAO (nmol/min/mg)	2.9	73	25
Cyt c oxidase (units /mg)	178	93	0.5

Table 3.4. Malonyl-CoA sensitive CPT activity before and after solubilisation with Tween 20 expressed as % inhibition of un-treated membranes

	malonyl-CoA	KCl	% inhibition
KCl pellet (control)	37	100	63
KCl supernatant (control)	5	4	0
Tween 20 pellet	22	95	76
Tween 20 supernatant	20	19	0

3.3 INHIBITION OF CPT I BY 2-BROMOPALMITOYL-COA

2-Bromopalmitoyl-CoA (BrP-CoA) is known to be a slowly reversible inhibitor of CPT activity (Grantham and Zammit, 1986). It competes competitively for the same CPT I binding site as palmitoyl-CoA. The inhibitor action was studied by comparing the activity of highly purified membranes before and after incubation with BrP-CoA (section 2.15.1). The maximum inhibition of CPT activity (90%) was obtained when the membranes were incubated with 5 μM BrP-CoA in the presence of 1.25 mM carnitine. After the incubation with BrP-CoA and carnitine the membranes were washed extensively to remove the cold carnitine which otherwise could affect the radioactive assay for CPT by diluting the [^{3}H]-carnitine substrate. The quality of the purified outer membranes also affected the extent of the inhibitory effect of BrP-CoA on CPT so that only in the purest preparations was inhibition at a maximum.

3.4 BINDING OF TRITIATED CARNITINE BY OUTER MEMBRANES IN THE PRESENCE OF BRP-COA

The binding of [^{3}H]-carnitine by highly purified membranes was measured in the presence and absence of BrP-CoA. The incubations were carried out as described in section 2.15.3. The samples were then washed in albumin (3 mg/10 ml of 500mM KCl), until negligible counts were observed in the washings. It was apparent from the assays (Table 3.5), that membranes bound 50% more carnitine in the presence of 5 μM BrP-CoA, than in controls (7.6 nmol of CPT active protein in rat liver outer membrane fragments bound 43 pmol of carnitine in the presence of BrP-CoA, and 31 pmol of carnitine in the absence of BrP-CoA). This suggests that BrP-CoA does not compete with carnitine or inhibit its binding, but inhibits the formation of the ester bond between the palmitate and the carnitine probably through steric interference by the bromide group.

Table 3.5. The effect of bromopalmitoyl-CoA (BrP-CoA) on the binding of [^{3}H]-carnitine by highly purified outer membranes

	control(dpm)	BrP-CoA(dpm)
Wash 1	*********	*********
Wash 2	67760	56261
Wash 3	1313	1726
Wash 4	633	919
Wash 5	797	746
pellet	6485	10107
ratio of [^{3}H]-carnitine bound in presence and absence of BrP-CoA	1	1.5

Mitochondrial outer membrane fragments were adjusted to a protein concentration of 2.5-3.0 mg/ml with 500 mM KCl. Incubation of the membranes with [^{3}H]-carnitine in the presence of 10 μM BrP-CoA was performed as described in section 2.15.3. All experiments were carried out with outer membranes prepared from 48 hour starved rats. The washes were carried out by resuspension of the pellet in a solution of albumin (3 mg/10ml of 500 mM KCl), followed by centrifugation at 100000 X g (Beckman Air-fuge). Opti-fluor 12 scintillant was then added to the supernatants and the final pellet. The samples were counted in a scintillation counter. This is a typical example of many similar experiments in which the increase of bound [^{3}H]-carnitine caused by BrP-CoA ranged from 45% to 60% giving an average of 50%.

3.5 INHIBITION OF CPT I BY MALONYL-COA

Highly purified membranes were treated with 100 μM malonyl-CoA and used for measuring CPT activity as described in section 2.15.2. 100 μM Malonyl-CoA inhibited the highly purified outer membrane CPT activity by 67% when compared to untreated membranes (Table 3.6). When the membranes were solubilised with Tween 20 the malonyl-CoA inhibited the CPT activity in the remaining membrane pellet by 86%. The enzyme activity in the supernatant was not inhibited. In the comparison between the Tween 20 treated membranes and distilled water treated membranes, there was no notable difference in the results. It was noted that if the rats, from which the membranes had been prepared, had been starved for 48 hours, the maximum binding of the malonyl-CoA was increased, (Kolodziej and Zammit, 1990a) but the inhibitory effect of malonyl-CoA was reduced by 40% (Table 3.7).

3.6 EFFECTS OF DETERGENTS ON CPT I ACTIVITY

The following detergents were all tested to determine their inhibitory effects on the activity of CPT I as described in section 2.14.1. These tests were performed as preliminary work before solubilisations were performed. If the detergents showed adverse effects on the activity of CPT I then, regardless of their solubilisation properties, they would be useless in the purification of the enzyme. Tween 20, octyl glucoside and Mega 8 produced relatively low levels of inhibition of CPT I activity at all concentrations with 40 mM Tween 20 producing negligible inhibition. CHAPS almost totally inhibited the CPT I activity even at the lowest concentration which was tested (10 mM). Mega 10 also caused total inhibition, but was only semi-soluble in low ionic strength buffers such as KCl and KPi (Table 3.8).

3.7 SOLUBILISATION OF CPT I BY DETERGENTS

Having established the inhibitory profile of the detergents the next stage was to determine whether the detergents had solubilised CPT I from the membranes. This was carried out as described in section 2.14.2.

Table 3.6. Inhibition of CPT activity in Tween 20 treated rat liver mitochondrial outer membranes by 100 μM malonyl-CoA

	control	+malonyl-CoA	% inhibition
supernatant	25	26	0
pellet	78	11	86

Mitochondrial outer membrane fragments were prepared from fed rats as described in section 2.11 and assayed for malonyl-CoA inhibition of CPT activity (Section 2.15.2). Tween 20 solubilisation of the membranes (Section 2.14.2), was carried out inorder to extract all CPT II activity which is malonyl-CoA insensitive (McGarry and Foster, 1980). The results shown here are an average of three experiments where the variance of malonyl-CoA inhibition was no more than 8%.

Table 3.7 Inhibition of CPT activity in Tween 20 treated rat liver mitochondrial outer membranes, prepared from 48 hour starved rats, by 100 μM malonyl-CoA

	control	+malonyl-CoA	% inhibition
supernatant	7	8	0
pellet	85	49	42

Mitochondrial outer membrane fragments were prepared from starved rats as described in section 2.11 and assayed for malonyl-CoA inhibition of CPT activity (Section 2.15.2). Tween 20 solubilisation of the membranes (Section 2.14.2), was carried out inorder to extract all CPT II activity which is malonyl-CoA insensitive (McGarry and Foster, 1980). The results shown here are an average of three experiments where the variance of malonyl-CoA inhibition was no more than 6%.

Table 3.8 The Effect of Detergents on CPT I Activity as reported by dpm of sample and percentage inhibition from control

Blank : 129 dpm Control : 6610 dpm

	OG		CHAPS		MEGA 8		MEGA 10		Tween 20	
Conc (mM)	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
40	296	95	140	98	3575	46	721	89	5948	8
20	4148	37	90	98	5473	17	295	95	5125	20
10	5858	11	65	99	3877	41	712	89	5563	14

These results are typical of five experiments to determine the effect on CPT activity of the presence of detergents.

Table 3.9 Solubilisation of CPT I by Detergents as Determined by Activity (dpm)

Control Supernatant : 244 dpm

Pellet : 5303 dpm

	OG		CHAPS		MEGA 8		Tween 20	
Conc (mM)	sup.	pel.	sup.	pel.	sup.	pel.	sup.	pel.
40	171	123	213	180	532	2584	171	4832
20	1026	484	194	169	179	5107	109	4789
10	511	1023	304	458	153	5509	98	4874

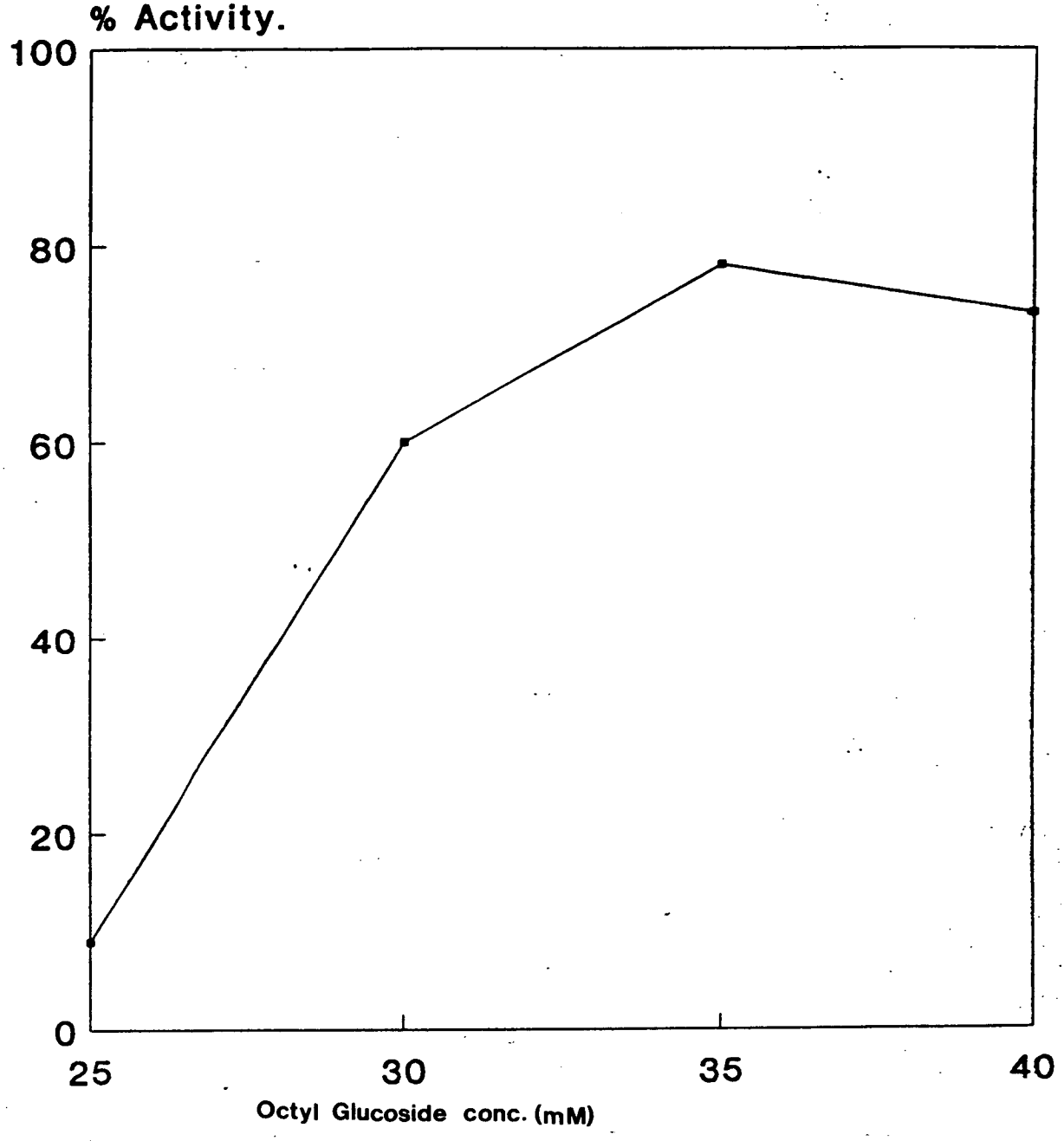
These results are typical of numerous experiments to determine the solubilisation effect of the detergents on CPT I from rat liver mitochondrial outer membranes.

From Table 3.9 it can be seen that Tween 20 and Mega 8 had little solubilisation effect on CPT I at any of the concentrations used. CHAPS (20 and 40 mM) however solubilised the enzyme but caused a total loss of activity in the supernatants. Octyl glucoside (20 and 40 mM) solubilised the enzyme and supernatants produced after treatment with 20 mM octyl glucoside contained some enzyme activity. The concentration of octyl glucoside was varied and it was found that 35 mM octyl glucoside in 500 mM KCl was the concentration which caused optimal solubilisation, while still retaining CPT I activity in the supernatant (Fig. 3.2). It was also noticed that there was a reproducible loss of total CPT activity in octyl glucoside treated outer membranes (i.e. supernatant plus the respective pellet) irrespective of octyl glucoside concentration. This inhibition was in the order of 62% when compared to the control, which in turn was only 42% of the original membranes before treatment. Using the [^{3}H]-carnitine to detect solubilisation it was found that under the conditions described by Murthy & Pande (1987b), 35 mM octyl glucoside in 500 mM KCl, 60% solubilisation could be obtained.

3.8 PURIFICATION OF CPT I FROM OUTER MEMBRANES

The aim of this experiment was to identify and locate CPT I by following the radioactivity in a complex with a labelled ligand on a non-denaturing polyacrylamide gel, made with trimethyl sulphone (sulphalane) (Vecchio *et al*, 1984). The relative mobility of the complex (Rf) could be calculated and the appropriate band cut from an identical acrylamide gel. This protein could then be run on a SDS polyacrylamide gel along with molecular weight standards giving an estimate of the molecular weight of CPT I. An outer membrane preparation was solubilised in Tween 20 to remove any residual CPT II activity. It was then mixed with BrP-CoA and 12.5 μM L-carnitine ($\pm$ 100 μCi [^{3}H]-carnitine) before being solubilised twice with 35 mM octyl glucoside in KCl (500 mM). Polyethylene glycol (PEG) was added to the combined supernatants to a final concentration of 20%. This was stored on ice for 20 minutes before centrifugation at 100000 x g for 1 hour (Beckman Air-fuge). The protein pellet was then resuspended in 50% sulphalane to give an approximate protein concentration of 1 mg/ml.

Fig 3.2 CPT I activity in the supernatant of octyl glucoside solubilised rat liver mitochondrial outer membranes



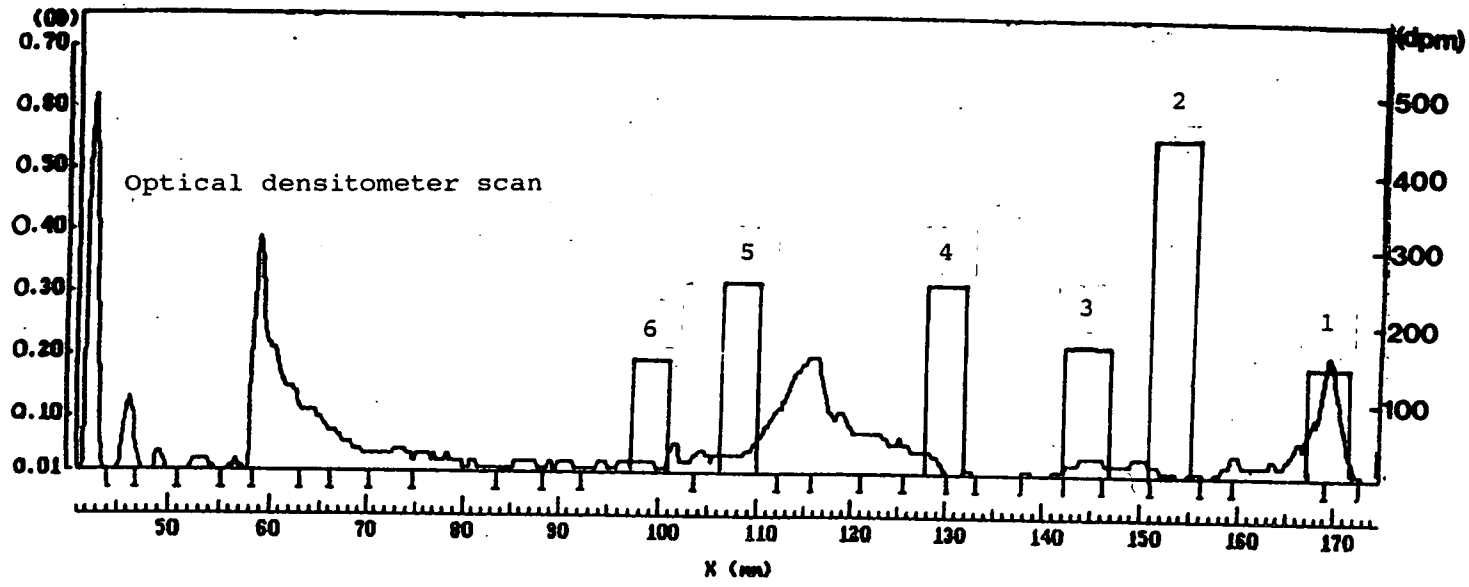
Membranes, devoid of CPT II activity (Section 3.2), were solubilised as in section 2.14.2. The membranes were then centrifuged at 100000 X g in a Beckman Air-fuge and the supernatants assayed for CPT activity (Section 2.12.4). The protein concentration of the membranes was 2 mg/ml and the highest concentration of octyl glucoside that caused solubilisation while retaining significant CPT activity was 35 mM.

This sulphalane suspension was then centrifuged at 8000 x g for 2 minutes (Beckman Air-fuge) to remove any particulate matter still present. The sulphalane suspension was then divided into two and run on a 5% sulphalane polyacrylamide gel (25 V for 8 hours at 4°C). The gel and its buffers were prepared as normal, (section 2.28) but sulphalane was substituted for SDS. After electrophoresis the gel was cut into two halves, one of which was stained for protein (Fig 3.3). The other half was cut into 120 slices each 2 mm wide. The slices were dissolved in 200 µl of soluene, then placed in 10 ml of toluene and the amount of bound [^{3}H]-carnitine in each slice was measured in a scintillation counter (Table 3.10).

3.9 DIALYSIS OF SOLUBILISED MEMBRANES TO ENSURE THAT SOLUBILISED CPT IS STILL BINDING [^{3}H]-CARNITINE

During the solubilisation of CPT I with octyl glucoside it was noted that most of the [^{3}H]-carnitine was being released from the enzyme. This was probably due to denaturation of CPT I. [^{14}C]-Glucose was introduced to the assay mixture in order to confirm that the CPT I was binding the BrP-CoA and [^{3}H]-carnitine. Glucose was used since it is of similar size to carnitine but should not be bound specifically to the membrane and will therefore give a control rate of dialysis of non-bound substrate. The membranes were then dialysed as described in section 2.16. The rates of dialysis of the two labelled compounds were measured. The results showed that a large proportion of the carnitine was in fact non-specifically associated with the membrane because the [^{3}H]-carnitine dialysed off the membranes at the same rate as the [^{14}C]-glucose (Fig. 3.4). Therefore carnitine did not remain specifically bound to CPT I during solubilisation.

Fig 3.3 Optical densitometer scan of non denaturing polyacrylamide gel elect.



Mitochondrial outer membrane fragments from starved rat livers (Section 2.11), incubated with BrP-CoA and 12.5 μM L-carnitine ($\pm$ 100 μCi [^{3}H]-carnitine) (Section 2.15.3) were solubilised twice with 35 mM octyl glucoside in KCl (500 mM) (Section 2.14.2). Polyethylene glycol (PEG) was added to the combined supernatants to a final concentration of 20%. This was stored on ice for 20 minutes before centrifugation at 100000 x g for 1 hour (Beckman Air-fuge). The protein pellet was then resuspended in 50% sulphalane to give an approximate protein concentration of 1 mg/ml. This sulphalane suspension was then centrifuged at 8000 x g for 2 minutes (Beckman Air-fuge). The sulphalane suspension was then devided into two and separated on a 5% sulphalane polyacrylamide gel. The gel and its buffers were prepared as normal, (section 2.28) but sulphalane was substituted for SDS. The gel was cut into two halves, one of which was stained for protein with page blue and scanned on an optical densitomiter.

