The Biochemistry of Lysyl Oxidase and TRAMP (Tyrosine Rich Acidic Matrix Protein)

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Abstract

Lysyl oxidase (E.C. 1.4.3.13) is a copper dependent enzyme of connective tissue that initiates cross-linking in collagens and elastin by oxidative deamination of certain lysine (or hydroxylysine) residues. The enzyme can be purified from 6M urea extracts of porcine skin. Tyrosine-rich acidic matrix protein (TRAMP) is a protein that co-purifies with lysyl oxidase and is the porcine equivalent of the human protein 'dermatopontin' and a protein (M_r = 22K) that co-purifies with dermatan sulphate proteoglycans from bovine skin. Polyclonal anti-serum was raised to purified TRAMP, and Western blotting showed that TRAMP is a widespread protein in murine tissues. Enzymatic studies showed that TRAMP is capable of releasing tritium labelled, low molecular weight material (<10 kDa) from [4,5-^{3}H] lysine-labelled collagen, but not from [4,5-^{3}H] lysine-labelled elastin, and that collagen β_{12} components were increased in the presence of TRAMP. TRAMP may therefore be a collagen specific lysyl oxidase.

In an attempt to remove the necessity for 6M urea during the purification of lysyl oxidase, and to allow genetic manipulation of the enzyme, work was undertaken to produce active mature lysyl oxidase using recombinant DNA technology. The cDNA sequence of the rat aorta lysyl oxidase precursor was used to design primers for amplification of a DNA fragment by the polymerase chain reaction (PCR), which was then radiolabelled and used to probe a human placental cDNA library for the human lysyl oxidase sequence. During the project, the cDNA sequence of the human placental lysyl oxidase precursor was published, and primers were then designed to amplify directly, a region of the human lysyl oxidase precursor cDNA, from the codon for Asp 169 to the termination codon. This sequence is thought to encode mature lysyl oxidase. The cDNA was obtained and sequenced. The amplified DNA was cloned into a yeast shuttle vector (pDP315), which was then used to transfect a strain of *S. cerevisiae* (JRY188) and attempts were made to express mature lysyl oxidase. Only one protein band of approximately 90 kDa was observed in

the yeast cell culture after running concentrated extracts on 12% SDS-PAGE.

Extracts of 6M urea from porcine skin were found that contained no endogenous lysyl oxidase activity, but were able to enhance lysyl oxidase catalysed tritium release from a [4,5-^{3}H] lysine-labelled elastin substrate. All activity was abolished in the presence of 2mM β–aminopropionitrile, a specific inhibitor of lysyl oxidase. Investigations were carried out on this material to determine its identity as a protein, its molecular weight was and its substrate specificity. The enhancer bound CM-Sepharose pre-equilibrated in 6M urea at pH 7.8, and could be eluted in 6M urea, 0.5 M NaCl pH 7.8. No enhancement was detected on lysyl oxidase activity when 1,5-diaminopentane or [4,5-^{3}H] lysine-labelled collagen were used as substrates. Enhancement was found to be greatest at low concentrations of radiolabelled elastin and the material responsible was inactivated by treatment with trypsin. The enhancer was stable at temperatures up to 80^{0}C, but was inactivated by boiling at 100^{0}C or by freezing.

Declaration

I declare that this thesis was composed by me, and that the work of which it is a record was performed by me, except where stated.

Euan G. Forbes

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Abbreviations

A	Adenosine
ACP	Aldol condensation product
Ao	Amine oxidase
BAPN	β–aminopropionitrile
BSA	Bovine serum albumin
bp	Base pair(s)
Bq	Becquerel(s)
C	Cytosine
^{14}C	Carbon-14
$CdCl_2$	Cadmium Chloride
cDNA	Complementary DNA
CM	Carboxymethyl
cm	Centimetres
CNBr	Cyanogen bromide
CO_2	Carbon dioxide
cpm	Counts per minute
dNTP	Deoxynucleoside triphosphate(s)
ddNTP	2',3' dideoxynucleoside triphosphate(s)
Da	Daltons
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
EDTA	Ethylenediamine tetraacetic acid
FACIT	Fibril associated collagens with interrupted triple-helices
FPLC	Fast protein liquid chromatography
g	Grams
g	Gravity
G	Guanosine
GTP	Guanosine triphosphate
^{3}H	Tritium
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
Hrs	Hours
IPTG	Isopropyl β-D-Thiogalactopyranoside
λ	Lambda
kbp	Kilo-base pair(s)
kDa	Kilo-daltons
LNL	Dehydrolysinonorleucine

LTR	Long terminal repeat
M	Molar
mA	Milliamps
mg	Milligrams
ml	Millilitres
mRNA	Messenger ribonucleotide acid
μg	Micrograms
MBq	Megabecquerels
MEM	Minimal essential medium
N	Normal
nm	Nanometres
ng	Nanograms
N_2	Nitrogen
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen orthophosphate
Na_2HPO_4	di-Sodium hydrogen orthophosphate
NaOH	Sodium hydroxide
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBU	Phosphate buffered urea
PMSF	Phenylmethylsulphonyl fluoride
PCR	Polymerase chain reaction
pg	Picograms
PQQ	Pyrroloquinolino quinone
RFLP	Restriction fragment length ploymorphism
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
S.E.	Standard deviation
T	Thymine
TBS	Tris buffered saline
TBS-T	Tris buffered saline/Tween
TEMED	N,N,N',N', tetramethylethylenediamine
T_m	Melting temperature
TRAMP	Tyrosine rich acidic matrix protein
Tris	Tris(hydroxymethyl)aminomethane
w/v	Weight per volume
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

AMINO ACIDS

Single letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Contents

This thesis is

dedicated to

Carol, Mum and Dad

Chapter 1

Introduction

1.1 Extracellular matrix

The extracellular matrix is a composite material of higher, multi-cellular organisms with major roles in the provision of tissue mechanical integrity, cell migration and cell differentiation (Brown *et al.,* 1991; Hay, 1991).

Extracellular matrix proteins include collagens (Hulmes, 1992), elastin (Mecham and Heuser, 1991), proteoglycans (Oldberg *et al.,* 1990; Kjelllen and Lindahl, 1991) and structural glycoproteins (Yamada, 1991). Collagens are a family of relatively rigid proteins characterised by the presence of a unique triple helical conformation. Elastin facilitates stretch and recoil and is therefore important for tissue flexibility.

The functions of both collagens and elastin are strongly dependent on the formation of lysine (or hydroxylysine) derived intra and inter-molecular crosslinks. The extracellular enzyme that initiates cross-linking, in both collagens and elastin, is lysyl oxidase (Pinnell and Martin, 1968).

1.2 Elastin

Elastin is a fibrous protein, present in most vertebrates, whose major function is to provide tissue elasticity and strength (Mecham and Heuser, 1991). Elastin is abundant in mammalian nuchal ligament and aorta (Davidson and Sephel, 1987), whereas relatively low levels are found in tissues such as foetal lung parenchyma (Rosenbloom, 1987)

1.2.1 Tropoelastin

The soluble precursor of mature elastin is tropoelastin (molecular mass

72-74 kDa; Rosenbloom, 1987). The structure of tropoelastin is unusual, consisting of hydrophobic stretches (which account for about 40 % of the molecule) interspersed with hydrophilic regions which contain potential cross-linking sites. These cross-linking sites are polyalanine sequences containing pairs of lysine residues (Hay, 1991) set two or three amino acids apart. In an α-helical conformation these lysine residues would be orientated on the same side of the helix, and hence be available for formation of multi-functional, interchain cross-linking. In contrast, the hydrophobic stretches are thought to form a random coil structure that gives rise to the elasticity of the molecule (section 1.2.3). Some hydroxylation of proline residues occurs in tropoelastin (Uitto *et al.,* 1976), although the significance of this is unknown.

1.2.2 Mature elastin formation

Newly secreted tropoelastin is targeted to specific sites on the extracellular surface of the plasma membrane where synthesis of mature, insoluble elastin occurs (Hinek *et al.,* 1988; Mecham *et al.,* 1989a). The assembly is thought to be regulated by specific elastin receptors that recognise the hydrophobic elastin sequence Val-Gly-Val-Ala-Pro-Gly (Mecham, 1991). The elastin receptor (molecular mass 67kDa) contains a cell binding component and an elastin / carbohydrate binding subunit (Mecham *et al.,* 1989a). This receptor is functionally and immunologically similar to the 67 kDa laminin receptor found in some tumour cells (Mecham *et al,.* 1989b).

Mature elastic fibres consist of a microfibrillar mass which surrounds an inner elastin core. The secretion of the microfibrillar component precedes

that of elastin (Farenbach *et al.*,1966), which suggests that the microfibrils create a scaffold on which the mature elastin fibres are formed. Maturation of elastin occurs by spontaneous inter- and intra-molecular cross-linking following oxidative deamination of specific lysine residues by the enzyme lysyl oxidase (E.C.1.4.3.13), to form α-aminoadipic-δ-semialdehyde (allysine). This aldehyde group is the functional precursor to the covalent cross-links desmosine and isodesmosine (Fig 1.1; section 1.4.3).

1.2.3 Elastin function

Elastin provides both structural integrity and ability to stretch and recoil without deformation. Elasticity is possible because of the unique hydrophobic structure of elastin. In mature elastin, the molecules are held together by stable cross-links. On stretching, the hydrophobic groups are exposed, leading to a loss of entropy (including that of the solvent), which raises the Gibbs free energy, and hence elastic recoil (Wainright *et al.*, 1976).

1.2.4 Elastin gene structure

The human tropoelastin gene is approximately 45 kilobase pairs (kbp) in length, containing 34 exons, and with an intron : exon ratio of 19:1 (Olliver *et al.,* 1987). Tropoelastin mRNA is 3.5 kbp in length, coding for 786 amino acids (Deak *et al.*, 1987). The hydrophobic and cross-linking domains of the elastin molecule are encoded by separate exons within the gene, which indicates that the gene structure is compartmentalised into

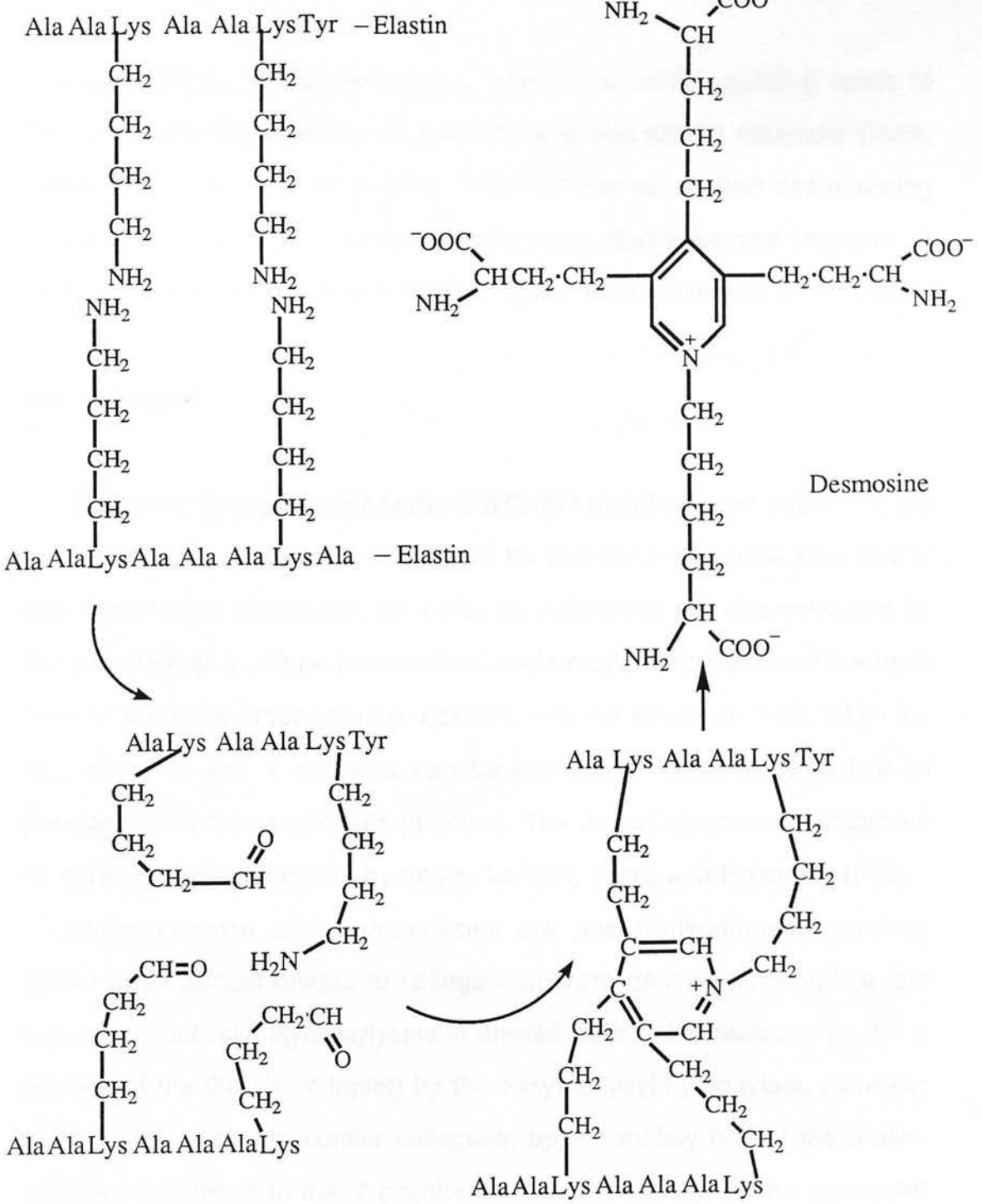


Figure 1.1. Stable cross-link formation in elastin. Desmosine is formed when allysine residues spontaneously condense with other allysine or lysine residues to form a pyridinium ring. (Adapted from Sandberg *et al.*, 1981)

functional areas. In human elastin, alternative mRNA splicing leads to tissue specific differences in the structure of the elastin molecule (Indik, 1988).Tissue specific differences in transcriptional control occur during development, as shown by the simultaneous decrease and increase in levels of aortic and skin elastin mRNA, respectively (Tokimitsu *et al.*, 1987).

1.3 Collagen

Vertebrate collagens (currently 18 different genetic types; Table 1.1) are a diverse family of proteins which can be sub-divided broadly into fibrillar and non-fibrillar categories. All collagen molecules are characterised by the presence of a unique triple-helical conformation. Formation of the triple helix requires the presence of a repeating primary structural motif, $(Gly - X - Y)_n$, where X and Y residues can be any amino acid, often proline (X position) or hydroxyproline (Y position). The helical structure is stabilised by extensive intermolecular hydrogen bonding (Berg and Prockop, 1973).