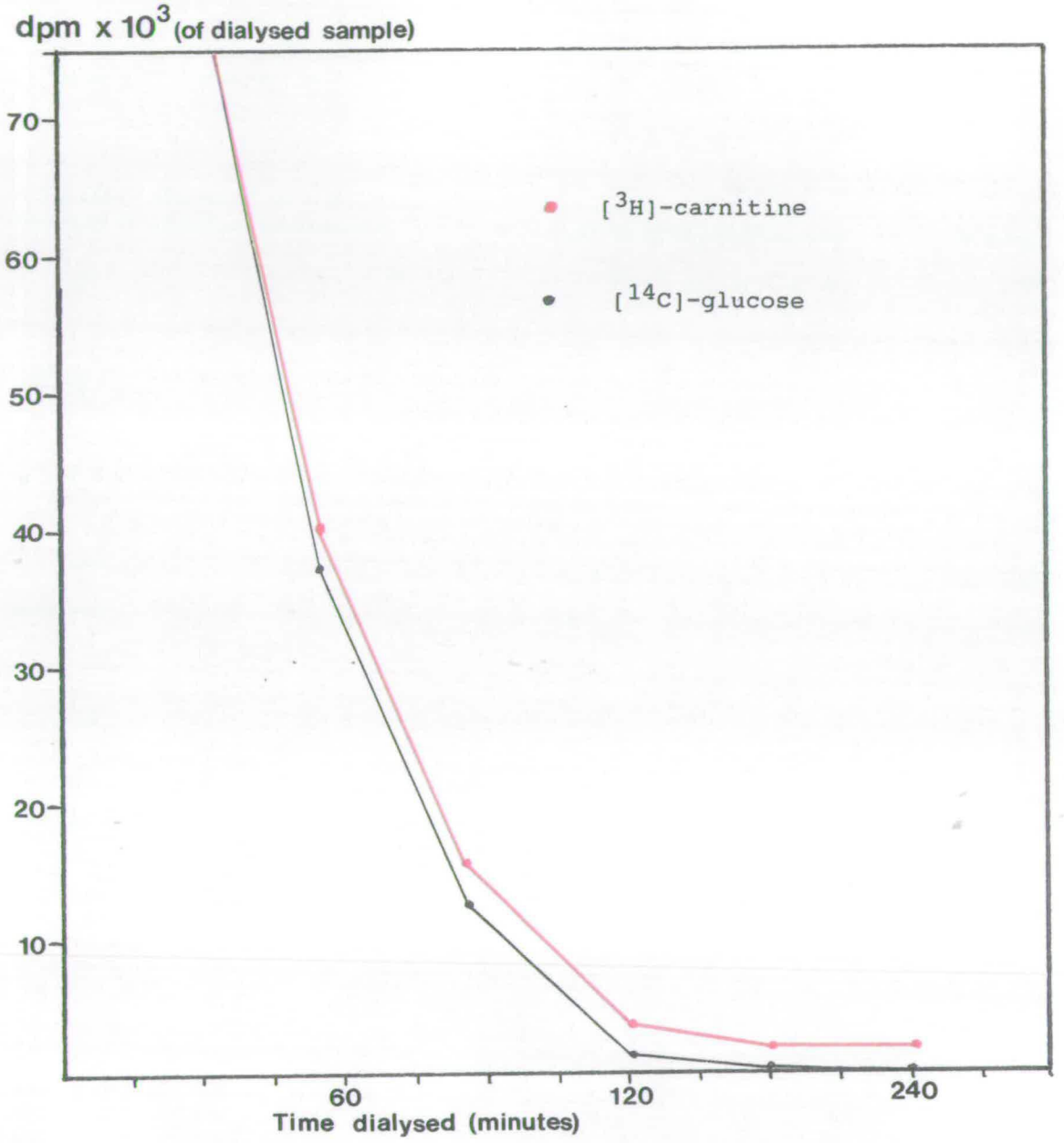
The other half was cut into 120 slices each 2 mm wide. The slices were dissolved in 200 μl of soluene, then placed in 10 ml of tolulene and the amount of bound [^{3}H]-carnitine in each slice was measured in a scintillation counter (Table 3.10). The radioactive peaks from Table 3.10 (1 to 6) have been superimposed onto the scan (right hand dpm scale).

Table 3.10 Rf values of gel slices with dpm significantly above control

	Rf value.	dpm
1	0.92	151
2	0.80	446
3	0.75	215
4	0.68	241
5	0.57	244
6	0.52	148

The results reported here are from one experiment which was unreproducible. The dpms reported are above background, but not significant enough to represent large quantities of [^{3}H]-carnitine bound by CPT I protein. For superimposition over the protein gel see Fig. 3.3.

Fig. 3.4 Rates of dialysis of both [^{3}H]-carnitine and [^{14}C]-glucose from bromo-palmitoyl-CoA treated rat liver mitochondrial outer membranes



Mitochondrial outer membrane fragments from starved rat livers (Section 2.11) were incubated with bromo-palmitoyl-CoA (BrP-CoA) and [^{3}H]-carnitine (Section 2.15.3), along with [^{14}C]-glucose (Section 3.9). The membranes (100 μl) were then dialysed using a micro dialyser (section 2.16). Samples were taken after periods of dialysis, counted in a scintillation counter and graphed as above. The total volume was measured at each sample time, allowing the total number of counts to be calculated for the sample.

3.10 DIGESTION AND N-TERMINAL AMINO ACID SEQUENCING OF PEPTIDES IDENTIFIED BY THE COVALENT MARKER 2-TETRADECYLGLYCIDYL-COA (TDG-COA) (Carried out in collaboration with Marian Koldziej, Hannah Research Institute, Ayr).

Kiorpes *et al* (1984), identified 2-tetradecylglycidyl-CoA (TDG-CoA) as an irreversible inhibitor of CPT I. It was later shown to also inhibit malonyl-CoA binding (Declerq *et al*, 1987). The authors therefore suggested that TDG-CoA bound to the same CPT I regulatory site as malonyl-CoA. If, therefore, the malonyl-CoA binding site for CPT I regulation, resides on CPT I itself, then protein purification from outer mitochondrial membrane fragments labelled with TDG-CoA could allow the purification of CPT I.

Using this principle Kolodziej *et al* (1992), partially purified an 84 kDa TDG-CoA binding protein. They raised antibodies to this protein and reported that they could immunoprecipitate CPT activity with the sera and therefore suggested that the protein was CPT I.

The TDG-CoA binding protein was subjected to N-terminal amino acid sequencing by Bryan Dunbar (Aberdeen University, Marischal College, Aberdeen). The N-terminal sequencing of the partially purified protein was unsuccessful because the protein was N-terminally blocked. This is a common problem in protein sequencing and is usually caused during the purification of the protein. Known blocking agents such as spectrophotometrically impure acrylamide can be avoided, but, other blocking agents are more specific to the protein being purified. Attempts to sequence the TDG-CoA bound protein were not reproducible and on occasions appeared to be derived from albumin which had been added during the purification (Table 3.11).

Attempts were made to digest the TDG-CoA binding protein with an endoproteinase (section 2.13.2.1). Care had to be taken during the digestion of the TDG-CoA binding protein with the endoproteinase because it was unstable and prone to rapid degradation. The digests of the TDG-CoA binding protein were carried out by M. Kolodziej, (Hannah Research Institute, Ayr) and myself. The enzymes used were, endoproteinase Asp-N (*Pseudomonas fragi* mutant) and endoproteinase Glu-C (*Staphylococcus aureus* V8).

Table 3.11 Sequences obtained from N-terminal amino acid sequencing of a 84 kDa TDG-CoA binding protein

Cycle	A	B
1	Thr	Ile/Lys
2	-	Val
3	Lys	Glu
4	Ser	Ile
5	Glu	Ala
6	Ile	Leu
7	Ala	Asp
8	Ala	Phe
9	Phe	Lys
10	Lys	Gly
11	-	Leu
12	Leu	Gly
13	-	Glu
14	Glu	Ile
15		Ala
16		Phe
17		Pro
18		-
19		Leu
20		Val
21		Leu
22		-
23		Phe

Table 3.12 The N-terminal amino acid sequences of peptides produced from an 84 kDa TDG-CoA binding protein after digestion with endoproteinase Asp-N at 37°C.

Cycle	A	B
1	-	Gly
2	Glu	Gly
3	Gln	-
4	Ala	-
5	Val	Glu
6	Ala	-
7	Phe	Val
8	Glu	-
9	Val	Glu
10	Val	Phe
11	-	Phe
12	-	
13	Asp	
14	-	
15	Ile	

Table 3.13 Sequences obtained by N-terminal amino acid sequencing of V8-endoproteinase fragments of a 84 KDa TDG-CoA binding protein

25 kDa fragment	15 kDa fragment	10 kDa fragment	42 kDa fragment	20 kDa fragment
Thr	Met	Leu	∇ Gln •	Pro/Val
Δ Gln	Leu	Phe	∇ Gln •	Ile
Δ Gln	Tyr	Asp	∇ Met •	Tyr
Δ Ala	Ile	Phe	∇ Gln •	Ala
Δ Val *	Thr	Glu	∇ Gln •	Ser
Δ Ala *	Pro	Lys	∇ Ile	Leu
Δ Phe *	Thr	Asn	∇ Leu	Ser
Δ Gln *	Asp	Pro	∇ Asp	Ser
Δ Phe *	Ile	Asp	∇ Asp	Ala
Δ Thr *	Gln	Tyr	∇ Pro	-
Δ Val *	Ala	Val	Ser	Leu
Δ Thr *	Ala	Ser	Glu	Leu
Δ Pro	Arg	Arg	Pro	Ala
Asp	-	Gly	Gln	Asn
Gly	Gly	Gly	Gly	Asp
Ile	Asn	Gly	Gly	Pro
Gln	Thr	Phe	Glu	Pro
Leu	Ile	Val	Ala	Pro
Gln	His	Ala	Lys	Pro
Gly	Ala	Asp	Lue	
Lys	Ile		Ala	
	Leu		Ala	
	Arg		-	
	Tyr		Gly	
	-		-	
	Arg		Ala	
			Asp	
			Arg	
			Val	

Digests of the TDG-CoA binding protein with Asp-N were carried out at temperatures between 22°C and 37°C (Noreau and Drapeau, 1979; Drapeau, 1980). These yielded peptides that were only just readable above background on the amino acid sequencer. The derived amino acid sequences were not reproducible on repeat experiments (Table 3.12).

The only enzyme that proved to be successful in the production of peptides suitable for sequencing was V-8 endoproteinase, using the method reported by Cleveland *et al* (1977), (M. Kolodziej, Hannah Research Institute, Ayr). The optimum recommended incubation temperature of V8 endoprotease is 37°C. Successful incubations with V8 endoproteinase were carried out at 0°C for 1, 5, 10, 20 and 40 minutes. The digestion products were separated by electrophoresis using a 15% polyacrylamide gel. The peptides were transferred to nitrocellulose, lightly stained with amido black, excised and used for N-terminal amino acid sequencing. Although there were many different sized fragments generated, only 5 different amino acid sequences were obtained by the sequencer (Table 3.13).

3.11 POLYMERASE CHAIN REACTION

(Section 2.22.4)

Using the peptides marked by (*) and (•) in Table 3.13, oligonucleotides (of both sense and anti-sense orientations) were generated incorporating total codon degeneracy. Non-degenerate 'guessmer' oligonucleotides (section 2.8) were also made from the sequences marked (Δ) and (∇) in Table 3.13, using known rat liver codon usage.

A = adenine; T = thymine; C = cytosine; G = guanine;
R = purine (A or G); Y = Pyrimidine (C or T);
N = A,G,C, or T.

Degenerate oligonucleotides (*) & (•):

sense a: 5' "GTNGCNTTYCARTTYACNGT" 3'
anti-sense a: 5' "ACNGTRAAYTGRAANGCNAC" 3'

sense b: 5' "CARCARATGCARCARAT" 3'
anti-sense b: 5' "ATYTGYTGCATYTGYTG" 3'

Non-codon degenerate oligonucleotides (Δ) & (∇):

sense c: 5' "CAGCAGGCTGTGGCTTTTCAGTTTACCGTGACCCCA" 3'

anti-sense c:

5'"GCCATCTGGGGTCACGGTAAACTGAAAAGCCACAGCCTGCTG" 3'

sense d: 5' "CAGCAGATGCAGCAGATCCTGGATGATCCA" 3'

anti-sense d: 5' "TGGATCATCCAGGATCTGCTGCATCTGCTG" 3'

The templates used were from the following sources; rat liver cDNA libraries in λgt10 and λgt11; rat liver mRNA; rat liver cDNA; and rat liver RNA/DNA duplex. Although mRNA is not itself considered as a template for PCR, it has been shown that PCR of mRNA is very successful (Tse and Forget, 1990). This method is very effective, even when subnanogram amounts of total cellular RNA are used, and is thought to be possible because of reverse transcriptase activity of *Taq* polymerase.

All the PCR products which were obtained by the above methods, were excised and purified from a 2% agarose gel. They were then phosphorylated, blunt-ended and cloned into pTZ19 or pTZ18. Single stranded DNA was then prepared and sequenced using the universal reverse sequencing primer.

A total of 24 clones was isolated in this way none of which had sequences corresponding to fragments of the malonyl-CoA binding protein (section 3.10). Positive identification can be made only when predicted amino acid sequence of the PCR product is identical to the amino acid sequence of the malonyl-CoA binding protein, beyond the sequence to which the oligonucleotide primer was generated.

When the λ cDNA was used, all of the PCR products were identified as stretches of out of phase λ DNA. In the other cases the clones were not necessarily identified (sequences unidentified by known sequences in data banks) but were not the malonyl-CoA binding protein. Fresh preparations of rat liver mRNA were prepared from 48 hour starved animals in order to increase the level of expression of CPT I and the malonyl-CoA binding protein. New clones were generated but were positively identified as non CPT I clones.

The conditions of the PCR reactions were also varied for all the different templates used. The temperature of annealing was varied between 32°C and 60°C as a control for non-specific binding, but this

did not affect the number of false positives being amplified simply because the primers, being heterogeneous, were binding to 100% complementary stretches of DNA, and therefore would not dissociate before true positives. Abundance of the target cDNA and distance between the two primers would then play a significant role in determining which products would be amplified. In this case the large abundance of multiple products from the primers swamped any true clones being amplified.

Extension time was also varied as the distance between the primers was unknown. The limit of *Taq* polymerase is known to be around 2 kb of DNA per 2 min extension time. This, however, is an optimum figure achieved in ideal conditions and does not include ramp times (the time taken for the temperature control unit to reach the desired temperature, in this case 72°C). It was found in control experiments using primers to amplify a 1.5 kb DNA fragment that the yield could effectively be doubled by increasing the extension time from 2 minutes to 5 minutes. For this reason extension times of 2-10 minutes were used. The magnesium ion concentration was varied from 0.5-3.0 mM. The magnesium ion concentration is fundamental to the specificity of the *Taq* polymerase and although at higher concentrations it can increase the amount of amplification dramatically, it can also increase the amount of non-specific amplification to the detriment of primer directed extension. These alterations of conditions still did not give rise to any sequence related to the malonyl-CoA binding protein. The presence and absence of gelatin in the reaction mixture was also a factor which was varied. This did increase the yield of primer-directed amplification but did not give rise to a positive clone. This suggested that either the degree of promiscuity portrayed by the oligonucleotide primers was too great to successfully amplify a fragment encoding the malonyl-CoA binding protein or the distance between the primers used was too great for *Taq* polymerase to effectively amplify.

3.12 IMMUNOGENIC STUDIES ON THE TDG-COA REACTIVE PROTEIN

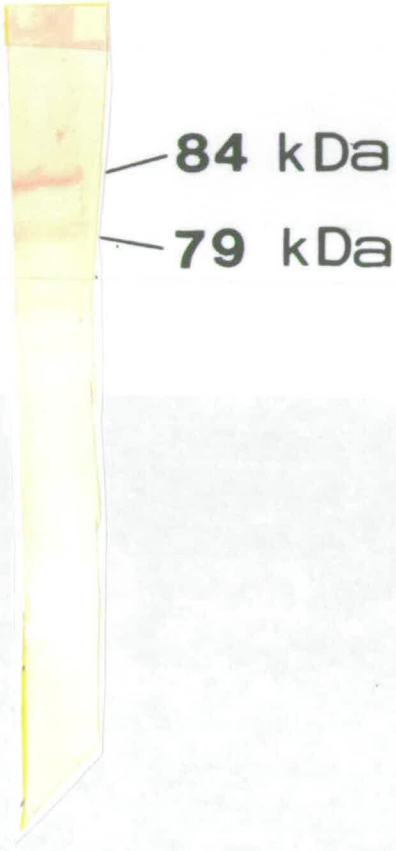
Aliquots of concentrated protein, eluted from gels similar to those used to perform the amino acid sequencing, were injected into a sheep. After 3 inoculations at 3 week intervals, immune serum was collected and IgG prepared (Kolodziej *et al* 1992).

Following affinity purification (section 2.31.3), the antibodies were checked for cross reactivity with rat liver mitochondrial outer membrane proteins. The affinity-purified antibody reacted strongly with an 88 kDa protein. There was also a weaker reaction with a 79 kDa protein (Fig. 3.5). The immune serum was checked for cross reactivity with rat liver mitochondrial inner membrane proteins, total rat liver mitochondrial proteins, rat liver peroxisomal proteins, yeast mitochondrial proteins and total yeast proteins. Only in total rat liver mitochondrial proteins was there an immunogenic reaction seen with an 84 kDa protein (Fig. 3.6). Cross reactivity was also checked against sera raised to the rat liver peroxisomal carnitine octanoyltransferase (COT), (gift from Dr R.R. Ramsay, Department of Biochemistry and Biophysics, University of California, San Francisco). There was no cross reaction of this serum with outer membrane proteins.

3.13 IMMUNOGENIC SCREENING OF A RAT LIVER CDNA LIBRARY IN λGT11, WITH SERUM RAISED TO AN 88 KDA TDG-COA BINDING PROTEIN

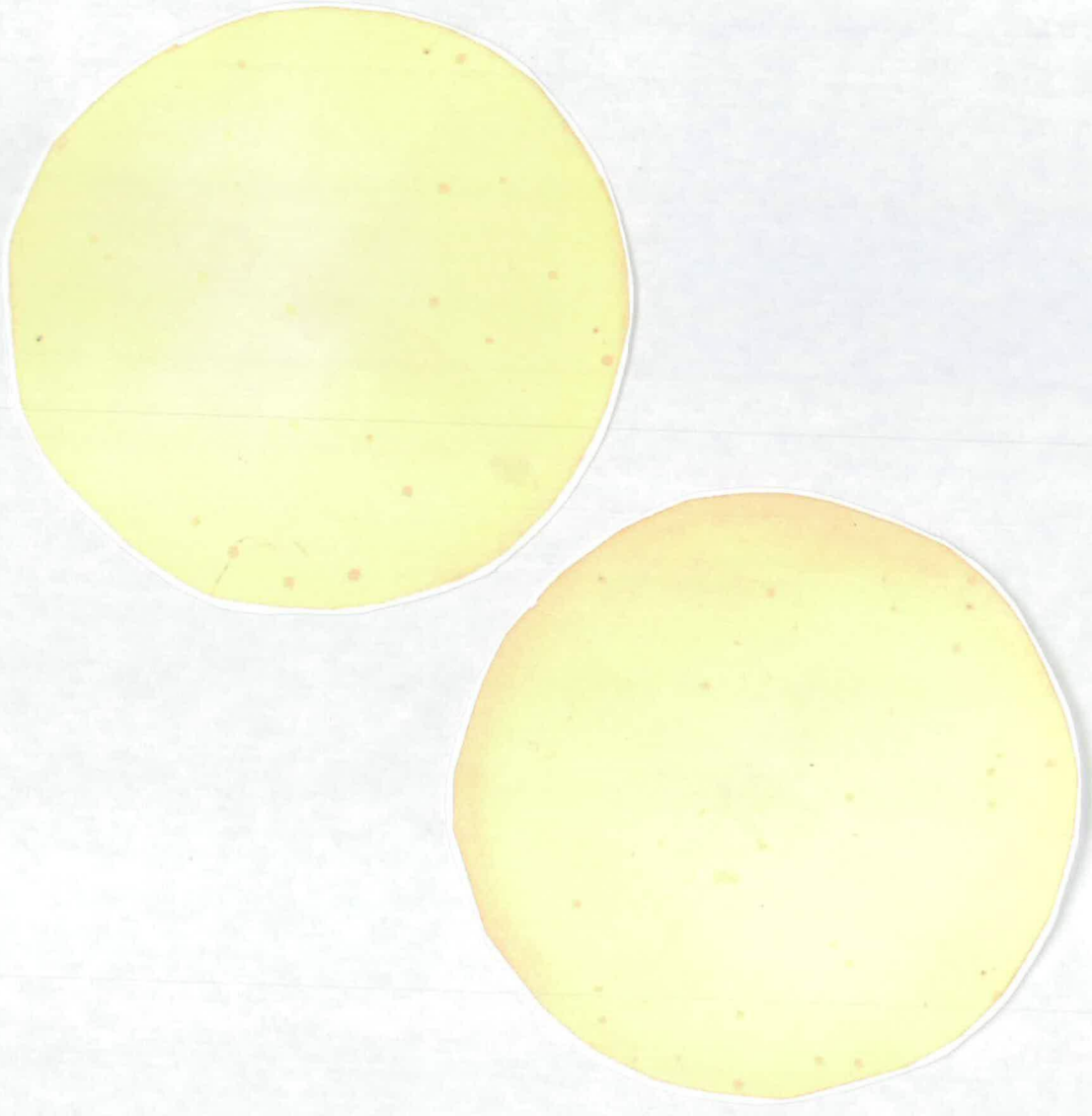
The affinity purified serum raised to the TDG-CoA binding protein was used to screen a λgt11 library (obtained from Clontech) as described in section 2.33.5. The positives, found from a primary screen at near confluent lysis, were picked with the large end of a sterile pasteur pipette. The plug was eluted in 1 ml of SM with 10 μl of chloroform, for 5-8 hours. The eluted phage were then plated out as before at a density of 10,000 pfu per 82 mm plates, and rescreened. This procedure was repeated 5 times until pure positives were obtained (Fig. 3.7).