Hydroxyproline and hydroxylysine are post-translationally modified amino acids almost unique to collagen, and are derived from proline and lysine, respectively. Hydroxylysine is formed from lysine residues (in the Y position of the Gly -X -Y triplet) by the enzyme lysyl hydroxylase (Kivirikko and Myllyla, 1985). In fibrillar collagens, approximately half of the proline residues (i.e. those in the Y position of the Gly-X-Y triplet) are converted into 4-hydroxyproline by the enzyme prolyl hydroxylase (Grant and Prockop, 1972; Prockop *et al.,* 1979). The presence of 4-hydroxyproline increases the stability of the collagen triple helix by increasing hydrogen bonding through water molecules. Hydroxylysine is required for collagen specific O-linked glycosylation (Kivirikko and Myllyla, 1984) and the

Table 1.1 : Vertebrate collagens

Type	α chains	Most common molecular form	Tissue distribution
I	α1(I), α2(I)	$[\alpha1(I)]_2\ \alpha2(I)$	Bone, skin, cornea, lung
II	α1(II)	$[\alpha1(II)]_3$	Cartilage, vitreous humour
III	α1(III)	$[\alpha1(III)]_3$	Skin, lung, vascular system
IV	α1(IV), α2(IV), α3(IV) α4(IV), α5(IV)	$[\alpha1(IV)]_2\ \alpha2(IV)$	Basement membrane
V	α1(V), α2(V), α3(V)	$[\alpha1(V)]_2\ \alpha2(V)$	Collagen I containing tissue
VI	α1(VI), α2(VI), α3(VI)	α1(VI)α2(VI)α3(VI)	Most connective tissues
VII	α1(VII)	$[\alpha1(VII)]_3$	Basement membrane anchoring fibrils
VIII	α1(VIII), α2(VIII)	$[\alpha1(VIII)]_2\ \alpha2(VIII)]$?	Descent's membrane Endothelial cells
IX	α1(IX), α2(IX), α3(IX)	α1(IX)α2(IX)α3(IX)	Collagen II containing tissue
X	α1(X)	$[\alpha1(X)]_3$	Hypertrophic zone of cartilage
XI	α1(XI), α2(XI), α3(XI)	α1(XI)α2(XI)α3(XI)	Collagen II containing tissue
XII	α1(XII)	$[\alpha1(XII)]_3$	Collagen I containing tissue
XIII	α1(XIII)	$[\alpha1(XIII)]_3$?	Endothelial cells?
XIV	α1(XIV)	$[\alpha1(XIV)]_3$?	Collagen I containing tissue
XV	unknown	unknown	Human placenta (from cDNA)
XVI	unknown	unknown	Human fibroblasts (from cDNA)
XVII	unknown	unknown	Human skin (from cDNA)
XVIII	unknown	unknown	Mouse kidney, lung and liver

References: Types I-XIV (Hulmes, 1992), Types XV-XVIII (Mayne and Brewton, 1993).

formation of stable, pyridinium cross-links (Reiser *et al.*, 1992).

1.3.1 Fibrillar collagens

The fibrillar collagens (types I, II, III, V and XI) are characterised by a molecular packing arrangement in which neighbouring collagen molecules are staggered along the length of the fibril by integral multiples of 67 nm, a distance (D) slightly less than the molecular length (300 nm; Hulmes, 1992). Gaps that appear as a result of this packing arrangement are called 'hole zones', and these give rise to the characteristic banding pattern that is seen when fibrils are stained and observed in the electron microscope.

Type I is the most abundant collagen, present in tendon, cornea, skin, bone and the vascular system. Each type I molecule is a heterotrimer of two αI (I) chains and one α2 (I) chain, both containing approximately 1000 amino acids. The α chains are each encoded by separate genes.

Type II collagen can be found in cartilage, embryonic cornea, and vitreous humour. Unlike type I collagen, type II collagen is a homotrimer, consisting of three αI (II) chains (Miller and Matukas, 1969). Type III collagen is also a homotrimer (Miller *et al.*, 1971) with glycine residues occupying some of the X or Y positions of the triplet repeat, leading to a more flexible molecular structure than other fibrillar collagens. Type III collagen is present in tissues that are relatively extensible e.g. skin and lung.

Types V and XI collagen are closely related to each other, and are associated with tissues containing types I and II collagens, respectively.

Fibrillar collagens are secreted into the extracellular matrix as procollagen molecules, with propeptides at both the NH_3 and COOH

termini. The propeptides must be removed before the collagen molecules can assemble. Detailed understanding of the proteolytic processing mechanism comes from the conversion of procollagen I to collagen I, by specific procollagen proteinases (Turderman *et al.,* 1977).The N-propeptide is removed by procollagen N-proteinase to give pC-collagen while the C-terminal propeptide is processed to pN-collagen by procollagen C- proteinase (Prockop and Kivirikko, 1984).

The mature collagen molecule consists of a central, triple-helical core flanked by two short non-helical telopeptides at the C-terminus (25 amino acids) and the N-terminus (16-18 amino acids). The telopeptides contain the lysine and hydroxylysine residues that are oxidatively deaminated by the action of lysyl oxidase (section 1.4.2) to produce allysine and hydroxyallysine respectively. These modified residues spontaneously form bifunctional cross-links with lysine and hydroxlysine residues from the helical regions of other collagen molecules, leading eventually to the formation of stable tri-functional cross-links (Reiser *et al.*, 1992).

1.3.2 Non-fibrillar collagens

The non-fibrillar group of collagens include the Fibril Associated Collagens with Interrupted Triple helices (FACIT). They include collagen types IX, XII and XIV and are found closely associated with fibrillar collagen fibrils, mediating interactions between fibrils and the rest of the extracellular matrix (Shaw and Olsen, 1991).

Network forming collagens (types IV, VII and VIII) are present near or as part of basement membranes, which are extracellular sheet-like structures that underly epithelial and endothelial cells (and some mesenchymal cells)

with functions that include filtration (e.g. glomerulus) or the provision of morphogenetic tracks (e.g. neuromuscular junctions; van der Rest and Garrone, 1991; Burgeson *et al.*, 1990).

Beaded filament (type VI) collagen (Timpl and Engel, 1987) is present in most connective tissues, including cartilage and cornea, and appears to be necessary for the maintainence of integrity of some tissues (e.g. skin and blood vessels; Kielty *et al.*, 1991). The protein has many cell adhesion (RGD) sites in its helical domain, and there are collagen binding domains contained in the extensive N and C-termini (Koller *et al.*, 1989).

Types VIII and X collagens are the so called short chain collagens, with molecular lengths approximately half the length of the fibrillar collagens. Type X collagen is specifically located in the hypertrophic zone during endochondral ossification (Schmid and Linsenmayer, 1987). Type VIII collagen is produced by endothelial cells (e.g. Descemet's membrane in the cornea) and by some transformed cell types (Sage and Bornstein, 1987). Types VIII and X collagen are closely related, and both can form networks with molecules in hexagonal array.

1.4 Lysyl oxidase

Lysyl oxidase (E.C.1.4.3.13) is the enzyme that initiates the formation of covalent cross-links in mature collagens and elastin. Lysyl oxidase activity is widespread in vertebrate tissue. Reports of lysyl oxidase-like activities have also been observed in sea urchin (*Lytechinus variegatus*; Wessel and McClay, 1987) and the cell envelope of *E. coli* (Mirelman and Siegel, 1979).

1.4.1 Molecular mass

Since Pinnell and Martin recognised the role of lysyl oxidase in the formation of cross-links (Pinnell and Martin, 1968), the molecular mass of the enzyme has been reported as 170 kDa in chick embryo cartilage (Seigel and Martin, 1974), 59-61 kDa in immature chick aorta (Harris *et al.*, 1974) and 32-33 kDa in bovine aorta (Kagan *et al.*, 1979 a), porcine skin (Shackleton *et al.*, 1990 a), rat skin (Romero-Chapman *et al.,* 1991) and human placenta (Kuivaniemi *et al.,* 1984). Multimers of lysyl oxidase have been observed up to 100 kDa (Kagan *et al.*, 1979a).

Recently, the cDNA coding for rat lung lysyl oxidase has been isolated from a λgt 11 library (Trackman *et al.*,1990). It codes for a 48 kDa precursor form of lysyl oxidase, which presumably is processed to become the active, mature enzyme .

1.4.2 Lysyl oxidase reaction

Lysyl oxidase catalyses the conversion of specific lysine and hydroxylysine residues in collagens and elastin to α-aminoadipic-δ-semialdehyde (allysine). The reaction requires molecular oxygen and releases H_2O_2 and NH_3 (Fig. 1.2). The pattern of the reaction follows a ping-pong kinetic course (Williamson and Kagan, 1986) where the substrate is oxidized to an aldehyde by the passage of two electrons from

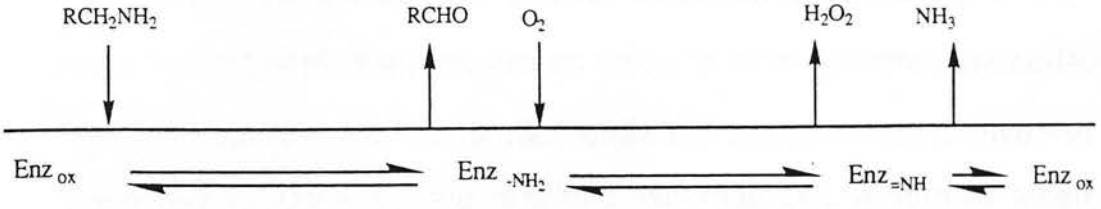


Figure 1.2. The proposed ping-pong kinetic course followed by lysyl oxidase. Peptidyl lysine is bound to the enzyme and becomes oxidatively deaminated. The reduced enzyme is reoxidised on binding of oxygen which receives two electrons and is released as hydrogen peroxide. Finally, the amine group is released as ammonia. Adapted from Kagan *et al.*, (1991).

the α-carbon of the amine substrate to a carbonyl cofactor. The enzyme is then required to be reoxidized by oxygen before it returns to a catalytically competent form (Kilgore *et al.,* 1989). These electrons are then released within hydrogen peroxide. Copper is also required for catalysis (Gaucheru *et al.,* 1990), although its role is still undetermined (section 1.4.4.1).

1.4.3 Cross-link formation

Following the conversion of lysine to allysine, a spontaneous condensation takes place in which various forms of cross-links are formed (Foster *et al.,* 1974; Davis, 1978; Eyre *et al.*, 1984). Allysine can condense with another allysine to form an aldol condensation product (ACP) or with the ε-amino group of an unoxidized lysine to form dehydrolysinonorleucine (LNL). In elastin, ACP is formed when the lysine residues involved are separated by three alanine residues, whereas LNL is formed when two alanines separate the lysines (Mecham and Heuser, 1991). These cross-links in turn condense to form stable pyridinium cross-links called desmosine and isodesmosine. ACP and LNL are common to both collagens and elastin.

The presence of hydroxylysine in collagens leads to the formation of additional cross-links, not observed in elastin, called dehydrohydroxylysinonorleucine and dehydroxydihyroxylysinonorleucine. Hydroxylated cross-links are capable of forming keto-amines which provide more stability to collagen (Robins, 1982), and can exist in glycosylated forms. In collagen ACP is also able to react with histidine to form aldol histidine. In cartilage, pyridinolines are formed that covalently

link collagen IX molecules to collagen II molecules (Eyre *et al.,* 1987).

1.4.4 Cofactors

1.4.4.1 Copper

Lysyl oxidase requires copper, as first discovered by feeding animals on copper deficient diets, which resulted in lathyritic-like disorders including bone fragility, aortic rupture, osteoporosis and joint deformities (Shields *et al.,* 1962; O'Dell *et al.,* 1961). Upon examination of tissue from these animals, tendon allysine levels were found to be decreased (compared to controls) while soluble collagen levels had increased (Chou *et al.*, 1969). In aorta and lung elastin, increased lysine and decreased desmosine levels were observed (Buckingham *et al.*, 1981). In chicks there was a significant reduction in lysyl oxidase activity (Rayton and Harris, 1979), and the observed effects were reversed when $CuSO_4$ supplements or serum copper proteins supplied with copper were administered. Only lysyl oxidase made *de novo* was able to incorporate copper (Rayton and Harris, 1979).

During the conversion of lysine to allysine, release of the aldehyde requires the presence of copper (Gaucheru *et al.,* 1990). The precise role of copper here is still undetermined; however the following two possibilities have been put forward. The metal ion may be involved in creating a complex with a putative carbonyl cofactor to form a catalytic semiquinone intermediate after the oxidation of the amine residue. The complex formed could then react with oxygen (Dooley *et al.*, 1991). Alternatively copper may stabilise a catalytic intermediate between the enzyme and its

substrate, necessary for this first stage of the reaction.

Recent studies suggest that copper may be incorporated into a copper-talon complex encoded by residues 284-301(in the human lysyl oxidase precursor) containing a single cupric ion (Krebs and Krawetz, 1993). A tyrosine residue at position 295 in this complex could be a candidate for modification to the putative carbonyl cofactor topaquinone (see 1.4.4.2) which would then be available for binding to the copper ion.

1.4.4.2 Carbonyl cofactor

Debate has surrounded the identity of the second cofactor of lysyl oxidase since copper amine oxidases were first shown to possess a carbonyl group capable of reacting with phenylhydrazine to form a chromophoric complex (Yasunobu *et al.,* 1976). Initially pyridoxal phosphate had been considered to be this cofactor because of its ability to reverse the actions of certain lathyrogenic agents such as ureides, hydrazines and hydrazides (Levene, 1961). A diet deficient in pyrodoxine has been shown to lead to a decrease of elastin cross-links in chick aorta (Starcher, 1969). It was then observed that derivatization of lysyl oxidase with phenylhydrazine failed to produce a peak corresponding to the phenylhydrazone of pyridoxal phosphate when isolated by HPLC (Williamson *et al* .,1986a). Derivatization of purified human lysyl oxidase with 2,4-dinitrophenylhydrazine was found to yield a product that was spectrally and chromatographically identical with a hydrazone derivative of pyrroloquinolino quinone (PQQ; Van Der Meer and Duine, 1986). Further supporting evidence was supplied from absorption and resonance Raman spectroscopy studies of phenylhydrazones derived from bovine aortic lysyl

oxidase. These were thought to be similar to hydrazones of PQQ (Fig 1.3 a), and therefore provided strong evidence that the carbonyl cofactor of lysyl oxidase was either PQQ or a similar molecule (Williamson *et al.*, 1986b). No direct structural evidence however has shown PQQ to be present at the active site of any known amine oxidase.

A recent study by Klinman *et al.* (1991) has implicated another carbonyl containing molecule as a possible cofactor. Topaquinone (trihydroxyphenylalanine quinone or 6-Hydroxydopa; Fig 1.3 b) has been isolated from a pentapeptide present at the active site of bovine serum amine oxidase, and identified by mass spectrometry, proton nuclear magnetic resonance and ultraviolet-visible spectroscopy (Janes *et al.*, 1990).

The phenylhydrazone of topaquinone is highly reactive when it undergoes cyclization, and it is likely that a reaction with glutamate may occur to yield a product that would subsequently co-elute (on HPLC) with PQQ. This result could give rise to confusion over the exact nature of the cofactor (Janes *et al.*, 1990).

The mechanism by which topaquinone is synthesised is unknown. Evidence points to a post-translational modification of a tyrosine residue, since the codon UAC (which codes for tyrosine) has been found to correspond to the topaquinone incorporation position at the active site of yeast amine oxidase from *Hansenula polymorpha* (Mu *et al.*, 1992) as demonstrated by amino acid analysis and mass spectrometry.