Fig. 3.5 Immunological detection of 84 kDa protein from rat liver mitochondrial proteins, using affinity purified antibodies raised to a 84 kDa malonyl-CoA binding protein in rat liver mitochondrial outer membrane



Rat liver mitochondria, prepared from starved rats, were solubilised in 35 mM octyl glucoside (Section 2.14.2). Solubilised proteins (30 μg) were then separated by 10% PAGE (Section 2.28). The proteins were then transferred to a nitrocelulose membrane by western blotting (Section 2.29), blocked with 2% albumin (Section 2.30.1) and detected using horse radish peroxidase and dianisidine (Section 2.32.1). It can be seen that there are two reactions on the western blot. The strongest is around 84 kDa and is assumed to be either CPT I or its regulatory malonyl-CoA binding protein. The second reaction, although much weaker, is around 79 kDa.

Fig 3.7 Plaque purified λgt11 clone, detected by the affinity purified antibody raised to the 84 kDa malonyl-CoA binding protein from rat liver mitochondrial outer membranes



Plaque purification was carried out as described in section 2.33. The membranes shown are from a fifth round screen of two out of three identical clones. The titre of plaque forming units (pfu) were adjusted to fall between 50 and 150 pfu per 82 mm plate. The positive plaques are visible as darker circles on the nitrocellulose membranes. Plaques were picked as areas in primary screens and then as single colonies in later screens until, as depicted above, all plaques on the plates reacted positively with the antibody.

3.14 INDUCTION OF β-GALACTOSIDASE FUSION PROTEIN

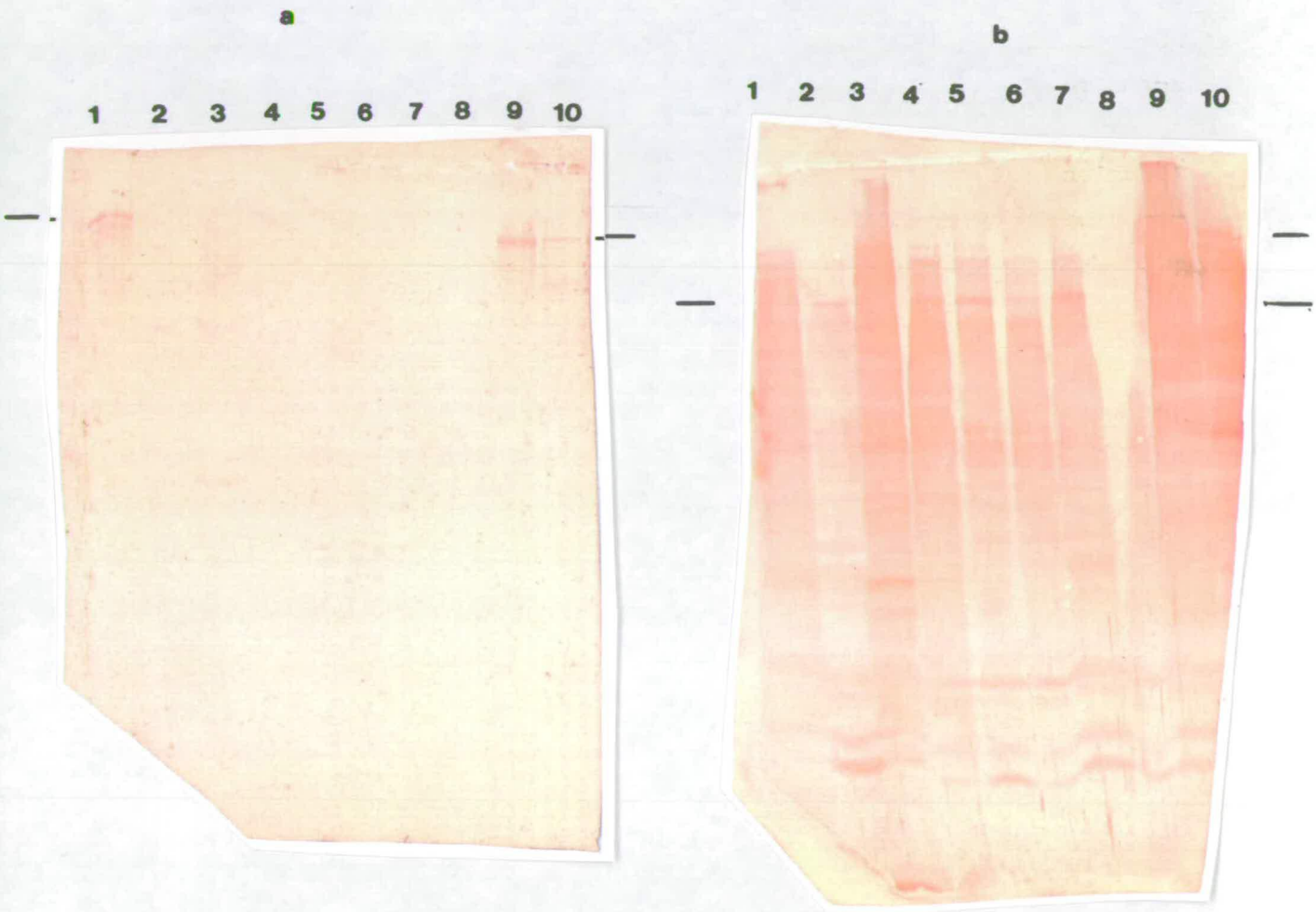
In order to prove that the insert encoded the protein responsible for the adsorption of the antibody, a method of induction had to be developed. This can be done by proving that the protein that is reacting with the serum is a fusion protein with β-galactosidase, the protein in whose gene the foreign DNA is inserted. This can be done by showing that the immunogenic protein can be induced for by isopropylthiogalactoside (IPTG), a non-metabolizable inducer of the lactose operon. This is usually done by inducing lysis of cultures infected with the λgt11 clone in the presence and absence of IPTG. Due to the sickly nature of the λgt11 clone, lysed cultures were unobtainable by normal procedures. Creation of a high frequency lysogen in *E.coli* strain Y1030 was also unsuccessful. Over 5,000 colonies were induced for lysogeny but all were negative. False high frequency lysogens were avoided by incubating the cells with a secondary λ, namely λ clear. This λ is deficient for the cI gene, which encodes the λ repressor protein. This means that λ clear always acts as a lysing agent unless another λ is also present which does contain an active cI gene. This would mean that on a large infection of λ clear only bacteria also containing the λgt11 clone already producing the λ repressor would be able to grow without lysing. Still no lysogens were produced. Therefore another method of induced and non-induced protein extraction had to be developed.

Plates were prepared for confluence as before but were not overlaid with nitrocellulose. Instead they were induced by spreading 20 mM IPTG (100 μl) over them carefully after a 4 hour incubation at 42°C. The plates were then incubated overnight at 37°C, before being extracted with protein loading buffer at 25°C for 4 hours on a rotating platform. The loading buffer was then drained, and aliquoted into microcentrifuge tubes. Samples of each aliquot were boiled for 5 minutes, spun in a microfuge (5 minutes, 15000 x g), and then the supernatants were run on a 10% polyacrylamide gel. The separated proteins were then transferred to a nitrocellulose membrane by western blotting, and tested for cross reactivity with both the screening serum and anti-β-galactosidase serum (Fig. 3.8). As seen from the western blots, the immunoreactive protein was induced by IPTG, and was visibly larger than the β-galactosidase protein.

Fig. 3.8 Isopropylthiogalactoside (IPTG) induction of both β-galactosidase from wild type λgt11 and β-galactosidase fusion protein in the λgt11 clone

a) Affinity purified antibody raised to rat liver mitochondrial outer membrane malonyl-CoA binding protein (84 kDa)

b) Antiserum raised to *E. coli* β-galactosidase



1, 9 & 10	λgt11 clone induced
3 & 8	λgt11 clone not induced
2, 5 & 7	Wild type λgt11 induced
4 & 6	Wild type λgt11 not induced

a; This western blot shows clearly the reaction between the affinity purified antibody and the induced λgt11 clones. Non-induced plates are devoid of detectable fusion protein.

b; The second western blot shows the reaction of anti-β-galactosidase antibodies, which react strongly with wild type λgt11 and faintly with the fusion protein on induced plates only.

This proved beyond doubt that the λgt11 clone contained a β-galactosidase fusion protein that reacted immunogenically with the affinity purified antibody raised to the malonyl-CoA binding protein.

3.15 CLONING OF THE DNA INSERT FROM THE λGT11 CLONE

λgt11 cDNA libraries are constructed by cloning cDNA inserts into a unique EcoR1 restriction site in the β-galactosidase gene. Therefore, when DNA is prepared from the λ clone, the inserts can be cut from the λ genome by digestion with EcoR1, and separated out on an agarose gel. The insert excised in this case, was estimated to be about 2.5 kb (Fig 3.9). This was then purified from the gel by Geneclean®, and cloned into the EcoR1 site in pTZ19 and pTZ18 to ensure that both orientations could be sequenced.

Single stranded DNA was made from pTZ19 by utilising the F1 origin of replication on the plasmid, along with M13KO7 helper phage. Single stranded pTZ19 DNA was hence incorporated into M13 phage heads. Single stranded DNA was then prepared as described in section 2.20.2).

3.16 SEQUENCING OF THE DNA INSERT FROM THE λGT11 CLONE

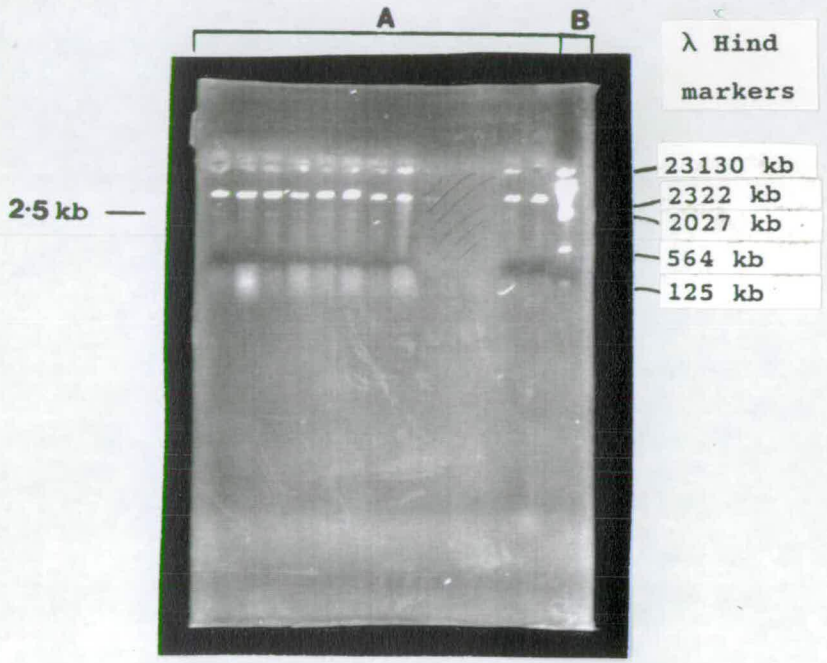
The single stranded pTZ19 DNA incorporating the λgt11 clone DNA insert, was sequenced using the reverse primer (section 2.23), situated at a site on the pTZ19 plasmid just behind the multiple cloning site. The insert was initially sequenced over the first 500 bp from both orientations (Fig. 3.10 and Fig. 3.11). This sequence was checked for similarity against known DNA sequence data bases. The result of this check was an identical match with the sequence encoding long chain acyl-CoA synthase, a gene from rat liver which had recently been cloned by Suzuk *et al*, (1990).

3.17 ANALYSIS OF RAT LONG CHAIN ACYL-COA SYNTHASE

In order to determine how best to sequence the cDNA clone, it was subjected to restriction endonuclease mapping (Fig. 3.12).

Once the true identity of the clone was discovered, no further sequencing was undertaken.

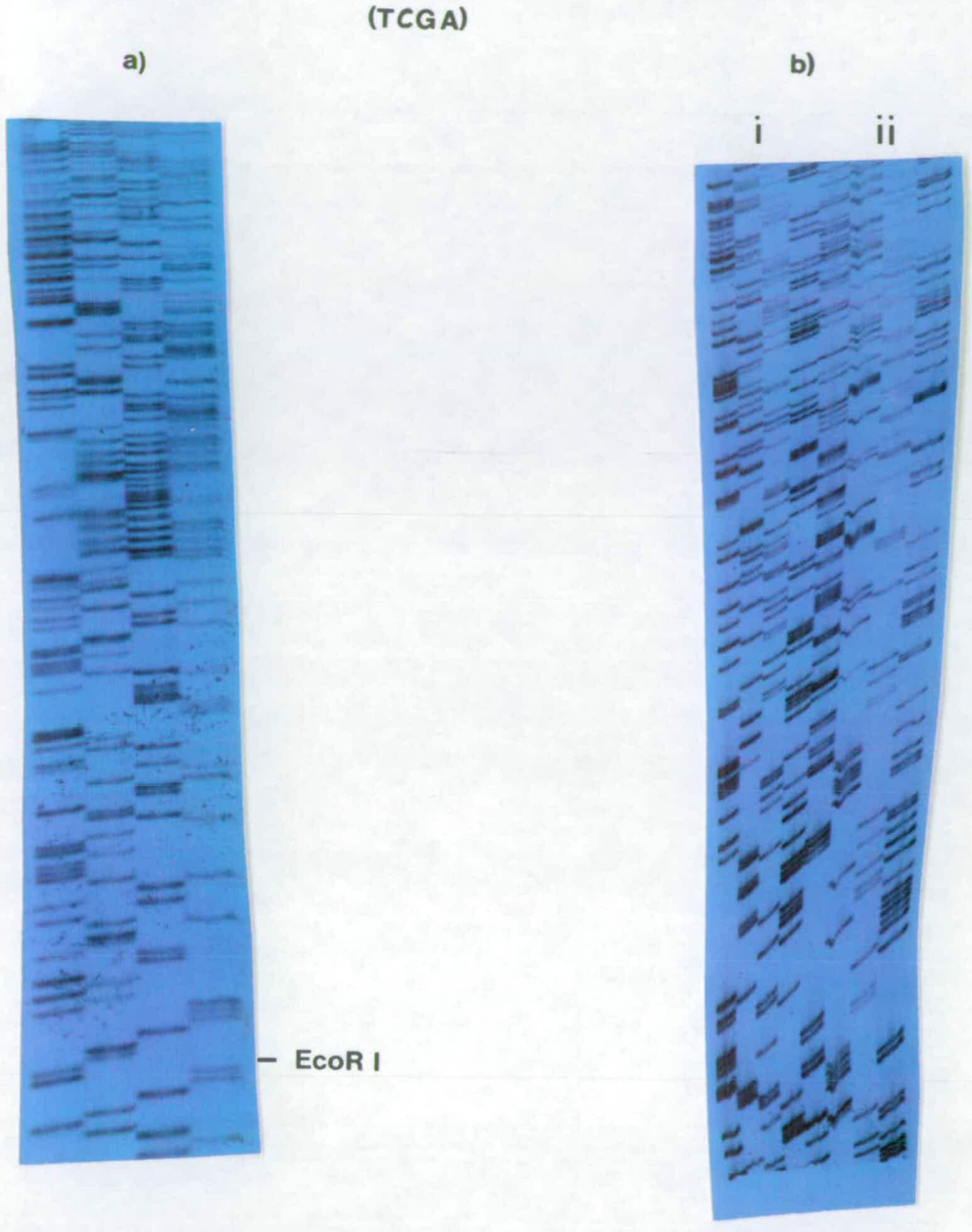
Fig. 3.9 EcoR1 digestion of DNA prepared from the λgt11 clone, obtained by isolation with the affinity purified antibodies raised to the malonyl-CoA binding protein from rat liver mitochondrial outer membranes



A ; EcoR I digested λgt 11 clone DNA
B ; λHind III markers

λDNA was prepared (Section 2.33.2) from recombinant λgt11 clones (Section 3.13) and digested with the restriction enzyme EcoR1. The digests were then separated on a 2% agarose gel (section 2.21.1) stained with ethidium bromide and visualised under ultra violet light. A fragment estimated to be around 2.5 kb was seen under the two unresolved λgt11 arms. All the tracks in A were prepared from the λgt11 clones and, where visible, gave rise to the same size of insert. Track B contains λHind III markers.

Fig. 3.10 Sequence data from the cloned 2.5 kb fragment of long chain acyl-CoA synthase



Sequence (a) shows the first orientation sequenced of the long chain acyl-CoA synthase, including the multiple cloning site of the pTZ plasmid up to the EcoR1 restriction site into which the cDNA was cloned. The sequencing was primed from the universal reverse primer.

Sequence (b) shows both orientations (i and ii) from inside the clone sequence which matched identically to the cDNA reported by Suzuki *et al* (1990).

Fig. 3.11 Alignment of sequence data form λgt11 clone with either end of the cDNA for long chain acyl-CoA synthase as reported by Suzuki *et al* (1990).