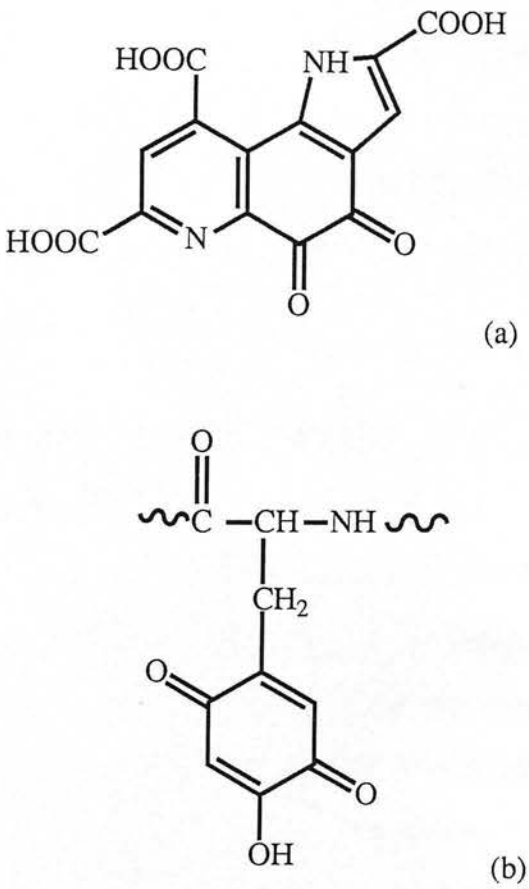


Figure 1.3. The structure of the putative carbonyl containing cofactors of lysyl oxidase. a) Pyrroloquinoline quinone b) Topaquinone.

A consensus sequence for the incorporation of topaquinone at the active site of copper-dependent amine oxidases has been postulated (Janes *et al.,* 1992). This sequence, Asn-Tyr-Asp/Gln, is present in bovine serum amine oxidase, porcine serum amine oxidase, pig kidney amine oxidase and pea seedling amine oxidase (Janes *et al.,* 1992). The consensus sequence is absent in lysyl oxidase, but Asn-Tyr-Tyr-Asp is present (residues 185-188) in the human lysyl oxidase precursor (Hamalainen *et al.,* 1991), and is conserved in the chick (Wu *et al.*, 1992), rat (Trackman *et al.*, 1991) and pig (A.D.Cronshaw; personal communication) enzymes.

1.4.5 Inhibition of lysyl oxidase

Lysyl oxidase is inhibited by 0.2 mM β-aminopropionitrile (BAPN), a naturally occurring lathyrogen found in *Lathyratus odoratus* (sweet pea). Other amine oxidases are inhibited by this reagent, though at much higher concentrations e.g. porcine plasma amine oxidase requires 5mM BAPN (Page and Benditt, 1967) for total inhibition.

Though first noted by Pinnell and Martin (1968) to be an irreversible inhibitor of lysyl oxidase activity, the mechanisms of BAPN and other lysyl oxidase inhibitors are not fully understood. BAPN binds covalently to lysyl oxidase, as shown by addition of ^{14}C-BAPN to the diet, and subsequent co-elution of lysyl oxidase and ^{14}C-BAPN by gel exclusion chromatography (Narayanan *et al.,* 1972).

With elastin or alkyl amine substrates, the initial reaction between lysyl oxidase and BAPN is competitive. After further incubation with the enzyme,

the reaction becomes irreversible, in a time and temperature dependent manner. Studies using [1,2-^{14} C] BAPN and [3-^{14} C] BAPN were used to examine the mechanism of inhibition of lysyl oxidase (Tang *et al.*, 1983). These studies suggested that BAPN is activiated by binding to the enzyme through Schiff base formation with a carbonyl group from the co-factor at the active site. An intermediate is then formed which becomes covalently attached to the enzyme, leading to inactivation (Tang *et al.,* 1983; Fig 1.4).

1.4.6 Lysyl oxidase purification

Lysyl oxidase activity was first demonstrated in a PBS (100mM phosphate buffer, 0.16M NaCl pH 7.4) extract of embryonic chick bone (Pinnell and Martin, 1968). The enzyme was found to be stable in 4-6M urea (Narayanan *et al.,* 1974), and this finding led to the use of DEAE-cellulose ion exchange (where in the absence of urea all activity was permanently bound) to achieve 50-100 fold purification of lysyl oxidase. Urea solubilisation of lysyl oxidase also allowed affinity chromatography on a collagen derivitized Sepharose 4B matrix, from which almost all activity was recovered (Narayanan *et al.,* 1974).

In the absence of urea, lysyl oxidase forms aggregates of up to 1000 kDa and displays low solubility (Jordan *et al.*,1977). The enzyme is only partially active in 6M urea, although activity is fully recovered after removal of urea by dialysis. Lysyl oxidase is stable in 6 M urea for several months at -20 ^{0}C.

Modifications to the procedure of Narayanan *et al.* (1974), using lathyritic collagen affinity columns and two separate DEAE-cellulose steps,

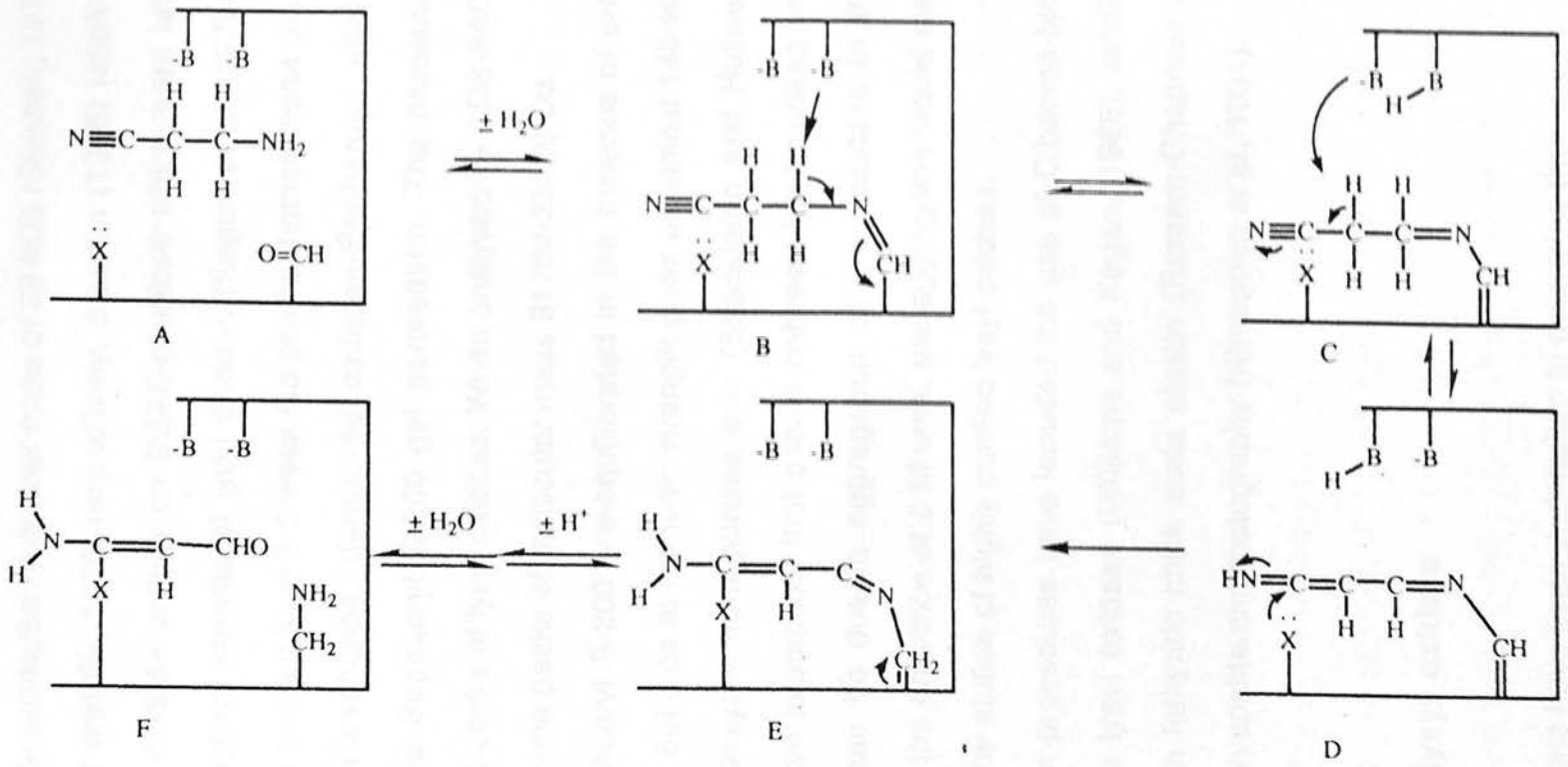


Figure 1.4. Proposed scheme for the mechanism of BAPN inactivation of lysyl oxidase (Tang *et al.*, 1983). In this scheme BAPN is initially bound to a carbonyl moiety on the putative cofactor (PQQ or topaquinone) in the active site by a Schiff base (B). An electrophilic ketenimine species is then formed (D) which is attacked by an enzyme nucleophile and becomes irreversibly bound in a covalent attachment to the enzyme residue X (E and F).

allowed purification of the embryonic chick cartilage enzyme to apparent homogeneity, with an estimated molecular mass of 28 kDa (Stassen, 1976) and 62 kDa (Siegel and Fu, 1976) respectively. Stassen (1976) resolved four peaks of lysyl oxidase activity on DEAE-cellulose which were later shown to be four distinct variants of lysyl oxidase (Kagan *et al.*, 1979a). These variants were purified from 2-6 week old bovine thoracic aorta using DEAE-cellulose ion exchange, denatured collagen-Sepharose affinity chromatography and Sephacryl S-200 gel permeation. The procedure yielded four different peaks of lysyl oxidase. When analysed by SDS-PAGE this yielded four enzyme peaks of molecular mass 31700-33100 Da.

The use of Sephacryl S-200 pre-equilibrated in the absence of urea and then eluted with 6M urea at low ionic strength gives an almost 140 fold purification of the enzyme from porcine skin (Shackleton and Hulmes, 1990a). The 'selective interaction' that occurs between lysyl oxidase and Sephacryl S-200 may be due to aggregation of lysyl oxidase in the absence of urea. In the presence of 6 M urea, the aggregates would then dissociate, followed by elution of highly purified lysyl oxidase.

Other purification procedures have included the use of Cibacron-blue dye, which binds to lysyl oxidase (Williams and Kagan, 1985), affinity chromatography with lathyritic chick aorta elastin (Romero-Chapman *et al.*, 1991) and hydroxyapatite chromatography (Almassian *et al.*, 1991).

1.4.7 Variants of lysyl oxidase

A number of studies have been undertaken to determine the differencies between the variants of lysyl oxidase. None of the variants

bind to concavalin-A Sepharose or stain with periodic acid / Schiff reagent (Kagan *et al.,* 1979a). Furthermore treatment of the four variants with alkaline phosphatase or neuraminidase does not affect their elution position on ion-exchange chromatography (Sullivan and Kagan, 1982). These observations indicate that the variation is not due to glycosylation. Furthermore, the variants persist after reduction and alkylation (Williams and Kagan, 1985), showing that they are not disulphide isomers.

Slight differences in amino acid composition, as well as small differences in the tryptic and *S.aureus* V8 protease digestion patterns, suggest that the primary sequence is the most likely source of the heterogeneity of these species (Sullivan and Kagan, 1982).

1.4.8 Regulation of lysyl oxidase

Regulation of the activity and expression of lysyl oxidase is of vital importance in connective tissue. Elastin and collagen synthesis, hence foundation and maintenance of the extracellular matrix is dependent on lysyl oxidase activity.

Steroid hormones are lipid-soluble molecules that can freely travel through plasma membranes. Steroid hormones commonly interact with a specific receptor in the cytoplasm. The receptors have a hormone binding site and a DNA binding site. When bound, the hormone-receptor complex migrates to the nucleus where it binds to specific sites on DNA and stimulates transcription (Weinberger *et al.,* 1986).

Oestrogen has been found to influence lysyl oxidase activity in different murine tissues (Ozasa *et al.*,1986). In mice, during pregnancy, lysyl oxidase activity was found to be high during oestrus and low during

dioestrus. An inverse relationship was also noted between the dilatability of the uterine cervix and the level of lysyl oxidase activity. Ovariectomy in these mice caused a decrease in lysyl oxidase activity of 75% after 3 days, which could be restored to levels observed at oestrus after injection of 1mg β-oestradiol (Ozasa *et al.*,1986). Skin collagen solubility is greatly reduced and lysyl oxidase activity significantly increased in bone and skin of gonadectomized 7 week old mice after treatment with 2mg oestrogen (Sanada *et al.*, 1978).

Testosterone (10-100mM) in the growth medium of calf aortic smooth muscle cells has been observed to stimulate lysyl oxidase activity 2.5 fold in the medium, and 5.5 fold in the cell layer (Bronson *et al.*, 1987).

Hypophysectomy in rats greatly reduces skin lysyl oxidase activity, which indicates an influence by hormones secreted by the pituitary (Shoshan and Finkelstein, 1976).

It is interesting to note that an untranslated 3' 258 bp region of the lysyl oxidase gene is 93% identical to a region in the 3' untranslated sequence of the elastin gene. These regions usually determine the rate of production and stability of the mRNA transcripts synthesised which suggests that expression of the two genes may in some way be co-regulated (Trackman *et al.*, 1990). Studies in chick aorta indicate that lysyl oxidase and collagen / elastin expression are indeed co-ordinated, such that the mRNA transcripts of lysyl oxidase peak at 12 days in chick aorta, followed by type I collagen and tropoelastin mRNA, which peak 2 days later (Wu *et al.*, 1992).

It has been noted that after neoplastic transformation of rat fibroblasts, both collagen and ras-recision genes (a phenotypic suppressor of the ras oncogene; Contente *et al.*, 1990) are down-regulated (Hajnal *et al.*, 1993).

Reversion of rat H-ras transformed fibroblasts was found to induce both lysyl oxidase and type I collagen αI chains, which also suggests co-regulation of expression (Hajnal *et al.*, 1993).

Finally, extracts of porcine skin have been found to enhance lysyl oxidase activity when elastin is used as substrate (D.R. Shackleton, unpublished observation). This may indicate that a lysyl oxidase regulating substance exists.

1.4.9 Assay of lysyl oxidase

For assay of lysyl oxidase activity on collagen or elastin substrates, the conventional procedure depends on the formation of tritiated allysine. When collagen or elastin is radiolabeled with [6-^{3}H] or [4,5-^{3}H] lysine, tritiated protons released during the reaction become incorporated into tritiated water (Fig 1.5). This labelling event is either direct, from the 6-position ([6-^{3}H] lysine), or occurs after keto-enol tautomerism of allysine ([4,5-^{3}H] lysine) which releases a proton from the carbon in the 5-position. During the course of the reaction, tritiated water is released in a linear fashion over several hours (Pinnell and Martin, 1968). The use of [4,5-^{3}H] lysine label is not ideal in collagen, since the conversion of lysine to hydroxylysine leads to the loss of one tritium atom on the 5-position (Popenoe *et al.*, 1965).

An alternative assay procedure measures allysine directly (Pinnell and Martin, 1968). [^{14}C]lysine labelled collagen or elastin substrate is converted to [^{14}C]allysine and then subsequently oxidised by performic acid to become aminoadipic acid which is then hydrolysed for 72 hrs in 6N

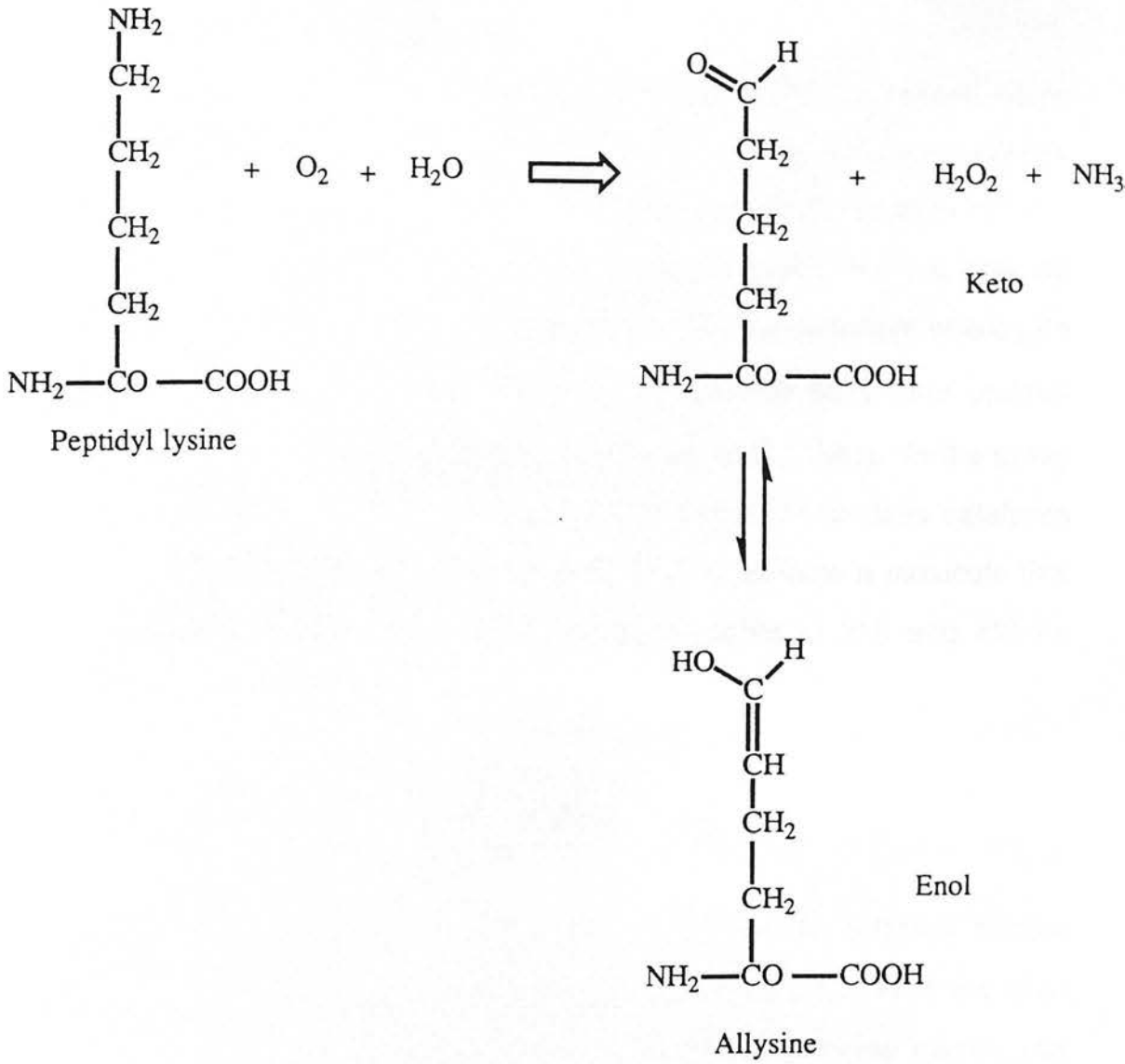


Figure 1.5. Lysyl oxidase assay using [4,5-^{3}H] lysine labelled elastin as substrate. Lysine is oxidatively deaminated to α-aminoadipic-δ-semialdehyde (allysine) which then undergoes keto-enol tautomerisation to release a tritum atom from the 5-position which is incorporated into water.

HCl at 107 °C.

Amino acid analysis of the hydrolysate is then used to separate and quantify the [^{14}C] aminoadipic acid.

The above reactions require long incubation periods of several hours and must be terminated before analysis can take place. They are therefore discontinuous and can provide no information about the kinetics of lysyl oxidase activity. In contrast, measuring H_2O_2 release using the artificial substrate 1,5-diaminopentane gives a continuous measurement of enzyme activity for up to 300 turnovers of the enzyme at 55°C (the optimal temperature for lysyl oxidase activity; Trackman *et al.,* 1981). In the assay developed by Trackman *et al.* (1981) horse radish peroxidase catalyses the reaction between H_2O_2 and homovanillate to produce a molecule that fluoresces at excitation and emission wavelengths of 315 and 425nm respectively.

1.4.10 Localisation of lysyl oxidase

Lysyl oxidase is secreted by cells in culture. In cultured human fibroblasts, the cell medium was found to contain almost all of the lysyl oxidase activity (Layman *et al.,* 1972). In rabbit aorta smooth muscle cell cultures, approximately 40 % of the lysyl oxidase activity was found to be in the medium (Gonnerman *et al.,* 1981). Subsequent immunolocalisation techniques have established that lysyl oxidase is present in the extracellular matrix of connective tissue septa from fibrotic rat liver and tendon sheaths of 17 day-old chick (Siegel *et al.,* 1978).

Two studies using polyclonal, anti-lysyl oxidase antibodies provide

apparently conflicting results (Kagan *et al.,* 1986; Baccarani-Contri *et al.,* 1989). Chicken anti-lysyl oxidase polyclonal antibodies, in association with gold conjugated rabbit anti-chicken immunoglobulin G, labelled the amorphous elastin / microfibril interface in calf aorta and newborn rat aorta (Kagan *et al.,* 1986). The gold label was absent from both the microfibrils and the amorphous elastin distal to this interface.This supports the hypothesis that the microfibrils are used as a scaffold around which the mature elastic fibres are formed. Cross-linking of tropoelastin molecules to produce mature elastin would therefore presumably occur at the elastin / microfibril interface.The antibodies utilized here did not cross-react with collagen bound lysyl oxidase, which may indicate possible substrate specificity of the enzyme. In contrast to the results with antibodies against bovine lysyl oxidase, anti-human placental lysyl oxidase polyclonal antisera reacted against elastin fibres outside the region of the elastin / microfibril interface, and also cross-reacted with collagen in all the human tissues tested (Baccarani-Contri *et al.,* 1989).

The anti-human placental lysyl oxidase antiserum cross-reacted with a 24 kDa protein that co-purifies with lysyl oxidase (Kuivanemi *et al.,* 1984). Furthermore, four monoclonal antibodies against human lysyl oxidase also cross-reacted with a similar 24kDa protein (Burbelo *et al.*, 1986).

1.4.11 Lysyl oxidase in embryogenesis

Lysyl oxidase is required for cross-link formation in collagens from an early stage of vertebrate development. Type II collagen for example is widespread in embryonic chick at stages 14 -19, where it is present in the basement membrane of notochord, gut endoderm surface ectoderm and

auditory vesicle (Kosher and Solursh, 1989). In the adult chicken, type II collagen is restricted to cartilage, intervertebral discs and vitreous humour, perhaps indicating a more complex role for type II collagen than simply the provision of strength to the developing tissue. This is also suggested by the ability of types I and II collagens to promote embryonic cell differentiation and migration in chick embryo (Bilozur and Hay, 1988).

Collagen involvement in invertebrate development has been observed in the sea urchin *Lytechinus variegatus* which requires the presence of cross-linked collagen before entering gastrulation (Wessel and McClay, 1987). The specific inhibitor of lysyl oxidase, β-aminopropionitrile (BAPN) can prevent this progression. Subsequent removal of BAPN by washing restores development (Wessel and McClay, 1987). Lysyl oxidase is therefore necessary for successful tissue development, the failure of which can have serious and widespread effects.

1.4.12 Role of lysyl oxidase in disease

1.4.12.1 Genetically inherited connective tissue disorders

Several inherited diseases of connective tissue are associated with reduced levels of lysyl oxidase activity (Danks *et al.*, 1983; Byers *et al.*, 1976; Kuivaniemi *et al.*, 1982; Peltonen *et al.*, 1983). These disorders are functionally and morphologically similar to lathyrism, a condition induced in by feeding animals the specific lysyl oxidase inhibitor BAPN.

Two forms of human Ehlers-Danlos Syndrome (types V and IX) have been linked with functional lysyl oxidase deficiency as manifested by

hyperextensible skin, tissue fragility, joint laxity and cardiovascular disorder. Lysyl oxidase activity in cultured skin fibroblasts was found to be reduced in one study of patients with Ehlers-Danlos type V (Di Ferrante *et al.,* 1975), though skin biopsies from patients and control groups in another study contained similar lysyl oxidase activity and antigenicity (Siegel *et al.*, 1979). Equivalent numbers of reducible cross-links in skin collagen between the groups also confirmed that lysyl oxidase deficiency is not responsible for this disorder (Siegel *et al.,* 1979).

Ehlers-Danlos Syndrome type IX (X-linked cutis laxa or occipital horn syndrome) is a recessively inherited disease affecting copper metabolism. Skin fibroblast cultures from these patients display greatly reduced levels of lysyl oxidase activity and reduced conversion of soluble to insoluble collagen (Kuivaniemi *et al.,* 1982). Concentrations of serum copper and ceruloplasmin (a copper transport protein) are reduced, whereas copper concentrations are increased within the skin fibroblasts (Kuivaniemi *et al.,* 1982). The metal ion within these cells does not appear to be available for incorporation into copper dependent enzymes such as lysyl oxidase. Abnormalities in cell copper metabolism may therefore be the primary defect responsible for the connective tissue disorders observed (Kuivaniemi *et al.,* 1982).

A similar inherited X-linked disease of human connective tissue, Menkes' syndrome, is associated with 'kinked' hair, retarded growth, neurological degeneration and a lifespan of around 3 years (Royce and Steinman, 1990). Copper and ceruloplasmin levels in serum are also reduced and lysyl oxidase activity is 6 - 12 % of normal levels (Royce and Steinman, 1990).

A parallel can be drawn here between occipital horn disease, Menkes'

syndrome and the "mottled" and "blotchy" phenotypes observed in mice. "Mottled" mice have around 50% of the normal lysyl oxidase activity in skin and die of cerebral abnormalities after about 14 days, while the connective tissue is morphologically normal. Subcutaneous injections of 50 mg copper in Cu^+ form in an alkylpolyether / sebacic acid solution at 7 days increases lysyl oxidase activity to between 84 - 150% of normal mouse levels (Royce *et al.,* 1982). Copper provided in the correct form seems necessary for incorporation into lysyl oxidase. "Blotchy" mice have lower lysyl oxidase activity than the wild-type (20 - 30% in skin) and die, at around 150 - 200 days, of aortic aneurysm. These two mouse disorders may be due either to defects in two separate genes positioned close to each other on the X - chromosome, or two different alleles of the same gene. Menkes' syndrome and occipital horn syndrome may be the human equivalents of "mottled" mouse and "blotchy" mouse genotypes, respectively.

1.4.12.2 Fibrosis

Fibrosis in rat lung can be induced with exposure to cadmium chloride (Almassian *et al.,* 1991). Fibrosis is associated with an induction of collagen synthesis and a large increase in the synthesis and activity of lysyl oxidase (Counts *et al.,* 1981). Carbon tetrachloride can similarly induce a fibrotic response in rat liver, with an accompanying four-fold rise in lysyl oxidase activity and three-fold rise of lysyl oxidase mRNAs present (48 Kbp; Wakasaki and Ooshima, 1990a).

Oral administration of β-aminopropionitrile and $CdCl_2$ to Syrian

hamsters simultaneously induces an emphysema associated phenotype, suggesting that the amount of collagen deposition in the lung tissue after injury determines whether a fibrotic or emphysematous pathology occurs (Niewoehner and Hoidal, 1982). Lysyl oxidase may therefore play a key role in the development of these two diseases during the healing process which occurs after injury.

1.4.12.3 Role of lysyl oxidase in cancer

Many human neoplasms are associated with the presence of retroviral genes called oncogenes, which are believed to have evolved from human genes (Weinberg, 1983). The 'ras' gene encodes the protein p21, which is a 21 kDa guanyl-nucleotide binding protein. The retroviral equivalents of this gene contain mutations which inhibit the GTPase activity of p21, as a result of which the GTP form is constantly active. The specific effect of this on the cell is unknown.

There are several examples of the mutations in oncogenes that lead to cell transformation i.e. in the lung carcinoma cell line (HS - 242), Ha-ras gene encodes leu instead of glu at position 61 and in lung cell line (Calu-1) Ki-ras oncogene encodes cys instead of gly at position 12 (Tanaka *et al.*, 1986).

Kuivaniemi *et al.*, (1986) have shown that lysyl oxidase activity is greatly reduced in a number of human sarcoma cell lines (fibrosarcoma, embryonal rhabdomyosarcoma) and other tumour cell lines such as trophoblastic choriocarcinoma and melanoma.

Rat lysyl oxidase cDNA has been sequenced, and found to have 89 % identity with a murine protein termed ras - recision gene (rrg; Kenyon *et*

al., 1991), which is a putative tumour suppressor gene or anti-oncogene of ras. The expression of rrg is greatly reduced after transformation of mouse NIH 3T3 cells with activated long terminal repeat (LTR) c-H-ras (Contente *et al.,* 1990). Treatment of these transformed cells with interferon reverted them to phenotypically non-transformed and non-tumorogenic cells which retained high levels of ras mRNA and p21 expression. Ras-recision gene expression levels were between 35 and 200% of normal NIH 3T3 cells (Contente *et al.,* 1990).

The blocking of rrg expression in itself is not enough to induce cell transformation (Contente *et al.,* 1990). It is therefore possible that transformation of cells induced by ras may require the down regulation of the rrg gene product, and therefore lysyl oxidase expression.

1.4.13 Lysyl oxidase precursor cDNA

Rat aorta lysyl oxidase cDNA has been cloned and sequenced (Trackman *et al.,* 1990). This was carried out by screening a neonatal rat aorta cDNA library in λgt 11 with anti-bovine lysyl oxidase polyclonal antiserum. The open reading frame of this sequence is 1227 bp, which corresponds to a calculated prepro-enzyme mass of 45,979 Da, consistent with reports of a 48,000 Da lysyl oxidase precursor in fibrotic liver cells (Wakasaki and Ooshima, 1990a).

The cDNA sequences of human chick and murine lysyl oxidase have also been determined. The human placental sequence has an open reading frame of 1215 bp and is 78% identical to the rat sequence at the DNA level, and 84% identical at the amino acid level (Hamalainen *et al.,* 1991).This sequence is also 89% identical to the murine ras-recision gene

(Kenyon *et al.,* 1991).

Chick aorta lysyl oxidase has an open reading frame of 1260 bp, giving a calculated prepro-enzyme molecular mass of 48,150 Da. This sequence is only 60% identical, in the first 150 amino acids, with the rat sequence; however, the latter 270 amino acids have 92% identity. This strongly conserved part of the sequence would be expected to code only for the mature enzyme (Wu *et al.,* 1992).

Rat, human and chick lysyl oxidase cDNA sequences contain a putative metal binding consensus sequence His-X_4-Cys-X_5-Cys-X_4-His. This sequence is not totally conserved in chick aorta where the final histidine is absent (Wu *et al.,* 1992). Copper binding consensus sequences YGYHRRFAC and YTGHHAY are conserved in rat, human and murine sequences but only partially conserved in chick.