5' end

Initiation methionine

CCAACACAGA ACTATGGAAG TCCACGAATT GTTCCGGTAT TTTCGAATGC CAGAGCTGAT
TGACATTCGG CAGTACGTGC GTACCCTTCC AACCAACACA CTCATGGGCT TCGGGGCTTT
TGCAGCGCTC ACCACCTTCT GGTATGCCAC CCGGCCGAAG GCCCTGAAGC CACCATGTGA
TCTGTCCATG CAGTCTGTGG AAGTAACGGG TGCTGTACTA CTACGACGAT GTCAGAACGA
TGTACGATGG GACAAGCTCT TGCTGTACTA CTACGACGAT GTCAGAACGA TGTACGATGG
CTTCCAGAGG GGGATTCAGG TGTCAAATGA TGGCCCTTGT TTAGGTTCTA GAAAGCCAAA
CCAGCCATAT GAGTGGATTT CTTACAAACA GGTTGCAGAA ATGGCTGAGT GCATAGGCTC
GGCGCTGATC CAGAAGGGTT TCAAACCTTG CTCAGAGCAG TTCATCGGCA TCTTTTCTCA
GAACAGACCT GAGTGGGTGA CCATCGAGCA GGGGTGCTTC ACTTACTCCA TGGTGGTTGT
TCCGCTCTAT GACACGCTTG GAACCGACGC CATCACCTAC ATAGTGAACA AAGCTGAACT
CTCTGTCATT TTTGCTGACA AGCCAGAAAA AGCCAAACTC TTATTAGAAG ----------
---

Termination codon

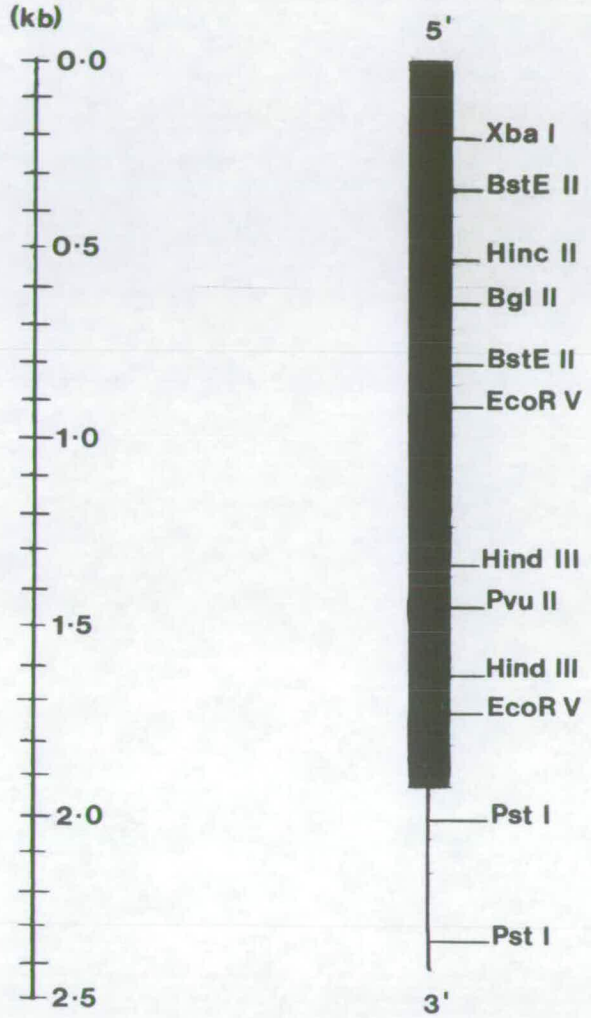
CATCAAGATT TAACATGAGG AAGAATCCTT AGAGGAAATG GCGCACCTCC ACAATCCTCC
TCGTACCAAT GGCCTTTGAG TTGGTAACTC TGCCTGCAGC GAGTGTGGGA AAGGAAATGC
CATGCTGGAC TTGTGCACAG GGTCTTACCA TAGGGATAGC AGAGGGCACG GAACACTGCC
TTACAGTCAC CTGTGTTGTA GCCCATGATC CTGGGGATAC ACAGTTTCCA AAACGAGCCT
TAAACATTGT AAAGGGGAAC CCATAAAAGT GCTAAGTTAT TTAAGACTTC AACCAATAAG
GTGGGTGTTA AAACTTCTGT CTCCTGTTTT TCTAACCAAG GGGTTAGGAC TTACTCTTTC
TGATACGTCT GCTGCTGGCT GCACACCCTG CAGCCGTCTG CTGCTCTGAA GAGTACAGTA
CACTGGAAGA AAGCTACCCC TTTAAGAGCA ACTGTCCAGG CTGAAGAAAG TCACAGTGGA
CCAGAGATTT CTCTTCTTAA CCCCTTCCCC TTGTCCCTTT CCCTCACCAC TCACATGCAT
TACATTTATG ----------

---An

3' end

The underlined bases represent the sequenced cDNA from either end of the λgt11 clone isolated using the affinity purified antibody raised to the TDG-CoA binding protein in rat liver mitochondrial outer membranes. The sequence matches the long chain acyl-CoA synthase sequence identically. The λgt11 clone was not full length and lacked 165 bases from the 5' end, which included the initiation methionine, and 1065 bp from the 3' untranslated sequence.

Fig. 3.12 Restriction map of cDNA isolated from λgt11 clone subsequently identified as a 2.5 kb fragment of long chain acyl-CoA synthase (Suzuki *et al*, 1990)



The cDNA clone was excised from the λgt11 bacteriophage and subjected to restriction endonuclease mapping. It should be noted that this map differs from the one reported by Suzuki *et al* (1990) since it is not full length. This was performed in order to plan a sequencing strategy for the TDG-CoA binding protein which was not undertaken once the sequence was positively identified as long chain acyl-CoA synthase.

3.18 DISCUSSION

Most of the described experimental procedures used for the purification of CPT I have relied primarily on the purity of the outer membrane preparation. Several groups have attempted similar work with contradictory results but this can often be attributed to contamination due to the presense of microsomes, mitochondrial inner membranes, or light mitochondria which are known to be heavily contaminated with peroxisomes. The CPT II and COT contamination in these respective organelles would swamp all CPT I activity and results could be misinterpreted. In the method used here (section 2.11), extreme care was taken, especially through the first few stages, to ensure accurate differential centrifugation of the mitochondria. The use of protease inhibitors was found to reduce the final yield of outer membrane in the preparation.

The Sprague-Dawley rats were starved for a period of 48 hours, (the legal limit for starvation of rats) in order to increase the amount of CPT activity present in the rat liver mitochondrial outer membrane. Starvation also had the effect of de-fatting the membrane preparation, hence increasing both the quality and the yield of CPT rich outer membrane fragments. The Percoll stage was designed to ensure the separation of the heavy from light mitochondria thus ensuring the absence of peroxisomes which are known to contain COT activity (60 kDa protein). COT is identical over some 25-30% of its residues with CPT II, and allowing for conservative amino acid changes has a homology of 50% (Chatterjee *et al*, 1988, Woeltje *et al*, 1990b).

The final stage in which the cytochrome c sepharose was prepared proved to be very variable and was vital in the quality of the final preparation. The only indication as to the quality of the activated sepharose was indicated by the purification results of the outer membranes. At all stages, in the purification of the outer membranes, samples were taken and assays performed to evaluate the degree of purification at each stage. Tables 3.1 - 3.4 outline the varying stages of purification. A bad batch of activated sepharose could result in a 30 to 60% loss in reactive protein and an irretrievable loss in quality. At this stage there is no further purification of CPT I from CPT II and therefore it could conceivably be left out without any marked effect on the purification of the preparation.

Such membrane preparations could be treated with Tween 20, in order to remove any CPT II protein, and then could be used for CPT I specific work. This method proved to be very successful and was used routinely after comparisons showed no significant difference from the activated sepharose method, apart from a marked increase in reliability.

The effect on CPT I, by starvation of the rats, was reported by Kolodziej and Zammit (1990a). They found as reported in Tables 3.6 and 3.7 that starvation diminished the sensitivity of CPT I to malonyl-CoA. The number of malonyl-CoA high affinity binding sites however increased almost 2 fold. This increase was very similar to that observed for specific CPT activity in outer membranes. In outer membrane preparations from starved rats, the maximum inhibition was 60%, as opposed to outer membrane preparations from fed rats which gave a maximum inhibition of 90%. No further increase in malonyl-CoA concentration could result in any further loss in CPT I activity. From this they postulated that there were other factors governing the activity of CPT I such as membrane fluidity. Membrane fluidity would be altered dramatically as the fatty acid content of the membrane decreased, or by changes in its cholesterol content. The fluidity was altered by using benzyl alcohol, isoamyl alcohol and 2-(2-methoxyethoxy)ethyl-8-(*cis*-2-n-octylpropyl)octanoate. All of these were shown by Kolodziej and Zammit (1990b), to have the affect of causing a decrease in malonyl-CoA inhibition of CPT I. They also suggested that the effect of increasing membrane fluidity on the malonyl-CoA sensitivity of CPT I may occur independently from other effects which act more directly with the active site.

In rat liver mitochondrial outer membrane preparations, the purity of CPT I with respect to CPT II was vitally important for the radiation inactivation studies performed by Zammit *et al* (1988); Zammit *et al* (1989). This method relied on the difference in CPT activity before and after irradiation for an estimation of the molecular size of the protein. These results can be linked to size since the larger the protein is, the more likely it is to be hit and inactivated by radiation. If CPT II was present (69 kDa protein), the activity after irradiation would be artificially higher than if all the CPT activity were due to CPT I (estimated 84 kDa protein). This would cause the weight of CPT I to appear considerably lower than it

actually is. The initial work performed on CPT I suggested an apparent molecular weight of 96 kDa, (Zammit *et al* 1988). In later studies however this was corrected to be approximately 84 kDa (Zammit *et al* 1989). The initial anomaly was most probably due to other impurities in the preparation of the outer membranes, hence as the purity of the preparations increased the accuracy of the estimation also increased. This method can be very accurate for some proteins but inaccurate for others. Zammit *et al*, (1988) also reported that radiation inactivation of CPT II gave an estimated molecular weight of 69 kDa. This size has been confirmed by sequence determination (Woeltje *et al*, 1990b).

In order to purify CPT I a method of detecting the protein had to be developed. CPT I is an integral membrane protein and is likely to be a very hydrophobic protein. It may even rely on specific phospholipid groups for activity (Sire *et al*, 1983). This would explain why extracting CPT I from the membrane resulted in a major loss of activity. The use of mild, non-ionic detergents was considered the obvious next choice. If CPT I could be extracted from the membrane without being denatured then perhaps activity, or at least some activity could be retained. The detergents were first tested for any inhibitory effects. The only detergent to show both a high degree of solubilisation while still retaining activity was octyl glucoside. The amount of activity retained was concentration dependent and a very fine balance had to be achieved (2 mg protein and 35 mM octyl glucoside in 500 mM KCl). It was also noted that once solubilised the activity was lost very quickly. Almost total loss of activity occurred after 8 hours at 4°C. Lund (1987), reported that two CPT activities could be separated by solubilisation. He used Triton X-100 (5% w/v), to solubilise a malonyl-CoA insensitive CPT, and then 30mM octyl glucoside to solubilise a malonyl-CoA sensitive CPT. In order to detect malonyl-CoA sensitive CPT activity, all detergents had to be removed. Even then activity was lost rapidly in its solubilised form. He also reported that activity could be partially protected by adding large quantities of glycerol but not to the extent that would allow further purification. For these reasons further purification of CPT I was very limited. Similar results were reported by Murthy and Pande (1987b), although they claimed that

activity could be restored after reconstitution in liposomes.

During the acylation of the carnitine by CPT I, 2-bromo-palmitoyl-CoA and carnitine can be tightly bound (non-covalently) in the reactive site of the enzyme. This is presumed to be due to the self catalysed formation of the S-carboxy palmitoyl-CoA ester of carnitine on the active site of the enzyme (Chase and Tubbs, 1972). Because of its bi-dentate nature this slowly reversible inhibitor binds tightly to CPT I along with carnitine which when labelled with [^{3}H] was shown by dialysis to remain associated with the membranes. It was thought that although the protein would lose activity after solubilisation even with non-denaturing detergents, perhaps the associated inhibitors would give a more sensitive detection system and further purification could be achieved. Again the main priority was to keep denaturation of CPT to a minimum otherwise the unfolded protein would merely release the ligand. Again solubilisation with octyl glucoside was initially successful but limited by the short time that the ligand remained associated to the protein. It was reported by Vecchio *et al*, (1984) that after initial solubilisation with a stronger detergent a more non-denaturing detergent could be introduced. The detergent used by Vecchio *et al* (1984) was thiophene, tetrahydro-1, 1-dioxy sulphanoyl (sulphalane). This detergent although not powerful enough to extract integral membrane proteins from their hydrophobic environment could, once the proteins had already been extracted with octyl glucoside, be used to limit any further loss in the activity of the CPT I by forming micelles which still favour protein structure. This method proved to be partially successful with CPT I, but detection of protein associated radioactivity was lost completely after 12 hours at 0°C. This extraction method did not allow the detection of CPT I as a single band on a polyacrylamide gel. Ten proteins were detected on a polyacrylamide gel. The protein of interest could be limited to one of six labelled proteins by examining the radioactive counts (Fig. 3.3). Kolodziej *et al* (1992) tried to solve the problem of identification by using a covalently bound radioactive marker, 2-tetradecylglycidyl-CoA (TDG-CoA). This compound is inhibitory and binds to the enzyme irreversibly (Declercq *et al*, 1987). Radio-labelling with TDG-CoA followed by PAGE were used by Kolodziej *et*

al, (1992) to identify outer membrane proteins which react with malonyl-CoA. One of these proteins was estimated to have a molecular mass of 84 kDa. It was therefore thought that this protein could be CPT I since the radiation inactivation size analysis suggested that this was the size of CPT I (Zammit *et al*, 1989). The full protein, along with partial digests of the protein, were then N-terminally sequenced (section 3.10), and also used to generate polyclonal antibodies (section 3.12). Sequences of the fragments were used to generate heterogeneous and guessmer DNA probes, the latter of which were elucidated from known rat liver codon usage. These sequences were used to screen λgt10 and λgt11 libraries but no true positives were found.

Polymerase chain reactions were also performed under many different conditions, but all sequences found, although clearly derived from the added primer, did not encode the CPT I peptides beyond the primer sequence. Out of a total of 24 bands purified, sub cloned into pTZ18 or pTZ19 and sequenced, none were found that resembled the TDG-CoA binding protein fragments. Initially the PCR was carried out on DNA prepared from the cDNA λ libraries, but due to the heterogeneity of the oligonucleotide all PCR products cloned were different and often contained λ DNA sequences. RNA, RNA/DNA duplex and Clontech cDNA were all used as templates for PCR, but no sequences resembling the peptides were found.

The immunogenic studies with the sera raised from the TDG-CoA binding protein suggested that there is no cross reactivity with CPT II, or the peroxisomal equivalent. Using an antibody against the peroxisomal COT (section 3.12) no cross reactivity was seen with the outer membrane preparations. Kolodziej *et al* (1992), reported that the anti-TDG-CoA binding protein sera, when checked against heart, skeletal muscle, foetal liver, adult liver, kidney, white adipose tissue, brown adipose tissue, mammary gland and brain, strongly cross reacted with a 84 kDa protein in foetal liver, adult liver and kidney cortex. Faint adsorptions were also seen in heart, skeletal muscle and brain all with a 84 kDa protein showing what the authors described as a slight degree of immunogenic similarity between the CPT I of these tissues. Similar results were reported by Esser *et al* (1993a). Reports of CPT I active malonyl-CoA binding proteins in these tissues suggests that the proteins are of different molecular

size, (Woeltje *et al*, 1987), different kinetic characteristics for Km of their reaction with carnitine, (McGarry *et al*, 1983), and different malonyl-CoA inhibitions, (Saggerson and Carpenter, 1981). Kolodziej *et al* (1992), however, did not comment on a 79 kDa protein which reacted strongly with rat proteins from white adipose tissue, brown adipose tissue, mammary gland and adult liver, and to a lesser extent with heart, skeletal muscle and foetal liver. This cross reaction is of great importance when it is taken into consideration that the molecular weight of the protein encoded for by the cDNA insert purified from the λgt11 clone is 78 kDa. This protein, long chain acyl-CoA synthase, was isolated from the λgt11 library by the same affinity purified antibody which was used for the previous western blots (section 3.12). Long chain acyl-CoA synthase has been shown to be localised in the microsomes (Konberg and Pricer, 1953); outer mitochondrial membrane (Norum *et al*, 1966; Garland *et al*, 1970) and peroxisomal membrane (Shindo and Hashimoto, 1978; Krisans *et al*, 1980). This would suggest that the protein was present in the original purified outer membrane fragments, but would not explain the cross reaction with the affinity purified antibody from the 88 kDa protein assumed either to be CPT I or its regulatory subunit. In either case the antibody was generated against a malonyl-CoA binding protein which, if it is CPT I, may also bind long chain fatty acids. The long chain acyl-CoA synthase is as its name suggests, a long chain acyl-CoA binding protein and so perhaps would display some identity at the active site. But for such a strong reaction as that demonstrated in Fig 3.5, the long chain acyl-CoA synthase must have a far greater similarity to the malonyl-CoA binding protein than expected. Purified rat liver long chain acyl-CoA synthase from microsomes, mitochondria (Tanaka *et al*, 1979), and the peroxisomal fraction (Miyamoto *et al*, 1981), are identical with respect to molecular and catalytic properties. They have the same molecular weights, amino acid composition, substrate specificities and kinetic properties. Suzuk *et al*, (1990) reported that the cDNA for long chain acyl-CoA synthase lacked a classical N-terminal targeting sequence for either endoplasmic reticulum or mitochondria. They also reported that *in vitro*, the initial translation product had the same molecular mass as the purified enzymes. The authors concluded from this that the primary structure of the protein was devoid of any signal

sequence. There is also no apparent peroxisomal targeting sequence visible at the C-terminus. This protein, being more widespread and abundant that CPT I, would obviously be more abundantly expressed in a rat liver cDNA library, which if the cross reactivity was caused by a true protein similarity, would hinder further attempts to isolate CPT I in this manner. The possibility that long chain acyl-CoA synthase was in fact CPT I can be ruled out since the peptide sequences (Table 3.13) taken from the same protein from which the serum was raised, are not encoded for by the cDNA which encodes long chain acyl-CoA synthase.

CHAPTER 4

PURIFICATION AND CLONING OF CARNITINE PALMITOYLTRANSFEASE II

CHAPTER 4

Purification and Cloning of Carnitine Palmitoyltransferase (CPT) II

4.1 INTRODUCTION TO THE PURIFICATION AND CLONING OF CARNITINE PALMITOYLTRANSFERASE II

It was originally thought, and indeed is still adhered to by some (Kerner and Bieber, 1990; Woldergiorgis *et al*, 1992), that CPT I and CPT II are identical polypeptides but that the former has associated with it a regulatory component to which inhibitors bind and cause allosteric inactivation of the enzyme. Implicit in this formulation is the assumption that detergents (as described in chapter 3) disrupt the link between the catalytic and regulatory components of CPT I allowing co-purification of active CPT I and CPT II as a single protein of 69 kDa. In some cases it is also thought that both CPT I and CPT II reside on either side of the mitochondrial inner membrane (Brady *et al*, 1986) explaining the lack of cross reactivity between serum raised to the 69 kDa CPT active protein and proteins resident in the mitochondrial outer membrane. The regulatory subunit, is however suggested by Woldergiorgis *et al*, (1992), to be the 87 kDa protein identified by Kolodziej *et al*, (1992). The 87 kDa protein is thought to reside in the mitochondrial outer membrane as an integral membrane protein, in close proximity to CPT I perhaps via a contact site. As described in section 1.11.3, contact sites are sites where the inner and outer mitochondrial membranes are in very close proximity to each other. They are thought to play a specific role in protein translocation across both mitochondrial membranes. The membranes are thought to be held in this configuration by integral membrane protein complexes between the two membranes. There is conflicting evidence for the single identity of CPT (discussed in chapter 3). As described in section 3.2, CPT II has already been shown to be easily solubilised from mitochondria while still retaining activity (Woeltje *et al*, 1990a), and therefore could be purified. This CPT active protein could then be used to clone the gene responsible, at least for one of the CPT activities in the mitochondria. Subsequent targeting studies could then be used to determine which location (or locations) the immature protein was targeted to.

4.2 PURIFICATION OF CPT II FROM RAT LIVER MITOCHONDRIA

The livers from 30 rats were removed and prepared as described in section 2.17. The purification was stopped after the heavy mitochondria were removed from the Percoll gradients. The heavy mitochondria were then pelleted 35000 X g (OTD-Combi), sonicated for 5 minutes, pelleted again and frozen (-80°C) overnight. After careful thawing the mitochondrial pellet was resuspended in an appropriate volume to give a final protein concentration of 10 mg/ml. Tween 20 (2 µl) was added per mg of protein along with antiproteases, the final concentrations of which were as follows; 100 µg/ml pepstatin; 1 µg/ml of phenylmethyl sulphonyl fluoride (PMSF); 200 µg/ml antipain; 4 µl/ml leupeptin. The solubilisation mixture was then sonicated on ice for 4 x 1 minute, (with 10 minute standing time) with constant stirring to disperse heat. It was then spun for 1 hour at 35000 X g (OTD Combi). The supernatant was then subjected to two polyethylene glycol (PEG) cuts. The percentage of PEG was calculated by trial and error techniques on sample volumes of the solubilisation mixture by measuring the activity of CPT in both pellet and supernatant in the same volume and using the same conditions. The first cut was made at 15% PEG, with the CPT activity remaining in the supernatent, and the second was made on the supernatant from the first cut at 50% PEG. In both cases the suspension was spun at 35000 X g 20 minuits (OTD Combi). The CPT active pellet, from the second PEG cut, was stored at -80°C overnight.