1.4.14 The lysyl oxidase gene and its expression

The lysyl oxidase gene has been characterised from several sources (Contente *et al.,* 1993; Kenyon *et al.,* 1993; Csiszar *et al.,* 1993; Mock *et al.,* 1992; Svinarichv *et al.,* 1992). The gene coding for murine lysyl oxidase is split into seven exons and six introns which span around 14 kbp of the mouse genome (Contente *et al.,* 1993). Two different sizes of mRNA transcript encode the enzyme. These are 4.8kb and 3.8kb in size, which differ in the length of their 3' non-coding regions through the use of separate poly A tracts (Contente *et al.,* 1993).The length of these mRNAs are consistent with human lysyl oxidase from one study which also displays a 2.0 kb transcript present in low abundance (Mariani *et al.,* 1992). In a separate study of the human gene however, transcripts were observed

at 4.3 kb, 2.5 kb and 2.0 kb (Svinarichv *et al.,* 1992).

Murine and human genes for lysyl oxidase have been mapped to chromosomes 18 and 5 respectively. A 1770bp cDNA fragment of human lysyl oxidase co-segregated with chromosome 5 when screening human x hamster somatic cell hybrids. Further in situ hybridization revealed that this fragment mapped to 5q 23.3-31.2 (Hamalainen *et al.,* 1991). Murine lysyl oxidase cDNA co-segregates with chromosome 18 in Chinese Hamster x Mouse somatic cell hybrids (Mock *et al.,* 1992).

A restriction fragment length polymorphism (RFLP) has been discovered within the coding sequence of the human lysyl oxidase gene. This mutation, a G to A transition, results in the appearance of a Pst 1 site into the sequence and results in arginine being replaced by a glutamine at codon 158. The RFLP occurred in one-third of a large population of apparently healthy, normal individuals (Csiszar *et al.,* 1993).

1.4.15 Lysyl oxidase post-translational modifications

Lysyl oxidase appears to be synthesised as a 47-50 kDa precursor protein which is N-glycosylated. Pulse-chase experiments with rat aortic smooth muscle cells have established that the 50 kDa precursor protein is processed to the mature 32 kDa form by removal of an N-terminal propeptide (Trackman *et al.,* 1992). There is also a putative, 21 residue, N-terminal signal peptide (Trackman *et al*., 1992). The putative cleavage site for removal of the propeptide has been predicted to be between Arg 134 and Arg 135 in the rat sequence, based on the calculated mass of the mature protein (Trackman *et al.,* 1990). However this predicted position of the proteolytic site is not consistent with laser desorption mass

spectrometry and amino acid sequencing data, which indicate that the site in human lysyl oxidase is between Gly 168 and Asp 169 (A.D. Cronshaw, personal communication).

Potential glycosylation sites within the lysyl oxidase sequence (Asn-Arg-Thr) occur at positions 91-93 and 138-140 in the rat sequence and position 97-99 in human lysyl oxidase. Incubation of the 50 kDa precursor form of rat lysyl oxidase with glycopeptidase F reduced its molecular mass to 45 kDa (Trackman *et al.,* 1992), while similar incubation of glycopeptidase F with the 32 kDa form had no effect on molecular mass. This indicated that only the 50 kDa precursor form was N-glycosylated even though the second potential glycosylation site lies beyond the processing site of the mature sequence as predicted by Trackman *et al.,* (1990; Arg 134-Arg 135).

1.5 Tyrosine rich acidic matrix protein

A protein that co-purifies with porcine skin lysyl oxidase has recently been isolated that has a molecular mass of 22kDa, and contains an abundance of tyrosine residues (11%; Cronshaw *et al.,* 1993). This protein, named Tyrosine Rich Acidic Matrix Protein (TRAMP), is the same as a protein isolated from bovine skin that co-purifies with dermatan sulphate proteoglycans (Neame *et al.,* 1989), and the human equivalent has recently been named 'Dermatopontin' (Superti-Furga *et al.,* 1993).

Other proteins about the same size as TRAMP have previously been observed to co-purify with lysyl oxidase. Sullivan and Kagan, (1982) observed a 24 kDa protein in extracts of bovine aorta. Proteolytic digests of this protein showed it to be very similar to lysyl oxidase, and the amounts of

the protein increased during storage of the enzyme. Lysyl oxidase from human placenta is also contaminated by a similar 23 kDa protein that cross-reacts with polyclonal antisera raised against the pure enzyme. The quantity of this protein also increased during storage of lysyl oxidase (Kuivaniemi *et al.,* 1984). Furthermore, a monoclonal antibody raised against bovine aortic lysyl oxidase has also been shown to cross-react with a 24 kDa protein (Burbelo *et al.,* 1986). Wakasaki and Ooshima, (1990b) also observed a 24 kDa protein that co-purified with lysyl oxidase from human umbilical cord. Amino acid analysis and peptide maps produced using *S. aureus* V8 protease showed that this protein closely resembled lysyl oxidase. TRAMP however has recently been found to contain no sequence homology with lysyl oxidase and is therefore a distinct protein (Cronshaw *et al.*, 1993).

TRAMP contains three large homologous domains, each with the repeating sequence Asp-Arg-Glx-Trp-Asn / Gln / Lys-Phe / Tyr. A scheme put forward by Neame *et al.* (1989), in which the disulphide bridge arrangement within TRAMP places one of these repeats in each of three loop structures, raises the possibility that these motifs interact with other molecules within the extracellular matrix.

Five variants of TRAMP have been resolved by mono Q ion-exchange FPLC (Cronshaw *et al.,* 1993). The five types have distinct isoelectric points, ranging from pI 4.1-4.4, but have identical electrophoretic mobility on SDS-PAGE and reverse-phase chromatography elution. TRAMP appears to be sulphated since it stains with Alcian blue, and sulphatase treatment reduces this staining significantly. The sulphation is probably associated with the tyrosine residues because acid hydrolysis reduces the staining, consistent with the susceptibility of the tyrosine ester bond to acid

hydrolysis (J.R.E. MacBeath, personal communication).

The biological significance of TRAMP is currently under investigation. It has been found to accelerate the formation of type I collagen fibril formation *in vitro* by reconstituting fibrils of lathyritic rats in warm start conditions (MacBeath *et al.,* 1993). This acceleration was observed at low concentrations of TRAMP, a molar ratio of 1 : 20 (TRAMP : Type I collagen). All the TRAMP variants were found to accelerate the fibril formation.

TRAMP contains the putative topaquinone incorporation consensus sequence Asn-Tyr-Asp, associated with copper dependent amine oxidases. The sequence Tyr-Asn-Tyr-Asp-Tyr incorporating the consensus sequence in TRAMP is the same as that in pig kidney amine oxidase (Janes *et al.,* 1992).

1.6 Aims of the present study

In the first part of the project, the aims were to determine whether TRAMP is a degradative product of lysyl oxidase, since proteins (22-24 kDa) that co-purified with lysyl oxidase in human umbilical cord were found to closely resemble the enzyme (Burbelo *et al.,* 1986; Wakasaki and Ooshima, 1990b); to find out how widespread TRAMP is in different tissues, and to discover whether TRAMP had any enzymatic activity on collagen, since previous studies had shown that TRAMP accelerates collagen fibril formation *in vitro* (MacBeath *et al.,* 1993).

A further aim of the project was to produce a radiolabeled fragment of rat aorta lysyl oxidase cDNA, which could be used to probe a human placental cDNA library, and obtain the sequence of human lysyl oxidase precursor DNA. Mature human placental lysyl oxidase could then be

amplified using PCR and cloned into a yeast shuttle vector for production and secretion of mature lysyl oxidase in *S. cerevisiae*. This work would remove the necessity for 6M urea during the purification of lysyl oxidase, and would allow genetic manipulation of the enzyme using recombinant DNA technology.

Finally, an investigation was to be undertaken of urea extracts from porcine skin that had been found to contain no endogenous lysyl oxidase, but were able to enhance lysyl oxidase catalysed tritium release from [4,5-^{3}H] lysine labelled elastin substrate (D.R. Shackleton, unpublished observations).

CHAPTER 2

Materials and Methods

2.1 Preparation of Lysyl oxidase and TRAMP

2.1.1 Materials

Stillborn piglets were obtained from the Animal Breeding Research Organisation, Dryden-Montmarle Field Laboratory, Roslin, Midlothian. Materials for chromatography, including columns and media were from Pharmacia / LKB, Milton Keynes. AG 501- X8 mixed bed resin was from Bio-Rad, Hemel Hempstead. All other chemicals (analytical grade), unless otherwise stated, were from BDH, Poole, Dorset.

2.1.2 Procedure

All the following steps were performed at 0-4°C. Lysyl oxidase was purified from the skins of stillborn piglets using a modification of the method of Shackleton and Hulmes (1990a).

The skins from five fresh stillborn piglets (wet weight 450g) were washed with water then minced with a hand mincer and homogenised into 500mls of 0.15 M NaCl, 1mM PMSF, 10mM sodium phosphate, pH 7.8 (PBS) using a 4 litre Waring blender. The homogenate was centrifuged in six 250ml Nalgene centrifuge tubes at 12400g for 20 minutes. Fatty deposits were removed, and the pellets were rehomogenised and centrifuged again.

The pellets were washed twice in 10mM sodium phosphate, pH 7.8 (PB) containing 1mM PMSF, and homogenised into 6M Urea, 10mM sodium phosphate, pH 7.8 (PBU) containing 1mM PMSF. Lysyl oxidase is

soluble only in the presence of high concentrations of urea (Narayanan *et al.,* 1974; to remove traces of cyanate, urea stock solutions were passed through a column of AG 501- X8 mixed bed resin (1.6cm x 15cm) immediately prior to buffer preparation). Three sequential extracts in PBU were made, over 16 hrs, 8 hrs and 16 hrs. Homogenates were centrifuged as above and the supernatants were pooled. After filtering twice through Whatman No.1 paper, the pooled extracts were loaded onto a DEAE-Sepharose Fast flow ion exchange column (2.6 x 40 cm), pre-equilibrated with PBU, at a flow rate of 30 mls / minute, followed by washing in PBU. Elution of the protein containing fractions with PBU containing 0.5 M NaCl at a flow rate of 25 mls / minute was monitored by absorbance at 280 nm on an LKB Ultraspec II spectrophotometer. Protein containing fractions were pooled and dialysed exhaustively against PB. The small precipitate formed during dialysis was removed by centifugation at 12400 g for 20 minutes and the remaining solution was loaded onto a Sephacryl S-400 column (2.6 x 40 cm) pre-equilibrated with PB. When run in these buffer conditions, the Sephacryl S-400 acts as an "affinity" column, giving purification of almost 140 fold (Shackleton and Hulmes, 1990a). The column was loaded at 10 mls / minute, washed in PB and eluted with PBU at a flow rate of 10 mls / minute.

The fractions produced from this step were then run on SDS-PAGE to identify lysyl oxidase and TRAMP. Protein containing fractions were assayed for lysyl oxidase activity. The protein concentration in the fractions was measured using the Bradford (1976) assay. Lysyl oxidase and TRAMP samples were stored at -20 ^{o}C.

This purification procedure produced lysyl oxidase fractions

contaminated by large quantities of TRAMP. Improvements in the procedure to separate these proteins are described in section 5.1.

2.2 Protein assay

Protein concentrations of each sample were determined using the Bradford assay (Bradford, 1976). A standard curve of protein concentrations was set up using 10-100μg of bovine serum albumin (BSA; Sigma) diluted in 0.15 M NaCl. To each protein sample (100 μl), 1 ml of Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol; 8.5% (v/v) phosphoric acid) was added, mixed and incubated at room temperature for 30 minutes. Absorbance at 595nm was then recorded using an LKB Ultraspec II spectrophotometer. Protein concentrations of samples were determined by comparing absorbance at 595nm with the BSA samples on the standard curve.

2.3 Preparation of [4,5-^{3}H] lysine labelled elastin substrate

2.3.1 Materials

Seventeen day old chicks were supplied by Ross Breeders, Newbridge, Midlothian. MEM Select-amine kits were from GIBCO, Paisley, Srathclyde. BAPN (fumarate salt) was from Sigma Chemical Co., Poole, Dorset. All other reagents (analytical grade), unless otherwise stated, were from BDH, Poole, Dorset.

2.3.2 Elastin purification procedure

The [4,5-^{3}H] lysine labeled elastin was prepared essentially as described (Pinnell and Martin, 1968). Modified-MEM was prepared without lysine, but supplemented with 50μg / ml ascorbic acid, glycine, alanine, valine and proline to increase the yield of elastin (Shackleton and Hulmes,1990b).The lathyrogen β-aminopropionitrile (0.2mM) was also added to the incubation medium to inhibit endogenous lysyl oxidase activity.

2.3.2.1 Dissection and radiolabeling

The thoracic arteries from 10 dozen 17-day chick embryos were removed and placed into petri-dishes containing incubation medium. The arteries were washed twice in fresh medium, then placed in 300 ml of modified MEM using a 500 ml autoclaved Erlenmayer flask (Nalgene). The flask was gassed for 1 min with 5% CO_2 / 95% air and then incubated (at 100 rpm in a Labtherm orbital shaker) for 1 hour to deplete the tissues of lysine. The arteries were then transferred to fresh medium containing 55 MBq [4,5-^{3}H] lysine per 100 mls. The arteries were then re-gassed and incubated overnight.

2.3.2.2. Extraction of elastin

The thoracic arteries were drained and washed with several changes of cold distilled water. They were then homogenised in 50 ml of 150 mM

NaCl on ice and centrifuged at 10,000g for 5 minutes at 2°C. The supernatant was discarded and the pellet re-homogenised as before. The pellet was then homogenised in 1N HCl, and centrifuged as before. This step was repeated. Finally, the pellet was homogenised in 150 mM NaCl, 100 mM sodium borate pH 8.0 (assay buffer) and centrifuged. This step was repeated.

A 100µl sample of the elastin produced was added to 900µl Ultima Gold liquid scintillation cocktail (Packard), and ^{3}H counts were determined using a Packard 1900 CA liquid scintillation analyzer, assuming 60 % counting efficiency. The concentration of the labeled elastin was adjusted with assay buffer to give 3 x 10^{5} dpm per 100µl.

During storage at -20°C, the elastin became increasingly insoluble and therefore it was re-homogenised in assay buffer immediately before use.

2.4.1 Preparation of [4,5-^{3}H] lysine labelled collagen substrate

The [4,5-^{3}H] lysine labeled collagen was prepared essentially as described (Siegel, 1974). Calvaria from 10 dozen 17 day old chick embryos were removed and incubated in lysine-free MEM supplemented with glycine, alanine, valine, proline, ascorbate and β-aminopropionitrile as above. The calvaria were washed in fresh medium, then pre-incubated and labelled with [4,5-^{3}H] lysine as above (section 2.3.2.1).

2.4.2 Extraction of collagen

The calvaria were washed three times in Hanks' balanced salt solution

(HBSS; GIBCO), and then homogenised with a Polytron homogeniser in 20 ml of 1M NaCl, 20mM EDTA, 10mM NEM, 1mM PMSF, 50 mM Tris, pH 7.5. The homogenate was then diluted to a total volume of 200 ml with water and extracted at 4^0C overnight, followed by centrifugation at 28,000g for 10 minutes. The supernatant was precipitated overnight after the addition of 3.5 M NaCl, and this was centrifuged at 28,000g for 10 minutes. The pellet was then washed in 200ml of 3.5 M NaCl, 50 mM tris, 20mM EDTA, 10mM NEM, 1mM PMSF, pH 7.5 and centrifuged at 28,000g for 10 minutes and then resuspended overnight in 10 ml of PBS (100 mM NaH_2PO_4, 0.15 M NaCl, pH 7.8), with continuous rotation. The solution was dialysed against PBS exhaustively, and finally centrifuged at 37,000g for 20 minutes. The concentration of the labeled collagen was adjusted with PBS to give 342,000 cpm per 100μl. The radiolabelled collagen was then aliquoted, and stored at -30^0C.

2.5 Lysyl oxidase assay

2.5.1 Introduction

The method used to assay lysyl oxidase is a modification of the procedure developed by Pinnell and Martin (1968). Lysyl oxidase activity is measured by the release of tritium from allysine, which is formed by lysyl oxidase catalysed oxidation of the [4,5-^{3}H] labeled lysine in the elastin substrate. The keto form of allysine produced during the reaction undergoes keto-enol tautomerism, with the result that a tritium atom is released from the 5-position to become incorporated into water. Tritiated

water is subsequently isolated and measured for radioactivity.

2.5.2 Lysyl oxidase reaction

Lysyl oxidase assays were routinely performed in duplicate (protocols 1a and 1b). Prior to each assay, the [4,5-^{3}H]lysine labeled elastin was homogenised in assay buffer. Aliquots of 100 μl of elastin substrate with a specific activity of 3000 dpm / μl were added to 700μl assay buffer in 1.5 ml Eppendorf plastic tubes. Typically, 2 μg of lysyl oxidase were added to the assay mixture and the total reaction volume was always adjusted to 900μl with PBU. Control assays were performed without enzyme to allow calculation of basal tritium release. The contents of the Eppendorf tube were mixed by vortexing, then incubated at 37°C for 16 hours. Trichloroacetic acid (50 % w/v) was then added to each tube to a final concentration of 5% (w/v), to precipitate high molecular weight, tritium labelled protein. The reactants were then centrifuged at 15400g for 5 minutes.

2.5.3 Isolation of tritiated water by ultrafiltration and microdistillation

Following centrifugation, two methods for isolation of tritiated water were utilized. The first method, ultrafiltration, is based on the filtration of tritiated water through the membrane of a Millipore Ultrafree-MC filter unit with a cut off point of 10,000 Da (Shackleton and Hulmes, 1990b; protocol 1a). The pore size of this filter also allows small, labelled peptides to pass

To a 1.5 ml Eppendorf tube, add:

Elastin: 100µl (3000dpm / µl) of [4,5-^{3}H] lysine labelled elastin.

Lysyl oxidase: 100µl

Assay buffer: 700µl

1. Vortex mix the reactants, and incubate for 16 hours at 37 ^{0}C.

   Add trichloroacetic acid 50% (w/v) to each tube to give a final volume of 5% (w/v).
2. Centrifuge the sample tubes at 15400g for 5 minutes. Transfer 400µl of the supernatant into a Millipore Ultrafree-MC filter unit (10,00 kDa cut off point), and centrifuge in a Beckman JA 18.1 rotor at 5000g for 90 minutes at 4^{0}C.
3. Remove 300µl of the filtrate from each filter unit and add 2.7 ml Ultima Gold liquid scintillation cocktail (Packard).
4. Count ^{3}H in a Packard 1900 CA liquid scintillation counter.

Protocol 1a. Lysyl oxidase assay using ultrafiltration (Shackleton and Hulmes, 1990b).

To a 1.5 ml Eppendorf tube, add:

Elastin: 100µl (3000dpm / µl) of [4,5-^{3}H] lysine labelled elastin.

Lysyl oxidase: 2µg

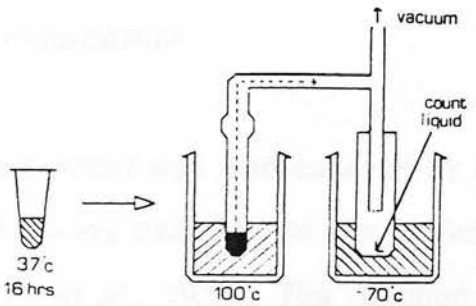
Assay buffer: 700µl

1. Vortex mix the reactants, and incubate for 16 hours at 37^{0}C.

   Add trichloroacetic acid 50% (w/v) to each tube to give a final volume of 5% (w/v).

2. Centrifuge the sample tubes at 15400g for 5 minutes. Transfer 800µl of the supernatant into a pyrex boiling tube with 5-10 anti-bumping granules (BDH).

3. Set up the microdistillation apparatus is set up as below under vacuum .



4. Heat the boiling tube at 100^{0}C for 5 minutes, and then seal the cooled collecting tube with Nescofilm until the distillate has thawed.

5. Take 300µl of the distillate and add 2.7 ml of Ultima Gold liquid scintillation cocktail (Packard).

4. Count ^{3}H in a Packard 1900 CA liquid scintillation counter.

Protocol 1b. Lysyl oxidase assay using microdistillation (Pinnell and Martin, 1968).

through, which may raise the radioactive background in the filtrate. This method is convenient however, and it was used when a large number of samples required to be assayed rapidly.

The second method of isolation involves evaporation of tritiated water in a microdistillation apparatus (Pinnell and Martin, 1968; protocol 1b). The reactants are heated to 100°C under vacuum in the presence of anti-bumping granules. The tritiated water then condenses in a separate part of the apparatus cooled to -70°C. Theoretically only tritiated water should be collected using this procedure leading to a lower background level of radioactivity. The lathyrogen BAPN (0.2 mM), which inhibits the activity of lysyl oxidase completely, was routinely included in separate assays as a control.

2.6 Peroxidase coupled lysyl oxidase assay

2.6.1 Introduction

Lysyl oxidase was also assayed by measuring the hydrogen peroxide released during oxidation of the artificial substrate 1,5-diaminopentane (Trackman *et al.,* 1981). The reaction was coupled to the peroxidase catalysed oxidation of homovanillate to a molecule that fluoresces at excitation and emission wavelengths of 315nm and 425nm, respectively. A Perkin-Elmer LS-3B fluorimeter was fitted with a heating block apparatus set at 52°C (the optimal temperature for the reaction; Trackman *et al.,* 1981).

2.6.2 Calibration

To calibrate the fluorimeter, horse radish peroxidase type VI A (20μg) and homovanillate (125 μg; both obtained from Sigma) were heated to 52 ^{0}C in 1ml assay buffer (section 2.4.2) in a cuvette. Aliquots of 0.2 nmoles H_2O_2 were then added to the reactants to obtain a standard plot relating fluoresence to the amount of H_2O_2.

2.6.3 Determination of activity

The lysyl oxidase substrate 1,5-diaminopentane (175μg) and lysyl oxidase (typically 2μg) were added to the reactants, and a fluoresence reading was recorded every three minutes. The baseline fluoresence in the absence of lysyl oxidase was also monitored and subtracted from the final activity curve obtained.

2.7 Preparation of polyclonal antibodies against TRAMP

TRAMP was purified by the procedure above (section 2.1) and by anion exchange Mono Q FPLC to separate the protein from lysyl oxidase (This final purification step and antibody preparation were done in collaboration with Mr J.R.E. MacBeath). Two female New Zealand white rabbits were obtained (City Farms, West Calder) and a sample of pre-immune serum was taken from each rabbit. Polyclonal antibodies were raised by the methods described by Harlow and Lane, (1988). Intradermal injection of a 1:1 mixture of 200μg purified TRAMP in 0.15 M NaCl and complete Freund's adjuvant (Sigma) was followed up four weeks later by a boost of

100μg of TRAMP mixed with incomplete Freund's adjuvant also in a 1:1 ratio. Thereafter, four, six week booster injections of 50μg of TRAMP mixed with incomplete Freund's adjuvant were administered.

Blood samples (10 mls) were taken from the rabbits at intervals of 10 days after each injection and screened for anti-TRAMP polyclonal antibodies. Exsanguination of the rabbits two weeks after the final boost, produced around 30 mls of serum from each rabbit. Blood was clotted at 37°C for 30 minutes, stored overnight at 4°C and centrifuged at 10,000g for 10 minutes. Serum was collected from this, and 100μl aliquots were stored at -30°C until required.

2.8 Preparation of crude samples for blotting

For the detection of TRAMP in tissue extracts by Western blotting, tissues were dissected from 21 day mice and stillborn piglets, and weighed. Approximately 1.0g of each tissue was homogenised into 2ml of PBU and extracted at 4°C for 24 hours. Bone tissue was pre-frozen in liquid N_2 and shattered into fine pieces using an impact pulverising device. The extracts was then centrifuged at 12400g for 20 minutes, then the supernatant was collected and measured for protein content in a spectrophotometer at 280 nm. Approximately 100 μg of protein from each sample was run on a discontinuous 12% SDS-PAGE gel before western blotting.

2.9 Western blotting

Rabbit antiserum was tested for its reactivity against TRAMP by Western blotting. Samples of pure TRAMP and crude extracts from porcine skin, and various murine tissues (described above) were run on SDS-PAGE. Proteins were then transferred to nitrocellulose by immunoblotting (Towbin *et al.,* 1979) .

The gel was sandwiched between Whatman chromatography paper and a nitrocellulose sheet (pore size 0.45μm ; Sartorius) in a Bio-Rad Trans-blot cell which allows complete immersion of both gel and nitrocellulose during protein transfer. The blotting apparatus was set up as follows. A locking gel holder was opened, and submerged flat in a tray containing blotting buffer. A Scotch brite porous fibre pad was placed on this and air bubbles were expelled. Whatman chromatography paper with slightly larger dimensions than the protein gel (15 cm x 18 cm) was placed on top. The protein gel was laid on top of this carefully avoiding any air bubbles. The nitrocellulose sheet was carefully positioned on the protein gel and a further sheet of chromatography paper and Scotch brite were laid on top before closing the gel holder. The trans-blot cell tank was filled with blotting buffer (0.16 M sodium phosphate, 20% methanol (v/v) pH 9.4) and the gel holder was locked into position inside. A constant current of 250mA was applied, and proteins were transferred to the nitrocellulose (negative to positive) over 16 hours.

Detection of the immobilized protein was carried out using an Amersham ECL kit and protocol. ECL detection relies on the oxidation of luminol (a cyclic diacylhydrazide) by H_2O_2, catalysed by horseradish peroxidase (attached to the second antibody). Light is emitted during the

reaction and in the presence of enhancers contained in the kit, the light output is increased up to 1000 fold and light decay is reduced substantially.

The nitrocellulose was removed from the tank after transfer, and subjected to temporary staining with 0.4% (w/v) Ponceau S, 3% TCA (w/v). Ponceau S allows confirmation of successful transfer, and can be removed by washing with several changes of TBS (137mM NaCl, 20mM Tris-HCl, pH7.6). The destained nitrocellulose sheet was blocked with TBS-T (TBS, 0.2% Tween 20 (v/v)) and 5% Marvel (w/v), for 1 hour. The nitrocellulose was then washed in TBS-T, once for 15 minutes and then twice for 5 minutes. The antiserum from the final rabbit bleed was diluted typically 1:10000 in TBS-T and the nitrocellulose was incubated in this for 1 hour. Pre-immune serum taken from the animals before immunization was used as a control. Three washing steps in TBS-T were followed by incubation with the second antibody, horseradish peroxidase coupled anti-rabbit IgG (Scottish Antibody Production Unit, Law hospital, Carluke, Lanarkshire) for 20 minutes. After washing again as before, the ECL reagents were mixed in a 1 : 1 ratio and a volume equal to 0.125 ml / cm^2 of nitrocellulose was added to the blot for exactly 1 minute. ECL mix was then allowed to drip off the blot before it was laid down on Saran wrap inside a Kodak X-Omatic cassette, and then covered with more Saran wrap being careful to exclude air bubbles. Care was also be taken to avoid letting the blot dry. Exposure was for 30 seconds (or more) to pre-flashed X-ray film (Amersham) which was developed in an X-Ograph Compact X2 film processor.

2.10 SDS-PAGE

2.10.1 Introduction

Protein separation and visualization is possible using discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as first described by Laemmli (1970). This technique separates protein molecules by size alone, as SDS binds to and denatures proteins causing them to migrate with a constant charge/mass ratio. Mercaptoethanol is used in the procedure to cleave disulphide bonds. SDS-PAGE is calibrated by running standards of known molecular weight, and plotting migration distance versus log molecular mass.

2.10.2 Procedure

Throughout the study 12% polyacrylamide gels were used, unless stated otherwise. This concentration of acrylamide is effective for the separation of proteins with molecular masses between 90 kDa- 15kDa. Gels were prepared as described in protocol 2.

Reducing sample buffer (5x) was added to the protein samples giving a final concentration of 25mM Tris-HCl, 2% SDS (w/v), 0.01% bromophenol blue (w/v), 10% glycerol (v/v), 3% β-mercaptoethanol (v/v) pH 6.8. These samples were boiled for 3 minutes and loaded on to the polyacrylamide gel, which was run in a water cooled LKB vertical gel electrophoresis apparatus at a constant current of 40 mA per gel, with an LKB 2002 power supply. Each gel was run for 3 hours or until the tracking dye front was 1

cm from the bottom of the gel.

2.10.3 Gel staining

Once run, gels were removed from the apparatus, fixed and stained with 0.25% (w/v) Coomassie Brilliant Blue-250, 50% (v/v) methanol, 10% (v/v) acetic acid for 12 hours, shaking slowly on a Denley reciprocal mixer. Unbound stain was removed with several changes of 7.5% (v/v) acetic acid, 5% (v/v) methanol over 12 hours. Coomassie blue stain was re-used several times. The lower limit of protein detection using this staining procedure is about 1μg of protein.

Silver staining was used when greater detection sensitivity was required. Silver staining can detect about 10ng of protein in a band (Andrews, 1986). The method is adapted from Morrissey (1981). Gels were fixed in three changes of 25% (v/v) methanol, 10% (v/v) acetic acid for 3 hours and then washed in distilled water for 1 hour. The gel was then incubated in 0.1% (w/v) silver nitrate for 2 hours and washed twice in distilled water. The gel was developed in 200 ml of 0.02% (v/v) formaldehyde, 3% (w/v) sodium carbonate until the stained protein bands became visible. To stop the developing reaction, 2.3 M citric acid was added for 30 minutes, and finally, the gel was placed into 2% (v/v) glycerol for 2 hours before drying.

2.11 TRAMP incubation with [4,5-^{3}H] lysine labelled collagen

[4,5-^{3}H] lysine labelled collagen (12μg) labelled with (100μl) in PBS

1. Add 20% (w/v) SDS to each protein sample to give a final SDS concentration of 2%.
2. Add 5x reducing sample buffer (50% (v/v) glycerol, 2% SDS, 15% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue, 125 mM Tris-HCl, pH 6.8).
3. Heat the samples to 100 ^{0}C for 3 minutes.
4. Load samples on to a discontinuous gel consisting of a12% separating gel (12% (w/v) acrylamide, 0.32% (w/v) bis-acrylamide, 0.2% SDS, 0.05% (w/v) ammonium persulphate, 0.0005% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED),125 mM Tris-HCl, pH 8.8) and 4.5% stacking gels (4.5% (w/w) acrylamide, 0.12% (w/v) bis-acrylamide, 0.2% SDS, 0.05% (w/v) ammonium persulphate, 0.0005% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED),125 mM Tris-HCl, pH 6.8).
5. Carry out electrophoresis with a water cooled vertical electrophoresis unit approximately 3 hours at a constant current of 40 mA per gel in running buffer (0.192 M glycine, 0.1% SDS, 0.025 Tris, pH 8.3). Turn the current off when the dye front is 1cm from the bottom.