The pellet was suspended in an appropriate volume at a concentration of 8-9 g/100 ml, and further purified by two ammonium sulphate ($(NH_4)_2SO_4$) cuts. The trial and error approach was adopted once more to establish the optimal percentages of $(NH_4)_2SO_4$. The first cut was made at 40% and the second (on the supernatant of the first cut) at 80%. Both $(NH_4)_2SO_4$ cuts were separated by centrifugation at 20000 X g (OTD Combi). The resultant CPT rich pellet was dialysed against 2 litres 500 mM KCl overnight.

The dialysed sample was then separated through an ion exchange Mono Q column 10/10 by a fast protein liquid chromatography (FPLC) (section 2.17.4). The active samples were collected, pooled and ultrafiltrated to a final volume of 8 ml in Centricon tubes (Amicon) with a 30,000 dalton cut off point. The concentrated sample was then

dialysed overnight against 10 mM imidazole pH 7.5 containing 0.5% Tween 20. The elution profile of the column can be seen in Fig. 4.1.

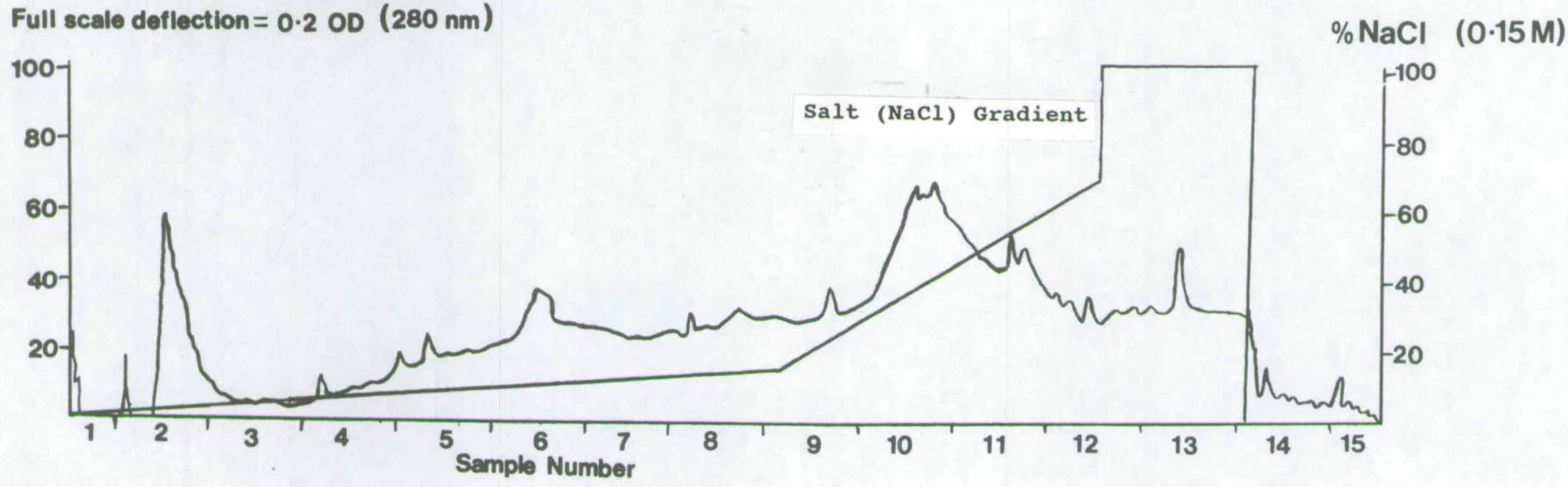
The dialysed samples were concentrated as before, to a final volume of 1.2 ml, which was loaded onto a chromatofocusing column (mono P), which had been pre-equilabrated to pH 8. The protein was eluted from the column with polybuffer 96 adjusted to pH 6 (section 2.17.5). The column was operated in conjunction with an FPLC system which produced an efficient and rapid separation of the sample. The activity was eluted as equal peaks at pH 6.2, which were pooled and dialysed against 20 mM KPi containing 0.5% Tween 20. The purified protein was then stored in aliquots at -80°C. The elution profile of the column can be seen in Fig. 4.2.

A typical purification table for the purification of CPT II is shown in Table 4.1. Although the CPT II activity is very low, when analysed by 22% polyacrylamide gel electrophoresis (PAGE), the purified sample consisted of a single protein of 69 kDa (Fig. 4.3). This CPT activity was not sensitive to malonyl-CoA. Reasons for the activity being so low are probably due to the denaturation of the protein during the column purifications which were carried out at room temperature.

4.3 N-TERMINAL SEQUENCING OF PURIFIED CPT II

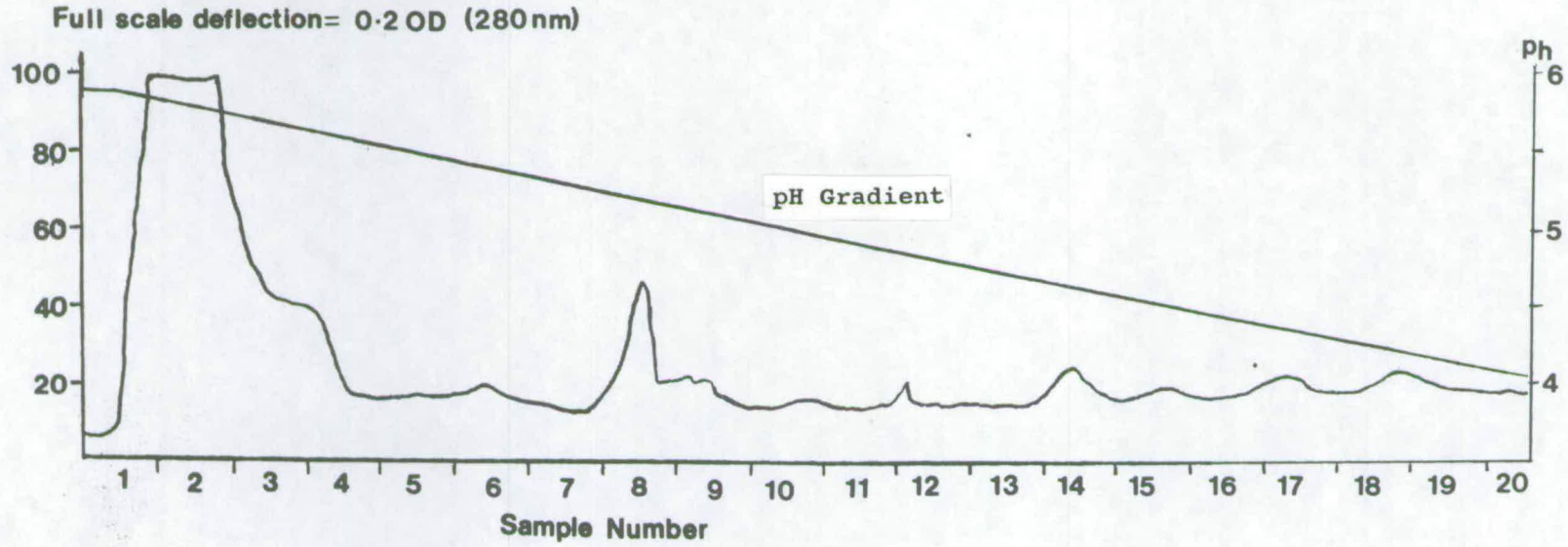
The purified CPT protein described in section 4.2, was subjected to SDS-PAGE and transferred to nitrocellulose by western blotting. The protein was the stained lightly with amido black, excised and subjected to N-terminal amino acid sequencing which was performed by Bryan Dunbar using the SERC protein sequencing facility in Aberdeen University. The yield of sequence was not large, probably due to a high degree of N-terminal blocking of the protein. Some sequence was however obtained (Table 4.2) for the first 20 amino acids. Although the reading was close to the limits of sensitivity for the protein sequencer, it was reproducible on all occasions of sequencing.

Fig. 4.1 Elution profile of ion exchange mono Q column, performed on fast protein liquid chromatography (FPLC) during the purification of CPT II (Section 2.17.4)



The trace shows a typical elution profile from the mono Q column, along with the salt gradient used in the elution of the proteins. The samples containing CPT activity were 4-8. These along with samples of an identical run were pooled, concentrated and measured for protein concentration.

Fig. 4.2 Elution profile of chromatofocusing mono P column, performed on fast protein liquid chromatography (FPLC), during the purification of CPT II (Section 2.17.5)



The trace shows a typical elution profile from the mono P column, along with the pH gradient used in the elution of the proteins. The samples containing CPT activity were 7 and 8. These samples were pooled, concentrated, measured for protein contcentration.

Table 4.1 Purification table of CPT II, purified from 30 non-starved rat livers

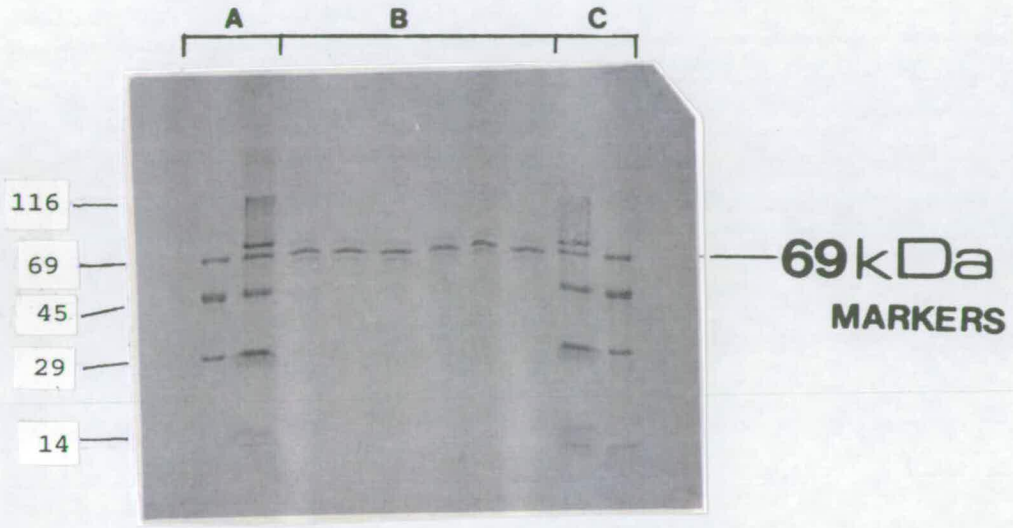
Sample	Total Activity (U)	Percentage yield
post Tween 20 solubilisation	3.91	———
15% PEG supernatant	2.31	100
50% PEG pellet	1.84	80
40% ammonium sulphate supernatant	1.27	55
80% ammonium sulphate pellet	0.72	31
post mono Q ion exchange	0.39	17
post mono P chromatofocusing	0.14	6

Although the final yield is low, both in terms of units activity (μmole/min), and percentage of starting activity, a single band of 69 kDa was obtained as visualised by polyacrylamide gel electrophoresis (Fig. 4.2). Before solubilisation by Tween 20 the total amount of mitochondrial protein present from 30 rat livers was 1 g. The final specific activity of the purified sample was usually around 2.5 - 3 μmol/min/mg. When compared to an initial specific activity of 0.006 μmol/min/mg, the purification was around 500 - 600 fold.

Table 4.2 N-terminal sequence of purified CPT II

Cycle	Ala
1	Val
2	Val
3	Gly
4	Pro
5	Asp
6	Asp
7	Tyr
8	Leu
9	Gln
10	-
11	His
12	Ile
13	Gly
14	-
15	Pro
16	Met
17	-
18	Tyr
19	Gln

Fig. 4.3 Examination of purified carnitine palmitoyltransferase II as determined by polyacrylamide gel electrophoresis



A&C ; markers
B ; purified CPT II

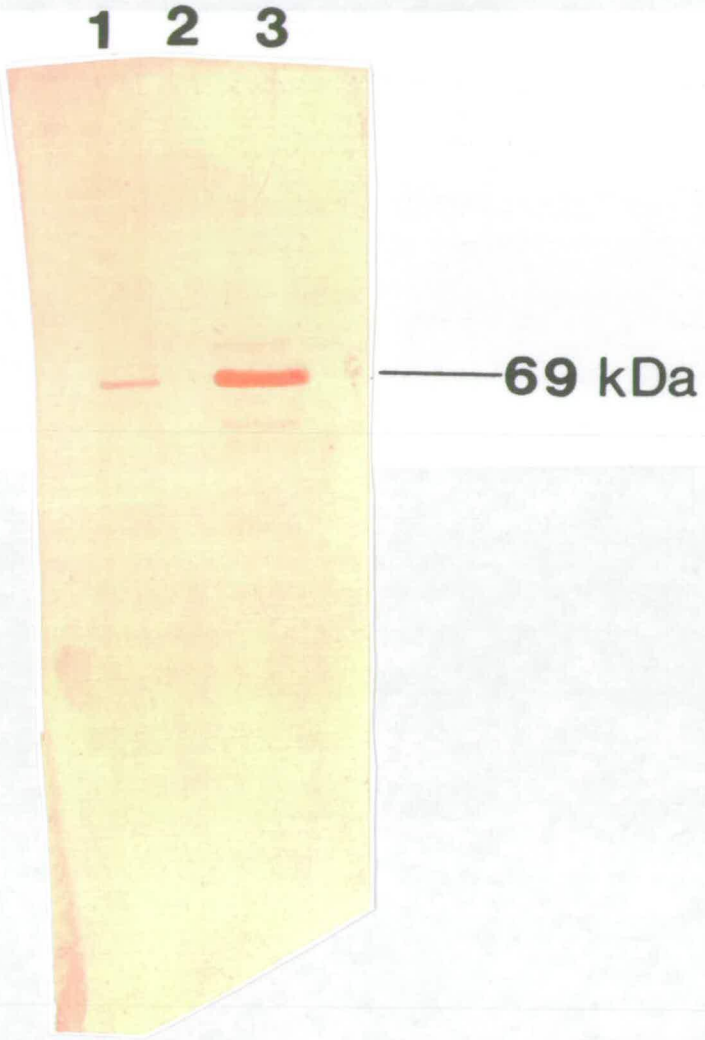
Approximately 10 μg of protein was loaded into each well of a 5% polyacrylamide gel which was electrophoresed as described in section 2.28. All the tracks in B are from the final purified sample of rat liver mitochondrial CPT II. The gel was stained with page blue and the purified CPT II estimated to be approximately 69 kDa.

Attempts were made to digest CPT II in order to generate peptides which may be used to provide further amino acid sequence data. The enzymes used for digestion were clostropain (section 2.13.2) and V8 endoproteinase, and chemical cleavage was produced by cyanogen bromide treatment (section 2.13.3). The digestions were carried out at 25, 30 and 37°C, for time periods of 1 to 30 minutes. Many apparent fragments were produced, when visualised by reverse phase high pressure liquid chromatography (HPLC). These fragments were analysed for amino acid content by Linda Kerr (WellMet sequencing facility, Department of Biochemistry, Edinburgh University) before being sequenced, but were found in all cases to be entirely devoid of protein.

4.4 IMMUNOGENIC STUDIES ON CPT II

Purified CPT II (section 4.2) was injected into a sheep, and 3 booster injections were administered at 3 week intervals. Serum was collected and IgG prepared as described in section 2.31. The serum was then affinity purified (section 2.31.3), and used for immunogenic comparisons between CPT II and CPT I. The serum reacted with a single protein prepared from rat liver mitochondria which had a molecular mass of 69 kDa (Fig 4.4). There was no observed cross reactivity between this CPT II antibody and any other mitochondrial proteins. There was no cross reactivity between the proteins and sera to the TDG-CoA binding protein (section 3.12) and the CPT II protein. The serum (gift from Dr.R. Ramsay, Department of Biochemistry and Biophysics, University of California, San Francisco) raised by Ramsay (1988) against rat liver mitochondrial CPT II (Ramsay *et al*, 1987) also reacted with the CPT II protein purified in section 4.2 (Fig. 4.5).

Fig. 4.4 Immunodetection of carnitine palmitoyltransferase (CPT) II on western blots using antibody prepared from purified CPT active protein of molecular mass 69 kDa



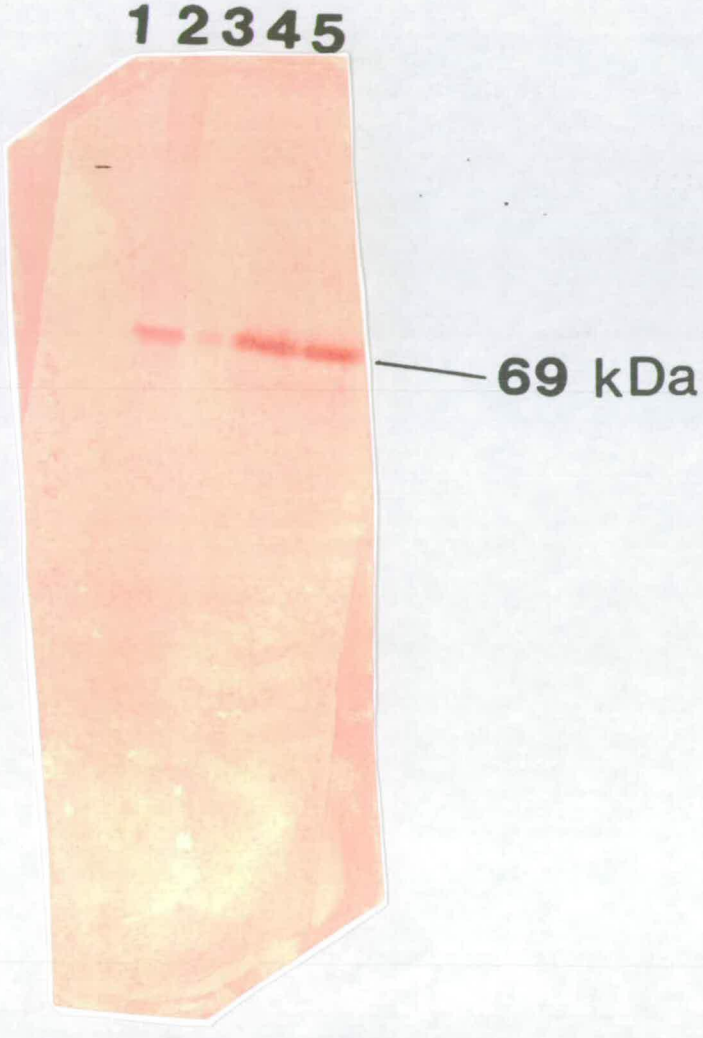
(1) Purified rat liver mitochondrial CPT II

(2) Rat liver outer mitochondrial membrane proteins

(2) Rat liver mitochondrial proteins

There is only one immunological reaction with rat liver mitochondrial proteins of 69 kDa from mitochondrial inner membranes. From this it can be deduced that the 69 kDa CPT II antiserum does not react with any other proteins in rat liver mitochondria ie. neither an 84 kDa or a 69 kDa protein in mitochondrial outer membranes, reacted with the serum.

Fig. 4.5 Confirmation that carnitine palmitoyltransferase II antiserum, raised by Ramsey (1988), cross reacts with the protein purified in section 4.1



(1) Rat liver mitochondrial outer membrane proteins

(2) Purified CPT II

(3) Blank

(4) & (5) Rat liver mitochondrial proteins

The antibody prepared by Ramsey (1988) reacts with the same CPT active 69 kDa protein prepared from rat liver mitochondrial inner membranes (section 4.2). It does not however react with any proteins in the mitochondrial outer membrane.

4.5 SCREENING OF λGT10 LIBRARY WITH OLIGONUCLEOTIDES

Brady *et al*, (1988) published a paper which reported the N-terminal sequence of a detergent-labile malonyl-CoA-insensitive mature CPT enzyme which had a molecular mass of 69 kDa.