Protocol 2. SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

was incubated with TRAMP (2 μg), in PBS pH 7.8 (final volume of 150 μl) overnight at 37 °C. A control sample without TRAMP was also incubated. Reducing sample buffer (5x) was then added to the reactants, followed by electrophoresis on a 6% SDS-PAGE gel. To visualise radiolabeled proteins by fluorography (Laskey and Mills, 1975), the gel was then soaked in 200 ml DMSO for 30 mins followed by a further 30 mins in fresh DMSO. The gel was then immersed in 200 ml of 20% (w/v) PPO in DMSO for 3 hours. A wash in 200ml of 10% methanol, 0.1% glycerol for 1 hour was followed by drying the gel under vacuum. Finally, the gel was placed in contact with Hyperfilm-MP pre-flashed X-ray film (Amersham) and exposed for 48 hours before being developed in an X-Ograph Compact X2 film processor.

2.12 Trypsin digestion

Lysyl oxidase stimulating fractions (each approximately 20 μg of protein) eluted from Sephacryl S-400 in 6M PBU were incubated with 25 μg of bovine pancreatic trypsin (GIBCO) in a total volume of 1.0 ml for 6 hours at 37 °C. After incubation, 200μg of Soybean trypsin inhibitor type I-S (Sigma) were added, and the incubation continued for 30 minutes to inhibit further trypsin activity. A control sample of PBU was also incubated with trypsin and Soybean trypsin inhibitor as above. Finally, trypsinized and non-trypsinized samples were assayed with lysyl oxidase to determine any activity change.

2.13 Preparation of the rat aorta lysyl oxidase cDNA probe LOF 1

All reagents, media, solutions, pipette tips, centrifuge tubes and Eppendorf tubes were autoclaved for 15 minutes at 15 psi before use.

2.13.1 Library titre determination

A Sprague-Dawley neonatal rat λgt11 cDNA library (Clontech) was obtained and titred. The manufacturer recommends that libraries are titred before use by infecting *E.coli* Y1090 cells (Clontech; Table 4.1) as follows (Manufacturers protocol). A streak culture of Y1090 was prepared on LB agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride and 2% (w/v) bacto agar) 2-4 days old, and incubated overnight at 37°C. One cell colony was used to inoculate 50 ml of LB-broth (1% (w/v) tryptone, 0.5%(w/v) yeast extract and 1%(w/v) sodium chloride) supplemented with 0.2% (w/v) maltose to induce the *E.coli* maltose operon which contains the lamB gene coding for the bacteriophage λ receptor, therefore allowing more efficient adsorption of λ. The inoculated LB-broth was shaken overnight at 37°C to produce a cell culture which was then centrifuged at 4000g for 10 minutes and the pellet produced was resuspended in 10mM magnesium chloride, and vortex mixed. The cells were incubated for 1 hour at 37°C.

Cell dilutions of 10^2-10^4 were set up in λ dilution buffer (0.1 M NaCl, 0.2% (w/v) $MgSO_4$-7 H_2O, 2% (w/v) gelatin, 20mM Tris-HCl, pH 7.5). Top agar (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride

and 0.7 % (w/v) bacto agar) was prepared and incubated at 50°C. Then 35μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside(X-Gal; 40mg/ml, dissolved in dimethylformamide) was added to 3ml of top agar, followed immediately by mixing with each cell dilution and pouring on to LB-agar plates. The plates were incubated at 37 °C for 12-16 hours.

2.13.2 cDNA library amplification

Amplification of the cDNA library is necessary to maintain its stock (Sambrook *et al.,* 1989). A quantity of cDNA library containing around 10^5 bacteriophage (2μl) was mixed with 600μl of *E.coli* strain Y1090 in LB-broth. This was incubated for 20 minutes at 37°C, and plated on to NZCYM agar (1% (w/v) casein enzymatic hydrolysate, 0.5% (w/v) bacto yeast, 0.5% (w/v) NaCl, 0.1% (w/v) $MgSO_4.H_2O$, 0.05% (w/v) casamino acids, pH7.0), which was incubated at 42°C for 12 hours to prevent lysogeny (in this λ vector, the repressor for lytic growth is temperature sensitive). The bacteriophage were then collected by adding 15 ml of SM buffer (100mM NaCl, 0.2 % (w/v) $MgSO_4.H_2O$, 0.01% (w/v) Gelatin, 50mM Tris.HCl, pH 7.5) on top of the agar and incubating this for 2 hours at room temperature. The SM buffer was then poured off and centrifuged at 7000g for 30 minutes to pellet the cell debris. The supernatant was collected, aliquoted and titred as before.

2.13.3 Polymerase chain reaction

2.13.3.1 Introduction

The polymerase chain reaction (PCR) is a method of quickly and efficiently amplifying a small amount of DNA, using a thermostable DNA polymerase (Saiki *et al.,* 1985; Mullis *et al.,* 1986; Faloona and Mullis, 1987). Primers are designed that flank the two ends of the double stranded DNA, from which Taq polymerase (a thermostable DNA polymerase) synthesises new DNA. The template DNA is denatured at high temperature (92-94°C), and then cooled to allow annealing of the primers to each of the single strands produced (the annealing temperature is usually calculated as 5°C lower than the melting temperature of the primers). The temperature is then raised to 72°C to allow optimal conditions for DNA synthesis using the thermostable DNA polymerase, and finally the temperature is raised to 94°C again and the cycle is repeated. After 30 -35 cycles, the thermostable DNA polymerase eventually loses activity and the reaction stops.

2.13.3.2 Procedure

From the sequence of rat aorta lysyl oxidase cDNA (Trackman *et al.,* 1990, 1991), primers for the PCR reaction were designed with the aid of Gene Jockey software (Biosoft; L1 and L2, Fig 4.1). A *Bam*HI restriction enzyme site was designed into the primers to flank the cDNA fragment produced. The melting temperature was calculated to be 68°C and 56°C

for the 5' and 3' primers respectively (from the formula, Tm = 4 x (C or G) + 2 x (A or T) ; Innis, 1990) and the PCR reaction was carried out (protocol 3) by the method of Friedman *et al.* (1988) using 5μl of neonatal rat λgt 11 cDNA library (Clontech), previously disrupted by heating at 70 °C for 10 minutes, as template DNA.

2.13.4 DNA gel electrophoresis

2.13.4.1 Introduction

Separation, identification and purification of DNA fragments (200 bp - 50 kbp) can be performed quickly and efficiently by electrophoresis in agarose gels (Aaij and Borst, 1972). Bands can be visualised by running the gels in the presence of ethidium bromide (1μg / ml), and exposure to ultraviolet light.

2.13.4.2 Procedure

For purification and separation of the lysyl oxidase cDNA fragment, 0.8%(w/v) agarose (ultrapure grade, BRL) supplemented with 1μg / ml of ethidium bromide was prepared in 50 ml TBE buffer (90 mM orthoboric acid, 2.5 mM EDTA, 90 mM Tris HCl, pH 8.0), melted in a microwave and poured in a Pharmacia LKB GNA 100 DNA electrophoresis apparatus. TBE was used as running buffer, supplemented with 1μg / ml of ethidium bromide. Bacteriophage λ DNA digested with *Bst*EII restriction

To a 500µl Eppendorf tube, add :

Template DNA: 5µl (λgt 11 cDNA library source, Clontech).

Buffer : 10µl (500mM KCl, 1% Triton X-100, 100mM Tris-HCl, pH 9.0; Promega).

Magnesium chloride: 10µl (25mM; Promega).

Deoxynucleotide triphosphates: 10µl (2 mM; Promega).

Primers: 1µl (700 µg/ml - 1500µg/ml; Oswel DNA service).

DNA Taq polymerase: 1µl (5 units/µl; Promega).

DNase and RNase free water: to 100µl total reaction volume (Sigma).

Mineral oil : 100µl overlaying the reaction (Sigma).

Temperature programme (Techne PHC-2 Dri-block cycler) 35 cycles:

94^{0}C for 1 minute (to denature the double stranded cDNA).

50^{0}C for 2 minutes (to anneal the primers to the denatured cDNA).

72^{0}C for 3 minutes (to extend the primers).

In the final cycle, increase extension time to 10 minutes.

Protocol 3. Cold start PCR, adapted from Friedman *et al.* (1988)

endonuclease (GIBCO) was used for size determination of the sample DNA. The gel was run at a constant 100 volts for 90 minutes.

2.13.5 Ligation of LOF 1 into the vector pK19

The gel band corresponding to the lysyl oxidase cDNA fragment (LOF 1) was excised from the agarose gel and isolated using a Geneclean kit (Bio 101 Inc; protocol 4) to remove contaminating DNA fragments smaller than 500 bp, e.g. PCR primers. The DNA was eluted in a final volume of 20μl TE buffer (1mM EDTA, 10mM Tris HCl, pH 8.0). All 20μl of LOF1 was then subjected to a restriction digest with *Bam*HI restriction endonuclease and incubation buffer B (both Boehringer Mannheim) in a 40μl reaction (20μl cDNA, 4μl buffer B, 1μl *Bam*HI, 0.1 mg / ml RNase, 14μl water; the RNase was supplied courtesy of Dr Alan Boyd, Biochemistry Dept., Edinburgh University). This was incubated for 90 minutes at 37°C. This cDNA was then purified with a Geneclean kit for the second time to allow ligation into a vector.

The vector used was pK19 (Pridmore, 1987; supplied courtesy of Dr Alan Boyd) which is derived from pUC19 (Messing, 1983) and encodes kanamycin rather than ampicillin resistance. The pK19 was digested with *Bam*HI (20μl pK19, 20μl buffer B, 160μl water, 1μl *Bam*HI, 0.1mg / ml RNase), and incubated at 37°C for 90 minutes. To remove contaminating protein this linearised vector DNA was then extracted in phenol : chloroform : isoamyl alcohol (25:24:1), and precipitated in ethanol (protocol 5). Both LOF 1 and pK19 were run on an agarose electrophoresis gel

1. Excise the band of DNA from the agarose gel visualized under ultraviolet light using a plastic scalpel (long wave UV light should be used for as short a time as possible to avoid damage to the DNA).

2. Weigh out the excised agarose slice in an eppendorf tube, and add 4.5 times the volume of the sodium iodide provided, and 0.5 times the volume of TBE modifier.

3. Incubate at 45-55^{0}C for 2 minutes, vortex mix and return to incubation for a further 5 minutes or until the agarose has completely melted.

4. Add 10µl of Glassmilk and vortex mix. Incubate on ice for 25 - 30 minutes, vortex mixing every 3 minutes.

5. Centrifuge for 15 seconds at 15400g, and resuspend the pellet in 500µl of New Wash (stored at -20^{o}c) and centrifuge again. Repeat twice.

6. Re-suspend the final pellet in 20µl of TE buffer pH 8.0 or water and incubate at 45-55^{0}C for 2 minutes. Centrifuge once more for 30 seconds, and collect the supernatant.

Protocol 4. Purification of DNA using a Geneclean II kit (BIO 101inc).

1. Vortex mix vector DNA with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1; Sigma) for at least 30 seconds.

2. Centrifuge the mixture at 15400g for 3 minutes and collect the aqueous layer.

3. Add volumes of cold ethanol and 3M sodium acetate pH 5.2 equal to 2.5 and one-tenth of the aqueous layer, respectively.

4. Incubate at -70 ^{0}C for 20 minutes, then centrifuge at 15400g for 10 minutes. Dry the vector DNA in a rotary evaporator and resuspend in TE buffer pH 8.0.

Protocol 5. Extraction and concentration of vector DNA.

to compare the concentrations of each. The ligation reaction was set up with 5μl LOF 1 cDNA, 1μl vector pK19, 1μl T4 DNA ligase, 2μl ligase buffer (Boehringer Mannheim) and 11 μl water, then incubated overnight at room temperature.

2.13.6 Transformation of competent NM 522 *E.coli* cells

E.coli cells can be transformed with plasmid DNA if treated with calcium chloride to produce a state of 'competence' (Cohen *et al.,* 1972). A strain of *E.coli* cells (NM 522; Table 4.1) were prepared for transformation (protocol 6) and incubated on ice for 2 hours before use.

The transformation reaction was then set up using 100μl of competent cells added to the total volume of the ligation reaction (section 2.3.5). This was incubated on ice for 45 minutes, then heat shocked for 2 minutes at 42°C followed by addition of 1 ml of LB-broth and further incubation, on a shaker, at 37°C for 1 hour.

LB-Agar plates supplemented with 0.005% (w/v) X-Gal, 0.003% (w/v) IPTG and 0.001% (w/v) kanamycin were prepared in advance, and 100μl of the transformed cells were then spread on to the plates using a sterile glass spreader. A concentrated sample of these cells was also plated by centrifuging the cells at 6500g for 2 minutes, and resuspending the cell pellet in 100μl of LB-broth. The plates were incubated at 37°C overnight (upside down to prevent condensation building up on the plate surface).

1. Inoculate LB-broth (10 mls) with *E.coli*, and incubate overnight. On the following day inoculate 50 mls of LB-broth with 0.5 mls of the overnight culture and grow until the culture reaches an optical density of 0.4-0.5 absorbance units at 600nm. Place the cells on ice.

2. Centrifuge at 6000g for 5 minutes, discard supernatant , and re-suspend cells in 20 mls of cold 100mM calcium chloride.

3. Centrifuge cells as above and resuspend in 10 mls cold 100mM calcium chloride. Leave on ice for 30 minutes then centrifuge at 4000g for 5 minutes.

4. Gently re-suspend in 1ml of cold 100mM calcium chloride, and incubate on ice for at least 2 hours before use. Ideally, the cells should be left overnight to maximise their competence.

Protocol 6. Preparation of competent *E.coli* cells.

2.13.7 Screening cell colonies

2.13.7.1 Introduction

Cell colonies can be screened for successful transfection with vector incorporating an insert, as follows. pK19 vector carries the first 146 amino acids of the β–galactosidase gene (lac Z), into which a polycloning site (including *Bam*HI) is incorporated. When this vector is grown inside a host cell strain that carries only the carboxy terminal of β–galactosidase, α-complementation results and functional β–galactosidase is produced, giving blue coloured cell colonies on X-Gal / IPTG agar plates. If however a DNA insert is ligated into the polycloning site, the amino terminus of the enzyme will be disrupted, and cell colonies produced will be white in colour.

2.13.7.2 Procedure

White colonies were screened for the correct insert by harvesting and lysing the *E.coli* cells in a minipreparation of vector DNA (protocol 7), and performing a restriction digest with *Bam*HI (section 2.13.5), to excise the fragment of interest, followed by electrophoresis on a 0.8% (w/v) agarose electrophoresis gel.