N- "Glu-Lys-Gly-Val-Gln-Thr-Gly-Gln" -C

This amino acid sequence was used to design degenerate oligonucleotides which were made by OSWEL DNA service, Department of Chemistry, University of Edinburgh. The oligonucleotides were used to screen a cDNA library contained in λgt11 (gift from Brian Burchell, Department of Biochemical Medicine, University of Dundee).

e1 3' ; 5' " GARAARGGNGTNCARAC " 3'

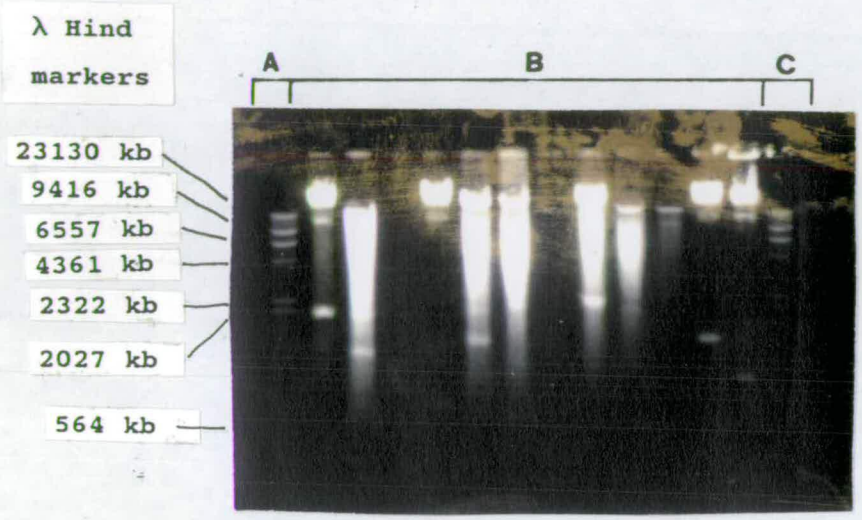
e2 5' ; 5' " GARAARGGNGTNCARACNGGNCAR " 3'

The oligonucleotides were phosphorylated with γ-labelled [^{32}P]-ATP (section 2.24.2.1). After comprehensive screening of this library, and 3 other such libraries, (Clontech λgt10, Clontech λ gt11, Clontech λgt11 stretch), with no success, efforts were stopped.

The libraries were checked for both inserts, and the degree of size variation between these inserts, by random single plaque purification. Twelve randomly purified plaques were amplified, and the DNA from them (section 2.33.2) was digested with EcoR1. The digests were analysed on a 2% agarose gel and the fragments were visualised using a UV transilluminator (Fig 4.6). The libraries were found to be of good quality and it was therefore thought that the lack of positive results in the screening was due to some other problem.

Northern blot hybridisation (section 2.24.1.2) was carried out using the degenerate probe e2 (Fig. 4.7). Although a band was visible at the size expected for CPT II mRNA (2.7 kb), many other bands were also visible. As the stringency of washing was increased, these bands did not disappear any more rapidly than the 2.7 kb band. This is probably due to the high degree of codon degeneracy built in to the probes.

Fig. 4.6 Agarose gel electrophoretic separation of EcoR1 fragments of DNA prepared from random single plaque purification of a λgt10 library



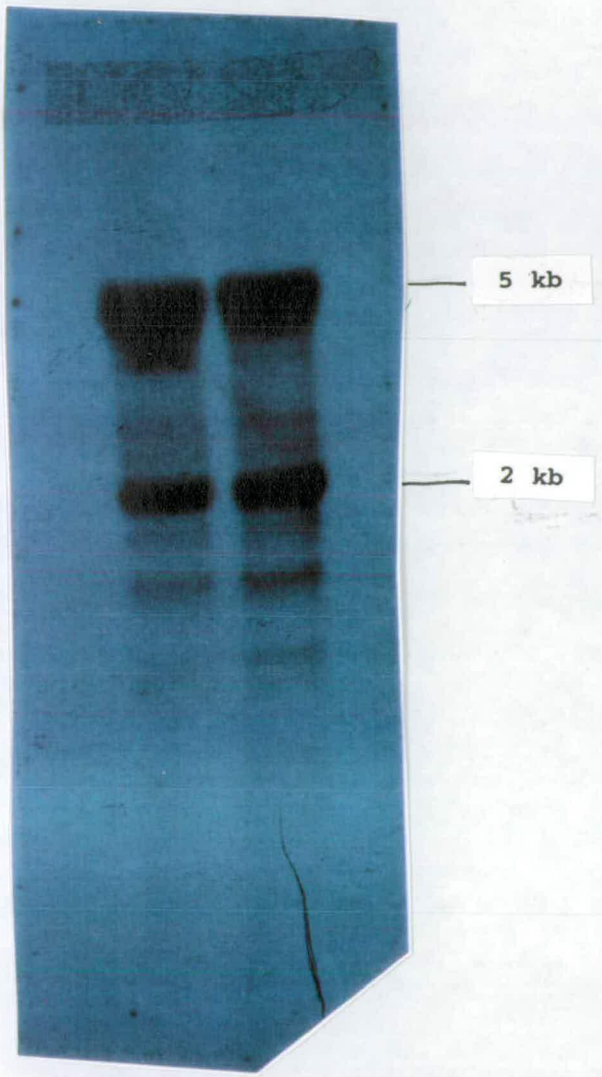
A & C ; λHind III markers
B ; EcoR I digested λgt10 DNA

λgt10 DNA was prepared (Section 2.33.2) from 12 randomly picked single plaques, taken from the λgt10 cDNA library (Section 4.5). The DNA was then digested with the restriction enzyme EcoR1, separated on a 1% agarose gel and stained with ethidium bromide. The photograph of the gel shows that the insert sizes of λgt10 cDNA library fall between 0.5-2.5 kb. All the plaques which were purified contained different sized fragments. This indicates that the library would appear to be of a good quality.

Fig. 4.7 Hybridisation of CPT II oligonucleotide, generated to N-terminal amino acid sequence reported by Brady *et al* (1988), to a Northern blot of rat liver mRNA.

Oligonucleotide sequence: 5' - GARAARGGNGTNCARACNGGNCAR - 3'

N = (A,G,C or T); R = (A or G)



RNA, prepared from 48 hour starved rat livers (Section 2.20.3.2), was separated (two identical samples) by agarose gel electrophoresis (Section 2.21.4) and Northern blot analysis (Section 2.24.1.2). At higher stringency of hybridisation and washing, all bands disappear at the same point.

4.6 CLONING OF CPT II BY POLYMERASE CHAIN REACTION

(Section 2.22.4)

The cDNA sequence for CPT II reported by Woeltje *et al* (1990b), was used to design non degenerate oligonucleotides to either end of the cDNA sequence. EcoR1 (GAATTC) and HindIII (AAGCTT) sites were incorporated into the two oligonucleotides respectively (section 2.8).

f 5' ; 5' "GATGAATTCATGATGCCGCGCCTGCTGTTTC" 3'

g 3' ; 5' "GTCAAGCTTCTAAGTTTTGATGGCTTTGCCT" 3'

These oligonucleotides were used in an attempt to amplify the cDNA for CPT II. The length of the cDNA for CPT II is just under 2 kb, which is the longest size of DNA that *Taq* polymerase can cope with in optimal conditions. This procedure, however is also reliant on a full length template being present in the amplification. The templates used varied from cDNA prepared from libraries contained in λgt10 and λgt11 vectors, cDNA purchased from Clontech, cDNA prepared from RNA (induced by starvation; section 2.20.3.2) and DNA/RNA duplex (prepared in a similar way). Many different conditions were used (section 3.11), including significantly longer extension times of 2 to 10 minutes, where, in the latter case, extra *Taq* was added after 7 hours. Although no fragments of the desired size were visualised after separation by 2% agarose gel electrophoresis and UV illumination, cloning procedures were still attempted. No inserts were evident from sequences of the 'cloned' fragments. Other oligonucleotides were therefore made to regions closer to the 3' end of the cDNA for CPT II (section 2.8).

h 5' ; 5' "CAGCACATGATGGCTGAGTGTTCCA" 3'

i 3' ; 5' "TACAGCTGCGACCTCACACTACCT" 3'

This pair of oligonucleotides were used in an attempt to amplify a smaller region of the CPT II cDNA at the 3' end of the gene. The products obtained from the PCR amplification were numerous but were not of the predicted size of the fragment encompassed between the two primers (563 bp).

The fact that the band was not visible on the gel, and that cloning of the total PCR reaction mix still did not yield the desired CPT II fragment, did not strictly mean that there was no amplification of the product. In order to check this, a second pair of oligonucleotides was made to generate a fragment from within the predicted fragment enclosed between the last pair of oligonucleotides.

j 5' ; 5' "GAAGCAGCGATGGGCCAG" 3'
k 3' ; 5' "TCGAAAATGTCTTCCAAGCA" 3'

After the first amplification was complete the DNA products would be purified and re-amplified using the second pair of primers. Hopefully this would select for a fragment 284 kb in size. This method however was unsuccessful in raising the desired fragment.

Different combinations of all the CPT II oligonucleotides were then used in PCR amplifications. Success was obtained with one of the full length oligonucleotides (h), and one of the newer oligonucleotides (g) made from the sequence situated 300 bp from the 3' end of the cDNA. The oligonucleotides generated a fragment of 300 bp (Fig 4.8).

The conditions used for the successful amplification were as follows. Denaturation of the duplex at 98°C for 10 minutes in the absence of *Taq*, followed by 35 to 40 cycles of;

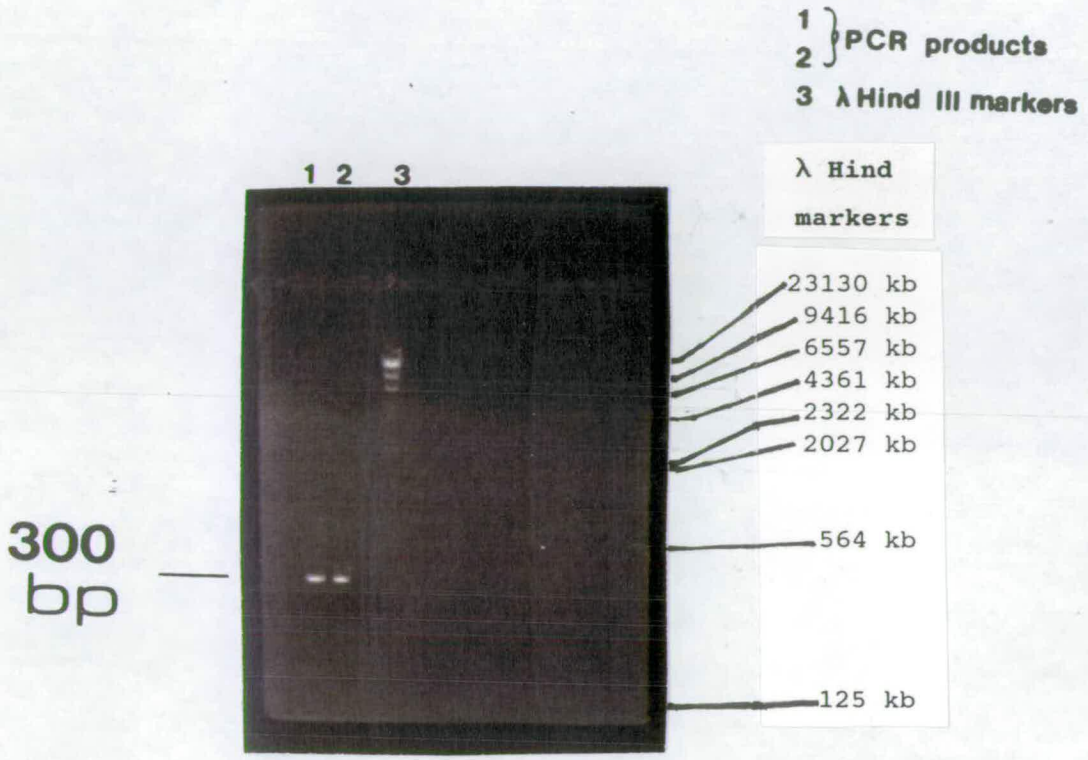
a) annealing at 50°C for 30 seconds
b) extension at 72°C for 2 minutes
c) denaturation at 95°C for 1 minute

It is important to note that the only ingredient absent during the initial denaturation was the *Taq* polymerase enzyme. It was vital that the dNTPs were present during this stage, because no amplification was obtained when they were absent. The reason for this is unclear.

The only template that generated the CPT II fragment, with these two primers, was RNA/DNA duplex. This duplex was reverse transcribed from RNA prepared from 48 hour starved rat livers.

This fragment was cloned into pTZ19, from which single stranded DNA was prepared and sequenced (section 4.7).

Fig. 4.8 300 bp fragment generated by polymerase chain reaction (PCR), using oligonucleotides generated to the C-terminus of carnitine palmitoyltransferase II



5' - CA$CACATGGCTGAGTGTTCCA - 3'

3' - CCGTTTCGGTAGTTTTGAATCTTCGAACTG - 5'

A 300 bp fragment was generated by PCR (Section 2.22.4) using the above oligos taken from the cDNA reported by Woeltje *et al* (1990b). The fragment was amplified from a template of RNA/DNA duplex prepared from 48 hour starved rat livers as described in section 4.6. The amplification products were the separated on a 2% agarose gel (Section 2.22.2) and stained with ethidium bromide.

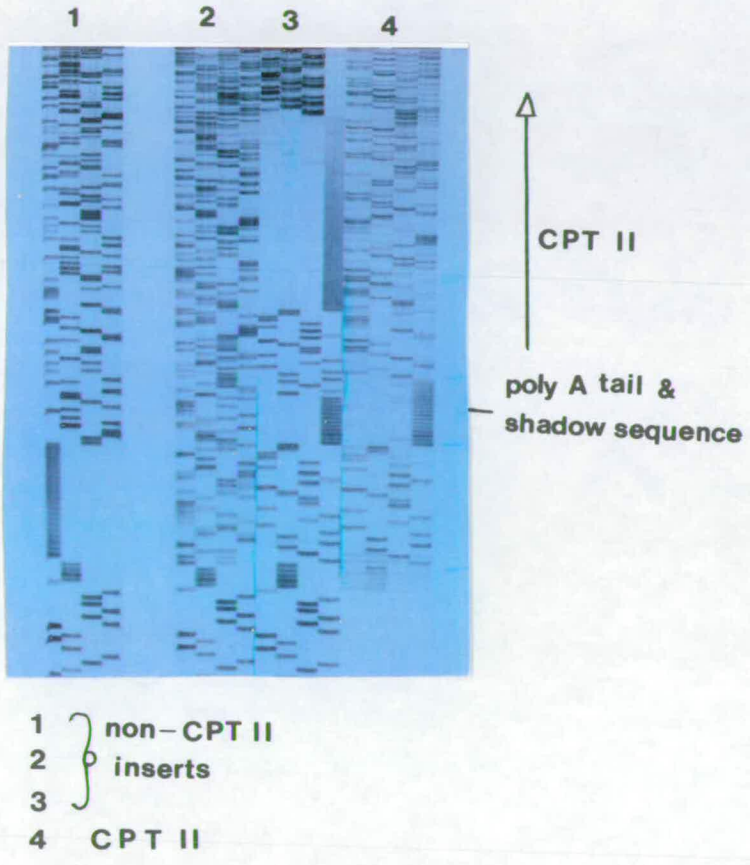
4.7 SEQUENCING OF THE 300 bp FRAGMENT

The single stranded DNA prepared from pTZ19 containing the 300 bp fragment was sequenced using both reverse primer and the two PCR primers, as described in section 2.23. When the oligonucleotides were used as primers, manganese was added to the sequenase reaction mixture in order to allow reading of the DNA sequence as close to the primer as possible. The sequences obtained (Fig. 4.9a) were 100% identical to the cDNA sequence reported by Woeltje *et al*, (1990b). It should be noted that in the sequence primed by the reverse primer there is also a shadow sequence leading off from the insert, and a strange feature of a poly A sequence enclosed by the PCR primers, before the CPT reading frame. This was removed by digestion with EcoR1 and Hind III followed by religation into pTZ19. However this did not get rid of the shadow sequence. Transformation of the plasmid DNA and picking of single colonies still did not get rid of the shadow sequence. This is confusing since the single transformants are caused by absorption of one plasmid by a individual cell, giving rise to a ampicillin resistant colony. It is unlikely that all the transformants absorbed two plasmids during transformation, but no other possibilities can be thought of. The shadow sequence was finally removed by PCR amplification of the CPT II fragment using the same primers but using the pTZ19 plasmid DNA as a template, followed by re-cloning into pTZ19 (Fig. 4.9b). The reason for this shadow sequence is unclear since it was not lost during re-transformation of the plasmid. Contamination in such a case was unlikely. The loss of the shadow sequence on re-amplification could only be due to it being amplified by different primers. This feature still remains slightly confusing.

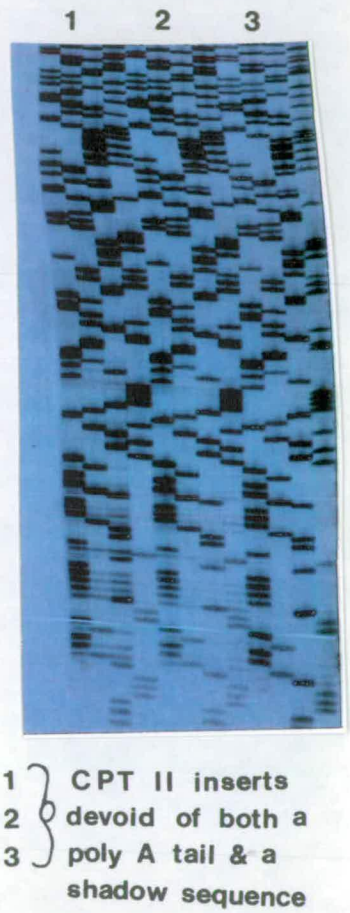
Fig. 4.9 Sequence data from the cloned 300 bp fragment of carnitine palmitoyltransferase II (CPT II)

Tracks read "TCGA"

a;



b;



a; The autoradiograph shows four sequences obtained from the 300 bp fragment, only one of which is identifiable as CPT II. Also visible in this sequence is a poly A tail between two of the primers positioned directly before the open reading frame of CPT II. There is also a shadow sequence visible over the CPT insert, suggesting the presence of a contaminating clone.

b; Sequence of excised and re-amplified (PCR) 300 bp fragment. This sequence is devoid of both a poly A tail and a shadow sequence.

4.8 SOUTHERN BLOT ANALYSIS OF THE 300 bp FRAGMENT

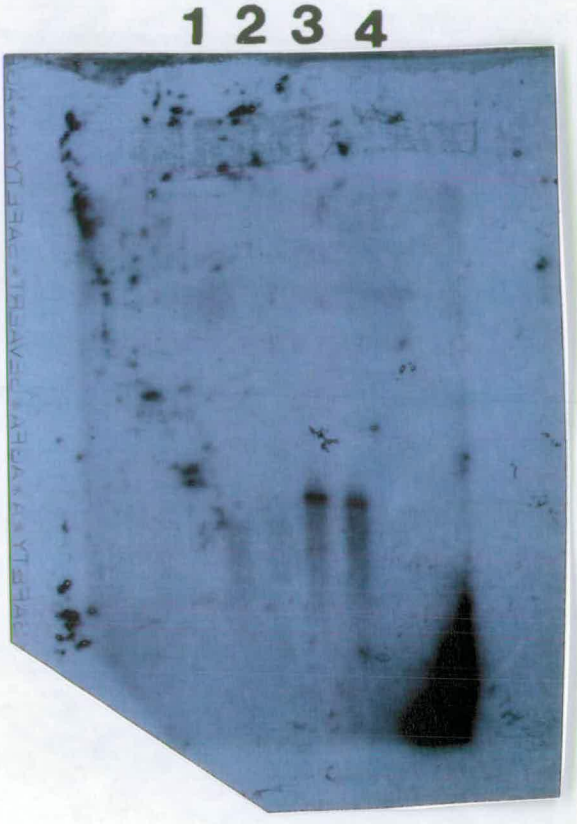
Southern blot hybridisation (section 2.24.1.1) was carried out under standard conditions using rat liver DNA digested with EcoR1 and HindII. The 300 bp PCR fragment (section 4.6) was used as a probe. It was labelled with [^{32}P] by either PCR incorporation (section 2.24.2.3) or by random priming (section 2.24.2.2). Hybridisation was carried out at 45°C and washings at a stringency of 55°C. The 300 bp fragment hybridised to a single band on the tracks containing the rat liver DNA digested with EcoR1 and HindII (Fig. 4.10). This suggests that the 300 bp fragment is hybridising specifically to one sequence only, which will presumably be CPT II. The probe therefore could be used effectively to screen cDNA libraries specifically for CPT II clones.

4.9 SCREENING OF A λGT10 LIBRARY WITH THE 300 bp FRAGMENT OF CPT II

(Sections 2.33.3 & 2.33.4)

The 300 bp fragment of CPT II was used to screen a cDNA library contained in λgt10, λgt11 and λgt11 stretch (Clontech). The fragment was labelled using random priming as described in section 2.24.2.2. However, screening has to date been unsuccessful, ie. positive clones of CPT II have not been found. The low expression of CPT II, especially when it has not been induced, makes the screening very difficult with the mammalian cDNA library. After screening approximately 500000 clones from each of the 4 libraries it is almost certain that the CPT II gene is not present in these libraries. Further success could only be achieved by acquiring another library, preferably one which had been prepared from rats which had been induced for CPT activity either by drug induced diabetes or starvation for 48 hours.

Fig. 4.10 Southern blot analysis, using 300 bp fragment from the C-terminus of carnitine palmitoyltransferase II, to probe EcoR1 and HindIII digested total rat liver DNA



1)&2) Control (*S. cerevisiae*) DNA digested with EcoR1

3) Rat liver DNA digested with EcoR1

4) Rat liver DNA digested with HindIII

DNA was prepared from rat liver as described in section 2.20 and DNA from *S. cerevisiae* was obtained from Dr. E. Ellis. Restriction endonuclease digestion of the DNA was carried out using EcoR1 and HindIII as described in section 2.22.1. Southern blot analysis was carried out as in section 2.24.1.1, and hybridised (Section 2.24.3) with a random primed (Section 2.24.2.2) 300 bp fragment of CPT II. At a stringency of 55°C washing all bands had disappeared from the autoradiograph apart from one on each of the rat DNA tracks.

4.10 DISCUSSION

Since the publication of the N-terminal peptide sequence of rat liver mitochondrial CPT by Brady *et al* (1988), followed by the publication of the full length cDNA of rat liver mitochondrial CPT by Woeltje *et al* (1990b), there has been much confusion as to the reason for the two different sequences. The peptide described Brady *et al* (1988) was not found by Woeltje *et al*, (1990b). Brady *et al* (1991) claimed that their sequence was part of the mature protein and was further upstream of the N-terminus of the CPT II clone reported by Woeltje *et al* (1990b). This is strange as the cDNA reported by Woeltje *et al* (1990b) does include a classical mitochondrial targeting sequence, and it would therefore be unlikely that further sequence associated with the active enzyme would be found further up stream.

Brown *et al* (1991) reported that *in vitro* studies on the clone found by Woeltje *et al* (1990b), efficiently targeted CPT II to the inner membrane of the mitochondria, and in so doing cleaved a leader peptide of 25 amino acids. They reported that the cleavage took place between leucine and serine which is positioned next to an alanine residue. This agrees with the N-terminal sequence of purified (mature) CPT II (Table 4.1) where the first identifiable residue is alanine. The sequence then agrees with the predicted amino acid sequence of Woeltje *et al* (1990b). There are a few differences that are due to amino acid sequencing errors, due the the low quantities of sequenceable CPT II protein.

```
 Initiation
 methionine
     ↓
    Met-Met-Pro-Arg-Leu-Leu-Phe-Arg-Ala-Trp-Pro-Arg-Cys-
                              Leader peptide clevage site
                                                    ↓
Pro-Ser-Leu-Val-Leu-Gly-Ala-Pro-Ser-Arg-Pro-Leu-Ser-Ala-
                                                      |
                                                    Ala-

Val-Ser-Gly-Pro-Asp-Asp-Tyr-Leu-Gln-His-Ser-Ile-Val-Pro-
  |       |    |   |   |   |    |   |           |
Val-Val-Gly-Pro-Asp-Asp-Tyr-Leu-Gln-***-His-Ile-Gly-***-

Thr-Met-His-Tyr-Gln-Asp-Ser-Leu-Pro-
      |       |    |
Pro-Met-***-Tyr-Gln-***-***-***-
```

The reason the first residue of the mature protein was not seen during sequencing, is probably because the N-terminal amino acid of a protein is often undetectable in the first cycle of sequencing. In view of this it is unlikely that the sequence described by Brady *et al* (1988) is part of the mature protein.

Similar results were reported by Finocchiaro *et al* (1991) on their elucidation of the cDNA encoding human CPT II. Since there is a high degree of homology between rat liver mitochondrial CPT II and human liver mitochondrial CPT II, this would seem to add further evidence against the claims made by Brady *et al* (1991). Another oddity about the sequence published by Brady *et al* (1988), was that the sequence was quoted from 3' to 5' and was for the antisense strand. No apparent reason or explanation was given for this.

The results obtained from the immunological studies (section 4.4) show that the affinity purified antibody reacted solely with a 69 kDa rat liver mitochondrial inner membrane protein. The protein recognised by this antibody was the same protein as that recognised by the anti-CPT II serum prepared by Ramsay (1988). Both sera did not recognise any proteins from rat mitochondrial outer membranes.

This evidence is contrary to reports by Ghadiminejad and Saggerson (1991) who did find a cross reaction. They reported that anti-CPT II serum reacted strongly with a 69 kDa protein in rat liver mitochondrial outer membranes. They could not however prove that it was a CPT active enzyme. This cross reaction is probably due to some mitochondrial inner membrane contamination during their purification procedures and not due to a immunological similarity between CPT II and CPT I.

The fact that there was no cross reaction between the anti-CPT II serum and any mitochondrial outer membrane proteins suggests that there is no immunogenic similarity between CPT I and CPT II. Similar results were reported in Chapter 3 for the anti-84 kDa TDG-CoA binding protein serum, which was believed to be either CPT I or its regulatory subunit.

The 300 bp PCR fragment described in sections 4.6 and 4.7 (Fig. 4.6), gave rise to more than one sequence (Fig. 4.7a). Out of 12 sequenced transformants, only one sequence was positively identified as CPT II, when compaired with the cDNA sequence reported by Woeltje *et al* (1990b).

The 300 bp fragment was shown by Southern blot analysis to react with only one sequence in total rat DNA (Fig. 4.10) which would suggest that it would be possible to screen for the full length clone by screening cDNA libraries held in λgt10 and λgt11.

However, attempts to use the 300 bp fragment to pull out a CPT II clone out of vector cDNA libraries proved to be unsuccessful. This was probably due to the naturally low expression of CPT II.

Future work aimed at cloning a full length cDNA of CPT II could best be undertaken by the preparation of a cDNA library prepared from rats livers which were pre-induced for CPT activity. This could be done either by using diabetic rats or 48 hour starved rats. This would increase the possibility of the message being present in the mRNA which could then be reverse transcribed into cDNA and ligated into a λgt10 library. The library could then be screened with the 300 bp fragment from the C-terminus of CPT II.

The 300 bp fragment could also be used to prime CPT induced mRNA for specific reverse transcriptase. This would result in one cDNA message being reverse transcribed only. This could the be amplified by PCR yielding a full length clone. The clone would have to be checked for sequence homology with the existing sequence for CPT II, since Taq polymerase is known to make mistakes (approximately 1 in 1000) as a result of its inability to proof read.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

General Discussion

The theory that CPT I and CPT II are identical proteins has been proposed by Woldergiorgis *et al*, (1992), Brady *et al*, (1986), and Kerner and Bieber (1990). The latter two groups also suggest that the two enzymes reside on either side of the mitochondrial inner membrane. If CPT I is identical to CPT II, then cross reactions would be expected between the serum raised to CPT II and a 69 kDa protein in the outer membrane. This was not observed (Fig. 4.4 and 4.5). Ghadiminejad and Saggerson (1991), reported that an antibody raised to rat liver mitochondrial inner membrane CPT protein, reacted with a 69 kDa protein in the outer membrane, but did not show that the protein was an active CPT enzyme. They also reported that CPT II could be associated with malonyl-CoA binding proteins from the outer membrane, and suggested that this was due to a high degree of similarity in structure between CPT I and CPT II. As described before, no evidence was found to substantiate the existence of an outer membrane 69 kDa protein of either immunogenic similarity to CPT II (section 4.4) or containing CPT activity (Zammit *et al*, 1989). This perhaps would strengthen the theory that CPT I resides on the outside of the inner mitochondrial membrane and was indistinguishable from CPT II upon detergent extraction (Woldergiorgis *et al*, 1992). The malonyl-CoA sensitivity of CPT I could therefore be induced by a regulatory subunit contained in the outer membrane but in close proximity to CPT I via a contact site. However, this purification procedure was used to isolate mitochondrial outer membranes which contained malonyl-CoA sensitive CPT activity, and furthermore did not contain a 69 kDa protein which could react with anti CPT II serum. So the work presented in Chapter 3 would tend to suggest that malonyl-CoA sensitive CPT activity in rat liver mitochondria was localised in the outer membranes only, which is strengthened by the immunological studies of Chapter 4.

The association of CPT II with malonyl-CoA binding proteins could be due to its similarity with the peroxisomal carnitine octanoyltransferase (COT) (section 3.18), which has been shown to be malonyl-CoA sensitive (Derrick and Ramsay, 1989) and has a molecular

weight of 60 kDa. So the association of CPT II with malonyl-CoA binding proteins in rat liver mitochondrial outer membranes could be due to a relationship with its peroxisomal counterpart rather than any similarity to CPT I. The COT was reported by Derrick and Ramsay (1989) to be inhibited by up to 90% by less than 10 μM malonyl-CoA. They also reported that malonyl-CoA sensitivity was lost after solubilisation, probably due to the dissociation of a malonyl-CoA sensitive regulatory subunit, and that a similar system was in operation in the case of rat liver mitochondrial CPT I. Skornin *et al* (1992) reported that COT was inhibited by both malonyl-CoA and its analogue TDG-CoA, but during such inhibition peroxisomal oxidation increased by 40% as measured by catalase activity. They suggested from this that the inhibition of COT decreased the amount of medium chain fatty acids being shuttled out of the peroxisomes therefore increasing the β-oxidation within the peroxisome. Although the COT was inhibited by the TDG-CoA, carnitine acetyltransferase (CAT) activity was seen to be increased by 40% (Skornin *et al*, 1992). The connection between CPT I inhibitors and peroxisomal oxidation of medium to long chain fatty acids is perhaps through production of acyl-carnitine for uptake into the mitochondria.

The specificities of CPT and COT are overlapping to some degree, but in general the preferred fatty acyl substrates of the two organelles are $C_{10.0}$, $C_{12.0}$ and $C_{14.0}$ for the peroxisome and $C_{16.0}$ to $C_{18.0}$ for the mitochondria. $C_{16.0}$ and $C_{18.0}$ are mostly oxidised in the mitochondria, which could perhaps in part explain why peroxisomal activity is increased when COT is inhibited by TDG-CoA. In other words the inhibition of the enzyme decreases the amount of activated long chain length fatty acid carnitine being transported to the mitochondrion, by inhibiting the shuttling of long chain fatty acids from the peroxisome to the mitochondrion. It does however, not affect overall peroxisomal oxidation.

The peroxisomal system is perhaps similar to the system observed in yeast where the mitochondria are devoid of CPT activity (Kohlhaw and Tan-Wilson, 1977). The activity of the fatty acid β-oxidation system has been detected exclusively in the peroxisomes (Kawamoto *et al*, 1978), but malate dehydrogenase, citrate synthase and aconitase, which are common to the tricarboxylic acid and glyoxylate cycles are localised in the mitochondria (Kawamoto *et al*,

1977). These results strongly suggest that acetyl-CoA necessary for the citrate synthase reaction in mitochondria could be supplied from the peroxisomes by a transport system for acetyl units. Several transport systems can be suggested: direct transport of acetyl-CoA; transport of acetate after hydrolysis of acetyl-CoA; and transport via the acetyl-carnitine shuttle mediated by carnitine acetyltransferase (CAT).

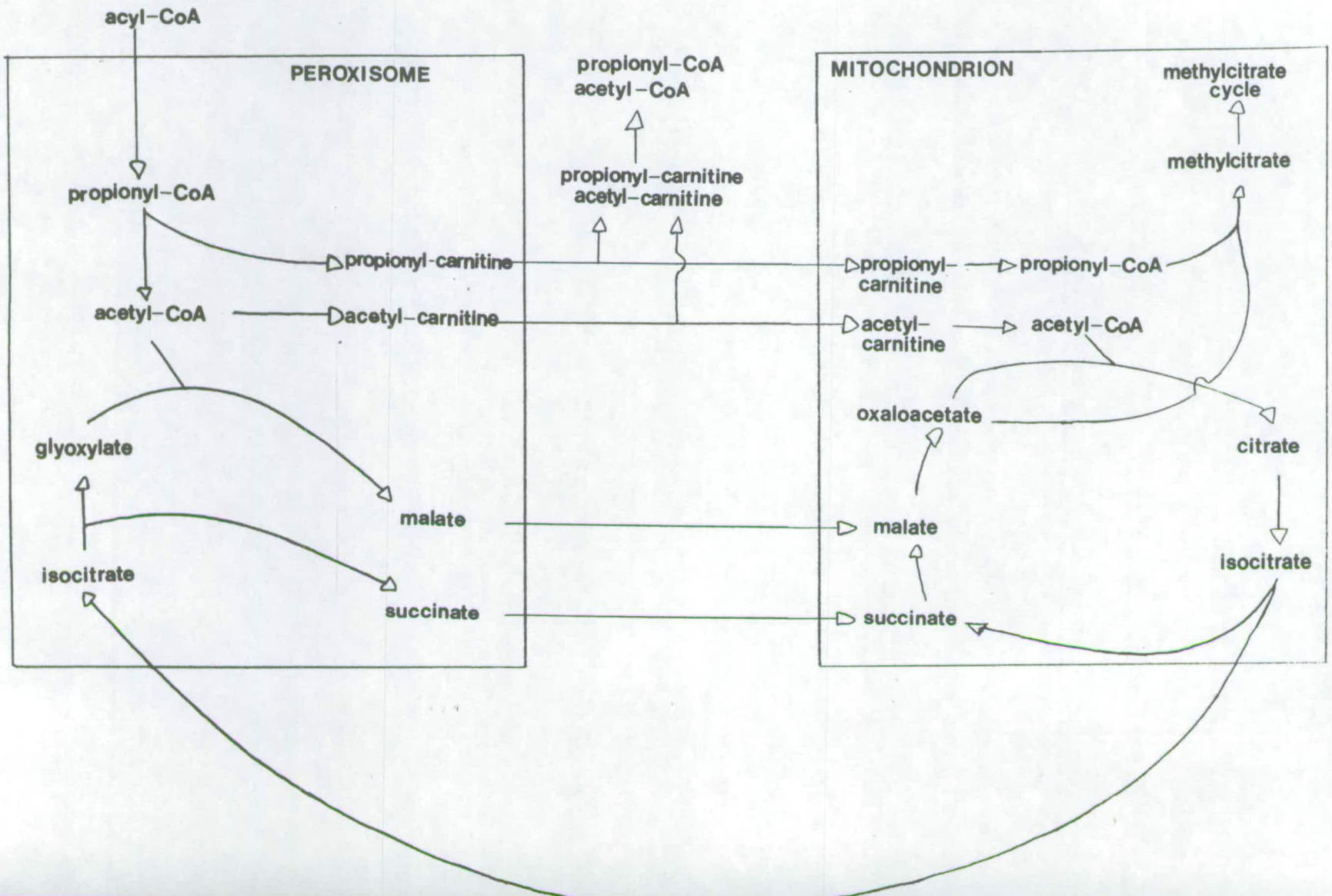
It was reported by Ueda *et al*, (1982) that since acetyl-CoA could not permeate the mitochondrial inner membrane and the activity required to hydrolyse acetyl-CoA could not been detected in yeast peroxisomes, the carnitine acetyltransferase shuttle system was the most likely candidate for the acetyl-CoA transport. They reported that the level of carnitine acetyltransferase in the cells was greatly enhanced when yeast were grown on alkanes.

The assumption that an 'acetyl-carnitine shuttle may play an indispensable role in the transport of acetyl units between the peroxisomes and the mitochondria is strengthened by reports by Kawamoto *et al*, (1978). They reported that CAT can be isolated from both peroxisomes and mitochondria in alkane grown yeast. The suggested shuttle system is shown in Fig. 5.1.

Recently a cDNA sequence was published which allegedly encoded *S. cerevisiae* mitochondrial CAT (Kispal *et al*, 1993). The predicted amino acid sequence showed highly homologous regions with choline and carnitine acetyltransferases, including rat and human mitochondrial CPT II and rat COT. The authors proposed that the CAT was possibly the evolutionary ancestral protein of all the acyltransferases.

The evolutionary role may be taken one stage further. The biological importance of peroxisomes in some of the higher eukaryotes is still unknown. In yeast however, one function of peroxisomes is to perform β-oxidation of medium to long chain fatty acids. This function can not be undertaken by yeast mitochondria since they are devoid of CPT. The peroxisomes then shuttle shorter chain fatty acids across to the mitochondria for entry into the Kreb's cycle, a metabolic pathway that does not exist in the peroxisomes. Perhaps during evolution, the CPT system was developed in higher eukaryotes, enabling the mitochondria to oxidise medium to long chain fatty acids. The peroxisomes in the higher eukaryotes, although still capable of oxidising fatty acids, would no longer be exclusively

Fig 5.1 Possible mechanism for the complete oxidation of long chain fatty acids via a peroxisome/mitochondrion carnitine dependent shuttle system in yeast.



required to do so. They would still operate as usual but would also supply the mitochondria with multi-chain length acyl-carnitine, via the carnitine shuttle system for both β-oxidation and entry into the Krebs cycle. This would make the physiological importance of peroxisomal β-oxidation redundant in higher eukaryotes.

From the binding studies of the different inhibitors and labelled substrates it can be seen that malonyl-CoA inhibits CPT I activity (section 3.5). The maximum binding of malonyl-CoA however, is unaffected by the binding of either carnitine or palmitoyl-CoA as reported by Kolodziej and Zammit, (1990a). The reasons for this have been much discussed in the literature to date. The binding site for malonyl-CoA could either reside on a separate binding site on CPT I, close to the active site of the enzyme, or on a entirely different protein. This protein would presumably act as a regulatory subunit of the active protein/phospholipid complex. This would however suggest that the protein that was labelled with the malonyl-CoA analogue TDG-CoA was a regulatory subunit and not CPT I. This would open further discussion as to the actual size of CPT I and whether in fact it is different from CPT II.

Kolodziej and Zammit (1990a) reported that the number of high affinity binding sites on outer membranes for malonyl-CoA was in the same order as the number of CPT I catalytic sites. During starvation, Kolodziej and Zammit (1990a), observed a co-ordinated rise in both the number of malonyl-CoA binding sites and CPT I active protein. They postulated that this was in order with a two fold increase in CPT I activity. It seems unlikely that a regulatory subunit would be co-ordinately induced during starvation, at the same time as CPT I was being desensitised to malonyl-CoA inhibition (section 3.5). So, when this starvation induced co-ordinated increase of binding is taken into consideration, it suggests that malonyl-CoA binds to CPT I and not a regulatory subunit.

Woldergiorgis *et al* (1992), reported that malonyl-CoA binding proteins, from rat liver mitochondrial outer membranes, could be purified and then reconstituted by dialysis in a liposome system. The authors reported that when these proteins were reconstituted along with detergent labile CPT II, reconstituted CPT activity could then be inhibited by up to 50% by 100μM malonyl-CoA. Hence claiming that CPT I and CPT II are identical proteins. They suggested that one of

the malonyl-CoA binding proteins that they purified, was the same as the 84 kDa protein purified by Kolodziej *et al* (1992). They also suggested that co-ordinated inducement of the malonyl-CoA regulatory subunit during starvation, was to enable the rapid inhibition of the CPT system once the starvation period was over.

Although the system described by Woldergiorgis *et al* (1992) is attractive, they made no attempt to reconstitute the malonyl-CoA binding proteins without the detergent solubilised CPT. This would have been useful in order to test the malonyl-CoA binding proteins for CPT activity.

Schemes for the two possibilities were suggested by McGarry *et al* 1991 (Fig. 5.2 & 5.3). These systems both work on the concept that rat liver CPT I is a mitochondrial outer membrane protein, as described in section 3.2, but show CPT I both as its own malonyl-CoA regulator (Fig. 5.3), and as an identical protein to CPT II, with a malonyl-CoA sensitive regulatory subunit (Fig. 5.2).

Murthy and Pande (1987b), reported that malonyl-CoA sensitivity of CPT I activity was restored from octyl glucoside solubilised outer membranes. These membranes had previously been depleted of all CPT II contamination by solubilisation with Triton X-100. Murthy and Pande (1990), also reported that two CPT activity peaks could be separated from total mitochondria on a hydroxyapatite column, hence strengthening the theory that CPT I and CPT II are distinct proteins.

An interesting addition to the conceptualisation of CPT I was reported by Murthy and Pande (1987a). They reported that when out-side out vesicles, containing reconstituted malonyl-CoA sensitive CPT active protein, were treated with proteases (nargase, papain, pronase and trypsin), malonyl-CoA binding was greatly inhibited. CPT activity however was not affected. The CPT I activity was degraded when the treatment with the protease was preceded by sonication of out-side out vesicles, which had been resistant up until then. They suggested from these findings that the malonyl-CoA binding site, on rat liver mitochondria, was on the outer side of the mitochondrial outer membrane, but the carnitine and palmitoyl-CoA binding site was on the inner side of the mitochondrial outer membrane.

Fig. 5.2 Diagram depicting malonyl-CoA regulation of CPT I through a separate regulatory subunit in rat liver mitochondria

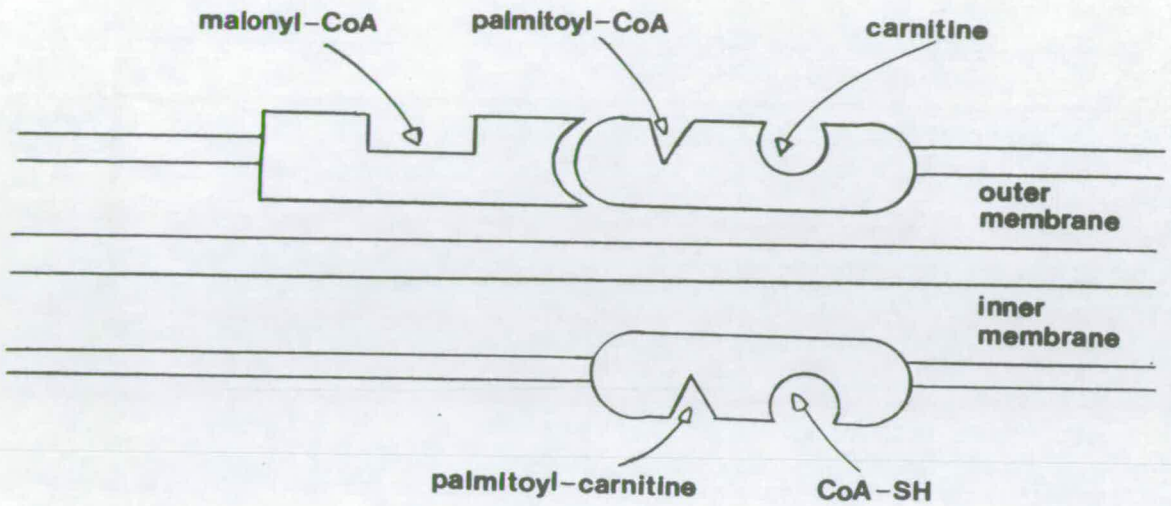
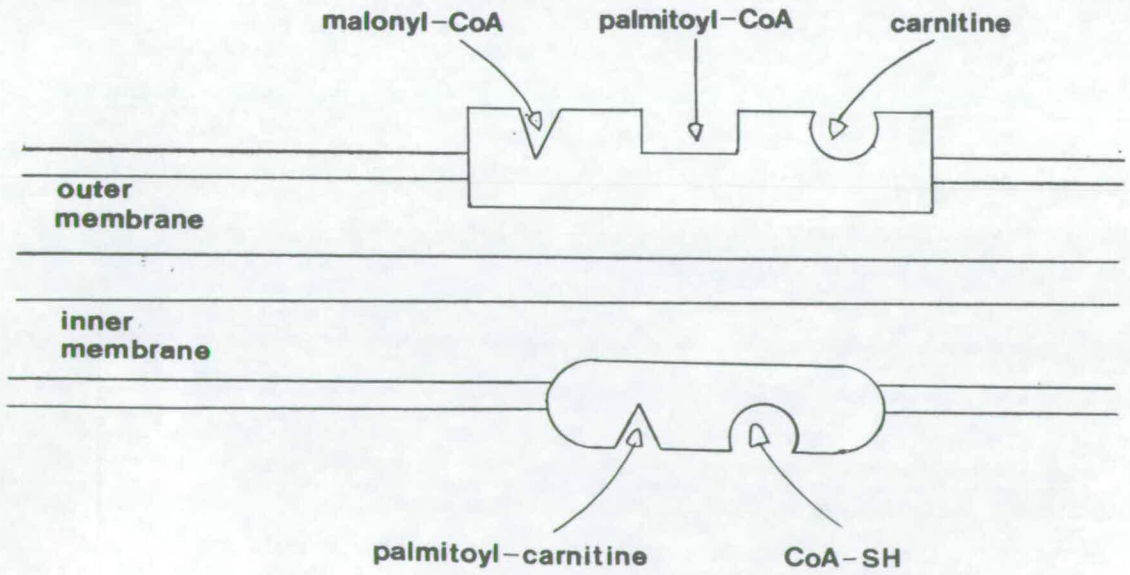


Fig. 5.3 Diagram depicting malonyl-CoA regulation of CPT I through a malonyl-CoA binding site on CPT I in rat liver mitochondria



McGarry *et al*, (1992) reported that the differential sensitivity of the two binding sites was not due to membrane topology but to the tertiary structure of the protein which responded to malonyl-CoA by inducing a conformational change. Esser *et al*, (1993a) reported that CPT I activity in mitochondrial outer membrane fragments could be protected from protease activity by the addition of malonyl-CoA. They showed, by polyacrylamide gel electrophoresis, that the CPT I protein was cleaved from 90 kDa to 82 kDa. The authors claimed that this 8 kDa cleaved portion of CPT I was unnecessary for catalytic activity. They also claimed that the protection of CPT I by malonyl-CoA from protease action strengthened the hypothesis that CPT I was the malonyl-CoA binding protein.

Further elucidation of the CPT system was reported by Esser *et al*, (1993b). Four fragments were obtained by carrying out partial chymotryptic and tryptic digests of CPT I while still in its membrane environment. The fragments were then purified and N-terminally sequenced. Oligonucleotides were generated to these amino acid sequences, and used to isolate a cDNA clone for CPT I (Esser *et al*, 1993b). The clone consisted of 102-nucleotide 5' untranslated region, a single open reading frame of 2319 bases, a 3' untranslated segment of 1957 nucleotides and a poly A tail. The authors reported that a fragment of the cDNA clone recognised a single mRNA of 4.7 kb in size. The predicted amino acid sequence of such a cDNA gave rise to 773 amino acids (Mr = 88150). The cDNA reported does contain the five peptides that were described in Table 3.13, indicating that the enzyme purified by the TDG-CoA (malonyl-CoA analogue) was CPT I, hence confirming that CPT I contains the binding site for regulation by malonyl-CoA.

Interestingly, one of the fragments reported in section 3.10, appears four amino acids from the N-terminus of the predicted translation product of the cDNA.

Gln-Ala-Val-Ala-Phe-Gln-Phe-Val-Thr-Pro-Asp-Gly-Ile-Asp-

It can therefore be assumed that the mature protein does not under go any proteolytic cleavage during its translocation into its final position in the mitochondrial outer membrane. This is very

common in known nuclear encoded mitochondrial outer membrane proteins.

It however disagrees with the prediction of the authors that a region (55 - 61 amino acids from the N-terminus) consisting of "-Pro-Ala-Asn-Pro-Ser-Ser-" was probably part of a cleavable mitochondrial import sequence, and that the existence of a cleavable mitochondrial targeting sequence was still to be established.

An interesting observation of the N-terminal sequence of the cDNA is that it is devoid of a classical mitochondrial targeting sequence. In order to clarify this situation, the full length clone of Esser *et al* (1993b) was examined for possible mistakes. From Fig. 5.4 it can be seen that in order for a classical mitochondrial targeting sequence to be included on the cDNA sequence errors would have to have been made. A number of scenarios exist for such errors.

The first could be the deletion of a thymine (T) between the adenine (A), at position 103, and the T at position 104. Inclusion of this T would alter the initiation methionine reported by Esser *et al* (1993b) to leucine, and would also alter the reading frame, before this point, from 'a' to 'b' (Fig. 5.4). The second error, would be the deletion of another T between the A at position 54 and the guanosine (G) at position 55. The inclusion of this second T would introduce a new inframe initiation methionine. These mistakes would add a further 50 bp on to the N-terminus of the open reading frame of CPT I. The predicted amino acid sequence of this new N-terminus would now include three hydroxylated amino acids (*) and three basic amino acids (∇), which is more similar to a classical mitochondrial targeting sequence. This sequence still includes two acidic amino acids (-), which are normally absent from mitochondrial sequences. The new N-terminal sequence would read;

```
     -   *          *       -
Met-Asp-Ser-Ala-Cys-Ser-Gly-Glu-Cys-
 ∇                  ∇   *   ∇
Arg-Ala-Ile-Gly-Pro-His-Ser-Arg-leu- - -
```

Another possibility would be that the T at position 64 was replaced by an A introducing a second initiation methionine in the same frame as the open reading frame (a) (Fig. 5.4). This would add a further 39 nucleotides to the open reading frame, and give a 13 amino acid extension to the predicted N-terminus. This leader peptide would

Fig. 5.4 N-terminus of the cDNA encoding CPT I (Esser *et al*, 1993) showing all three reading frames

The reading frame encoding the predicted amino acid sequence of CPT I is a. The initiation codon starts at nucleotide position 102. Unlike classical mitochondrial targeting sequences, the predicted N-terminal sequence contains very few basic or hydroxylated amino acids.

contain no acidic residues, three basic amino acids (*) and two hydroxylated amino acids (∇). This is also more similar to a mitochondrial targeting sequence and importantly contains no acidic residues;

```
          *              ∇      *  ∇      *
Met-Leu-Arg-Gly-Val-Gln-Ser-Asn-Arg-Ser-Pro-Lys-Met-
```

Incorporation of the third reading frame (c) (Fig. 5.4) would involve at least three sequence alterations. One to shift frames (deletion of Guanine (G) at position 101), another to introduce a new initiation methionine (substitution of cytosine (C) at position 60 with a A) and a third to delete the amber stop codon starting at position 87 (TAG) (substitution of T to A, altering the codon to code for Lysine). The predicted N-terminal amino acid sequence would contain one acidic amino acid (-), two hydroxylated amino acids (∇) and one basic amino acid (*). This would seem the less likely of the three possibilities because of the number of changes involved along with the inclusion of the acid amino acidic.

```
                ∇      -      *          ∇
Met-Ala-Pro-Gly-Ser-Ala-Glu-Gln-Lys-Val-Pro-Thr-Gln-
```

There are many other possibilities involving other possible sequence errors in the cDNA reported by Esser *et al* (1993b). Such errors, although possible, are not common and therefore unlikely.

The identity of the N-terminus of CPT I could easily be checked by PCR of the extreme 5' end of the cDNA clone. This could be done using primers generated to sequences either side of the reported N-terminus (Fig. 5.4). The amplified product could then be checked by DNA sequencing. Since the PCR amplification product would be from the 5' end of the template, a very high quality cDNA or mRNA would have to be used to ensure that most the messages present were full length. If reverse transcription was initiated from oligonucleotide dT, then the cDNA would have to include the large untranslated 3' sequence (1957 nucleotides). In order to alleviate this problem reverse transcription of the mRNA could be primed using an oligonucleotide generated to the 3' end of the reading frame. This could help in two ways, it would specifically generate RNA/DNA duplex for the CPT I

clone, and would shorten the length of the cDNA required to contain the N-terminus of the clone. It would also be important that the rat livers from which the mRNA or cDNA was prepared, were pre-induced for CPT I.

From the cDNA reported by Esser *et al* (1993b), there are 2 hydrophobic domains in CPT I. The more N-terminal of the 2 hydrophobic domains (residues 48-73), has 5 positively charged amino acid residues flanking its N-terminal side, and 3 positively charged amino acids on its C-terminal side. Applying the rules for membrane topology, described by Hartman *et al* (1989) and Parks and Lamb (1991) (discussed in section 1.11), a net positive charge of plus 3 is obtained in favour of its N-terminal side. This suggests that CPT I has an orientation where its N-terminus is exposed to the cytoplasm and its C-terminus is exposed to the intermembrane space (N_{cyt}-C_{in}).

It was suggested that the second hydrophobic domain also traversed the membrane (Esser *et al*, 1993b). However, when there is more than one transmembrane sequence, the orientation of them all is determined by the most N-terminal one (Hartmann *et al*, 1989). Therefore it seems more likely that the second hydrophobic region loops round through the membrane leaving the C-terminus still exposed to the intermembrane space.

CPT I, when compared to the other known acyltransferases, shows a similar degree of homology over much of its amino acid sequence. Allowing for conservative changes, the similarity in amino acid sequence is 50%. CPT I is significantly larger than the other recently analysed rat tissue acyltransferases. The main confirmed difference between CPT I and the other acyltransferases is that CPT I has an extra binding site for regulation via malonyl-CoA. Comparison of the known acyltransferase cDNA sequences suggests that there is least homology between the N-terminus of CPT I and the other acyl-transferases. It is therefore not unreasonable to predict that the N-terminal domain of CPT I will be responsible for malonyl-CoA binding.

The predicted N-terminal sequence protuding into the cytosol is only 48 residues in length. It is impossible to say whether or not this is large enough to act as a malonyl-CoA binding site.

An interesting picture is developed when this information is combined with both the protease studies of Murthy and Pande (1987a), and the predicted CPT I membrane topology described above. The

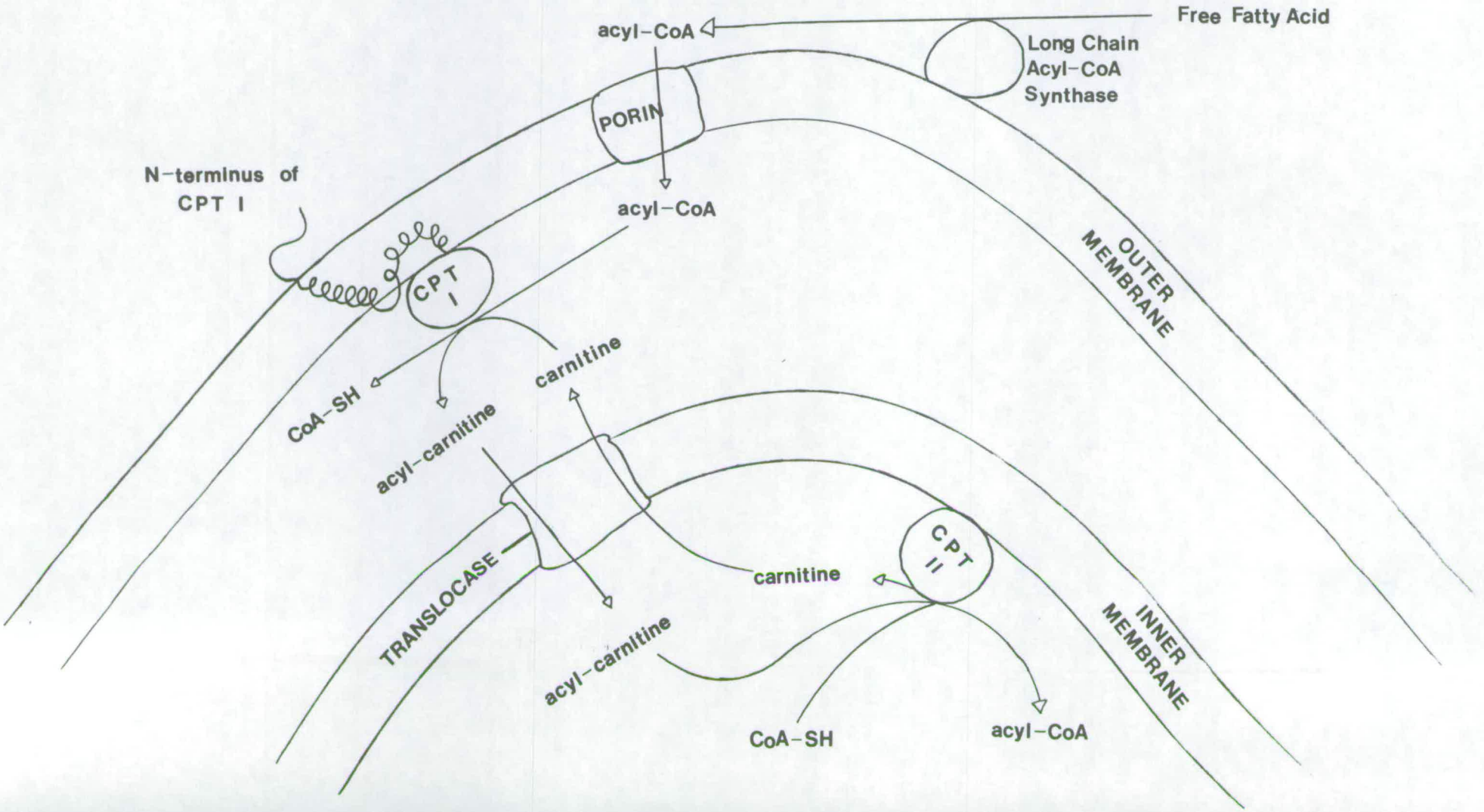
hydrophobic domains of CPT I are situated just after the predicted N-terminal domain and are therefore positioned perfectly to separate the two catalytic binding sites by spanning the outer membrane. The resultant picture of the CPT system would therefore resemble the diagram depicted in Fig. 5.5.

The translocation of such proteins has, to date, been little studied. The translocation of CPT I could possibly occur as follows; CPT I must be targeted to the mitochondrial outer membrane, probably to a receptor initiated by its N-terminus (devoid of a classical targeting sequence). This would normally be followed by insertion of the N-terminus into the outer membrane. Translocation of the N-terminus would then proceed up until the first hydrophobic sequence. This would then act as a stop transfer signal. The first hydrophobic sequence would then 'flip' round, because of the charge differential on the flanking regions (N-terminal flanking region being more positive). This would leave the N-terminus on the cytosolic side of the membrane. Translocation of the C-terminus however would continue, probably facilitated through unknown translocation machinery.

Another possibility is that the N-terminus aligns itself with the outer membrane bringing some other part of the protein in close proximity to the membrane. Translocation of the protein is then initiated through this second structure and not the N-terminus, which remains cytosolic at all times. Whether some other mitochondrial targeting system exists has yet to be discovered.

From the findings of this study along with careful examination of the recent publications, it is now apparent that rat liver CPT I and CPT II are distinct proteins, encoded by distinct genes. The proteins reside on the outer and inner mitochondrial membranes respectively. Their relationship to each other is no more close than that of all the acyltransferases, probably through an ancestral CAT. The targeting of CPT II to the inner mitochondrial membrane is facilitated through a classical N-terminal leader peptide of 26 amino acids, possibly through known translocation machinery. The method of targeting of CPT I to the outer mitochondrial membrane will be a subject of great discussion. It could be through traditional targeting sequences, or by another unknown method. The translocation of CPT I to its functional position in the outer membrane is also a matter for debate and can really only be elucidated once the

Fig 5.5 A possible schematic representation of CPT system in rat liver mitochondria as suggested by the evidence reported in this thesis along with other current opinions.



targeting experiments containing alterations in the protein have been carried out.

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