A large scale preparation of vector DNA was then carried out on the

1. Innoculate 5 mls of LB-broth with one cell colony, and incubate overnight at 37^0C.
2. Pipette 1.5 mls of the overnight culture into an Eppendorf tube and centrifuge at 15400g for 3 minutes.
3. Resuspend the pellet in 180µl of lysis solution 1 (50mM Glucose, 10 mM EDTA, 0.2%(w/v) lysozyme, 25mM Tris-HCl, pH 8.0) and add 400µl of lysis solution 2 (0.2 N NaOH, 1% w/v SDS).
4. Vortex mix and leave on ice for 5 minutes.
5. Add 300µl of 3M sodium acetate pH 5.2, mix and leave on ice for 10 minutes.
6. Centrifuge at 15400g for 10 minutes and remove 750µl of the supernatant, to which 500µl of propan-2-ol (isopropanol) is added and vortex mixed.
7. Centrifuge at 15400g for 5 minutes and resuspend the pellet in 200µl of TE buffer pH 8.0.
8. Add 400µl of phenol : chloroform : isoamyl alcohol (25:24:1), and vortex mix hard for at least 30 seconds. Centrifuge as before for 3 minutes and remove the aqueous layer which contains the DNA (this is usually the upper layer) to another tube. Add one tenth its volume of 3M sodium acetate pH 5.2, and 2.5 times volume of ice cold ethanol.
9. Incubate at -70 ^{0}C for 20 minutes and then centrifuge at 15400g for 10 minutes.
10. Freeze-dry the sample and resuspended in 20 µl TE buffer pH 8.0.

Protocol 7. Miniprep of vector DNA (Sambrook *et al.,* 1989; Birnboim and Doly 1979; Ish-Horowicz and Burke 1981).

cell colony containing the insert (protocol 8), to prepare sufficient vector DNA for sequence analysis.

2.13.8 DNA sequence analysis

The method of DNA sequencing utilized was the dideoxy-mediated chain termination method (protocol 9; Sanger *et al.*, 1977; Hsiao, 1991) . When a DNA chain is being synthesised by DNA polymerase, the inclusion of 2',3' dideoxynucleoside triphosphate (ddNTP) terminates chain growth due to the lack of a hydroxyl residue at the 3' position of deoxyribose, and hence the inability to form the required phosphodiester bond to the next dNTP. This property of ddNTPs is exploited in the Sanger method by including a small concentration of one of each type (ddATP, ddGTP, ddTTP, ddCTP) in four separate DNA synthesis reactions. During the reactions, there is competition between the dNTPs and ddNTPs for inclusion into the growing chain, which results in terminations at each site of possible ddNTP incorporation along the chains produced. If the reactions are labelled with [^{35}S]-d ATP then the different lengths of chain synthesised in each reaction can be visualized on a polyacrylamide gel after exposure for 48 hours to Hyperfilm MP pre-flashed X-ray film (Amersham), developed in an X-O graph Compact X2 film processor.

DNA primers are required for the sequencing reactions to take place. When sequencing a vector insert, a universal primer is often used if the vector contains the corresponding sequence: 5'-GTAAAACGACGGCCAGT-3'. Although pK19 has the universal priming site, the PCR primer (L1) was utilized in the sequencing of pLOF 1.

1. Inoculate 100 mls of LB-broth with one cell colony, and incubate overnight at 37^0C.

2. Centrifuge the cells at 6000g for 10 minutes, and resuspend the pellet in 2 mls of lysis solution 1 (50mM glucose, 10 mM EDTA, 0.2%(w/v) lysozyme, 25mM Tris-HCl, pH 8.0). Add 4 mls of lysis solution 2 (0.2 N NaOH, (1% w/v) SDS), mix by inversion and leave on ice for 10 minutes.

3. Add 3 mls of ice cold 3M sodium acetate pH 5.2, vortex mix and leave on ice for a further 10 minutes.

4. Centrifuge the cells at 6000g for 15 minutes, remove the supernatant to another tube, and to this add an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). Vortex and centrifuge again at 6000g for 10 minutes.

5. Remove the aqueous layer which contains the DNA and add two volumes of ethanol. Leave this at room temperature for 5 minutes.

6. Centrifuge at 6000g for 10 minutes and remove all supernatant. Resuspend the pellet in 5 mls of 70% ethanol, re-vortex and centrifuge again as above.

7. Remove the supernatant, and dry the pellet in a freeze-drying apparatus. Re-suspend the pellet in 500µl TE buffer pH 8.0.

Protocol 8. Midiprep of vector DNA (Sambrook *et al.,* 1989; Birnhoim and Doly 1979; Ish-Horowicz and Burke 1981).

Polyacrylamide gel preparation

1. Prepare a 7M Urea, 5 % polyacrylamide gel solution in 150 ml TBE buffer (90 mM Tris, 90 mM Orthoboric acid, 2.5 mM EDTA). Degas the solution under vacuum for 20 minutes.
2. To seal the underside of the Bio-Rad IPC gel assembly, combine10ml of the gel solution with 50µl of 25% (w/v) ammonium persulphate, and 50µl of 0.005% (v/v) N,N,N',N' - tetramethylethylenediamine (TEMED). Pour this solution on to a sealing strip, and clamp the IPC assembly to this. Let the sealing strip set (2 minutes).
3. To prepare the polyacrylamide gel, combine 60 ml of gel solution with 60µl of 25% (w/v) ammonium persulphate, and 0.001% (v/v) TEMED. Pour into the IPC assembly, leave to set for 60 minutes and then pre-run at 2300 volts (40 Watts) for at least an hour before loading samples.

DNA sample denaturation:

1. Adjust a sample of 1-3ng of double stranded DNA from a midi-prep to a final volume of 10 µl with TE buffer pH 8.0
2. Add 2µl 2M ammonium acetate pH 4.5 and 55µl of cold ethanol. Centrifuge for 5 minutes at 15400g.
3. Re-suspend the pellet in 200µl of cold 70% ethanol, centrifuge again for 2 minutes, dry the pellet in a rotary evaporator and then resuspend in 7µl TE pH 8.0.

Protocol 9 (part 1). Sequence analysis of double stranded DNA using a Bst DNA sequencing kit (BioRad).

Primer annealing:

1. In an Eppendorf tube, mix 2µl 5x reaction buffer (100mM $MgCl_2$, 100mM Tris-HCl, pH 8.5), 7µl denatured DNA (from above) and 1µl of universal primer (5'-GTAAAACGACGGCCAGT-3'; 2.5 ng/µl) and incubate at 75^0C for 5 minutes. Allow the mixture to cool.
2. Add 1.5 µl [α^{35}S] dATP and 1µl Bst DNA polymerase (1 unit / µl) , vortex and centrifuge for 2 - 3 seconds.

Sequencing:

1. Label four eppendorf tubes A, G, C, T , and add 2µl of one of the pre-mixed nucleotide solutions to each tube i.e. A mix contains 620 nM dATP, 62 µM dCTP, 62 µM dGTP, 62 µM dTTP, 25 µM ddATP in 0.15 mM EDTA, 1.5 mM Tris, pH 8.0).
2. Add 2.5 µl of the annealing mix from above to each and incubate for 2 minutes at 65^0C.
3. Add 2 µl of chase solution (1 mM each dNTP) to each and continue incubation at 65^0C for 2 minutes
4. Add 4 µl of stop solution (95% deionised formamide, 10 mM EDTA, 0.05% xylene cyanole FF, 0.05% bromophenol blue) to the side of the Eppendorf, and centrifuge to stop the reaction.
5. Prior to loading onto the polyacrylamide gel, heat the samples to 90^0C for 2 minutes.

Protocol 9 (part 2). Sequence analysis of double stranded DNA using a Bst DNA Sequencing kit (Bio-Rad).

2.13.9 LOF 1 labelling

Approximately 8μg (visually determined) of LOF 1 was cut from pK19 using *Bam*HI as described (section 2.13.5). LOF 1 (0.1μg) was heated to 100 ^{0}C for 3 minutes and then labelled by the method of Feinberg *et al.* (1983) in a reaction with 2μg BSA (Sigma), 2 units of Klenow DNA polymerase (Boehringer Mannheim), 2 units α [^{32}P]-dATP (Amersham), 5μl oligo-labelling buffer (0.11 mM dATP, dTTP, dGTP, 30mM $MgCl_2$, 1M Hepes, 12μM EDTA, 280 mM Tris-HCl, pH 8.0). The reaction was incubated overnight at room temperature, and desalted in a 1ml column of Sephadex G-50 pre-equilibrated in STE buffer (1mM EDTA, 100mM NaCl, 10mM Tris-HCl, pH 8.0). The column was centrifuged at 1000g for 90 seconds to elute the labelled DNA, which was then heated to100^{0}C for 7 minutes.

2.13.10 Human placental cDNA library plating and screening

A human placental 34 week cDNA library in bacteriophage λ (Clontech) was obtained and used to transfect Y1090 cells. LB-agar plates were innoculated with the Y1090 cells, and one colony from this plate was used to innoculate LB-media supplemented with 0.2% maltose and 10mM $MgSO_4$ and this was cultured overnight. Cells were collected by centrifuging at 3000g for 10 minutes and were then resuspended in 5 ml of 10mM $MgSO_4$. The human placental cDNA library was then diluted in λ dilution buffer 1:10 and 10μl of this was added to 1 ml of the cells and 35 ml of molten top agarose (1%(w/v) tryptone, 0.5%(w/v) yeast extract, 1% (w/v) sodium chloride and 2% bacto agar) which was then poured onto petri-

dishes (30 cm x 30cm) containing L-bottom agar (1% (w/v) tryptone, 0.5%(w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar) and these plates were incubated overnight at 37 ^{0}C.

Nitrocellulose (Sartorious) cut into sheets (30cm x 30cm) were placed on top of the plates for 1 minute, and the corners of the filter were marked asymetrically with a syringe needle allow later identification of orientation. Duplicate plaque lifts for 2 minutes were also taken . The sheets were lifted off the plates and placed into denaturing solution (1.5 M NaCl, 0.5 M NaOH) DNA side up for 1 minute, then placed into neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.5) for 5 minutes and lastly, washing solution (0.3 M NaCl, 20mM NaH_2PO_4, 2 mM EDTA pH 7.4) for 5 minutes. The DNA was cross-linked to the nitrocellulose using ultra-violet light in a Stratagene UV Stratalinker 2400 at full power for 30 seconds.

The filters were placed into a revolving cylinder in an oven with 50 ml of pre-hybridisation solution (0.9 M NaCl, 90mM tri-sodium citrate, 0.25% (w/v) bovine serum albumin, 0.25% (w/v) Ficoll, 0.25% (w/v) polyvinyl pyrrolidone, 0.5 mM EDTA, 10% SDS) for 50 minutes at 65^{0}C with 2 mg / ml boiled herring sperm DNA and then add the probe DNA and continuing the incubation overnight.

The pre-hybridisation solution was poured off and the filters were washed twice in SSC solution (0.9 M NaCl, 90mM tri-sodium citrate, pH 7.4) for 30 seconds. The filters were then wrapped in cling film and developed using Hyperfilm MP X-ray film (Amersham) and processed in a X-Ograph compact X2 film processor.

2.14 Production of human placental cDNA lysyl oxidase clone VLO1

2.14.1 Introduction

From the published sequence of human placental lysyl oxidase (Hamalainen *et al.*, 1991) new primers (V1 and V2; Fig 4.6) were designed to clone human lysyl oxidase using the polymerase chain reaction (protocol 3) from valine 167 to the termination codon at position 418.

2.14.2 Hot start PCR

'Hot start' (D'Aquila *et al.*, 1991; protocol 10) is a modification to the PCR procedure that was used to reduce the amount of spurious DNA appearing in some amplifications. According to this procedure, the PCR reactants (except for the Taq DNA polymerase) are pre-heated to 95^{0}C for 5 minutes to completely denature the double stranded cDNA template, before the reactants are cooled to the annealing temperature of the reaction and Taq DNA polymerase is added. The reactants are then heated to the denaturing temperature again, and the reaction continues as for 'cold start' PCR.

To a 500 µl Eppendorf tube, add:

Template DNA: 1µl (0.3ng/µl; human placental Quickclone cDNA, Clontech).

Buffer : 3µl (500mM KCl, 1% Triton X-100, 100mM Tris-HCl, pH 9.0; Promega).

Magnesium chloride: 3µl (25mM; Promega).

Deoxynucleotide triphosphates: 3µl (2 mM; Promega).

Primers: 1µl (700 µg/ml - 1500µg/ml; Oswel DNA service).

DNase and RNase free water: to 30µl total reaction volume (Sigma).

Mineral oil : 40µl overlaying the reaction (Sigma).

Temperature programme (Techne PHC-2 Dri-block cycler)

1 cycle of:

95^{0}C for 5 minutes (to completely denature all cDNA)

60^{0}C for 2 minutes (add 1µl DNA Taq polymerase; (5units / µl; Promega)

then 35 cycles of :

94^{0}C for 1 minute (to denature the double stranded cDNA)

60^{0}C for 2 minutes (to anneal the primers to the denatured cDNA)

72^{0}C for 3 minutes (to extend the primers).

After 15 cycles, add a further 1µl of Taq DNA polymerase to each tube.

In the final cycle, increase the extension time to 10 minutes.

Protocol 10. Hot start PCR (D'Aquila *et al.,* 1991).

2.15 Production of VLO 2

2.15.1 Introduction

New primers were designed (V3 and V4) to amplify lysyl oxidase from valine 167 to beyond the termination codon TAG at position 418 (Fig 4.10).

The template cDNA used was human placental Quickclone cDNA (Clontech) which is more suitable for use with polymerase chain reactions than cDNA libraries because there is less non-specific DNA to cause unwanted amplifications, and the cDNA included is selected to contain more full length cDNAs, therefore reducing amplifications of partial cDNA. The primers were flanked by restriction enzyme sites *Bbr*I (5') and *Hin*dIII (3') to allow directional cloning into a vector.

2.15.2 Procedure

The reaction was carried out as described (protocol 10) in a total volume of 30μl, using 1μl of Human placental Quickclone cDNA (Clontech) as template, diluted 1 in 3 with TE buffer pH 8.0, giving approximately 300 pg / μl of cDNA per reaction.

The mineral oil was removed from the reactants and 20 μl of the PCR reaction was run on a 0.8% agarose gel. The 750 bp DNA produced was excised from the gel and purified using a Geneclean kit (protocol 4).

2.15.3 Ligation of VLO 2 into pGEM-T

2.15.3.1 Introduction

For more efficient cloning of the lysyl oxidase cDNA, a vector specifically designed for incorporation of PCR products was used. pGEM-T is an ampicillin resistance carrying Promega vector based on pGEM-5Zf (Promega), but modified by cutting with *Eco*R V restriction endonuclease and adding a terminal thymidine to the two ends. PCR amplified DNA produced by Taq polymerase always contains an extra adenosine base at each end not specified by the template, which means that these products can be directly cloned into the pGEM-T vector without the use of restriction endonucleases.

2.15.3.2 Procedure

The amplified PCR DNA was purified with a Geneclean kit (protocol 4), and 7μl (10 ng) was added to 1μl pGEM-T (50 ng) in a standard ligation reaction (section 2.12.5). This was incubated at 15°C for 3 hours and then heated to 72°C for 10 minutes.

2.15.4 Cell transformation

Competent cells (JM109; Table 4.1) supplied by Promega were transformed by the total volume of ligated PCR product. The competent cells were thawed from -70 °C (storage temperature), and 100μl were

added to the ligation reaction. The cells were gently mixed and put on ice for 20 minutes, heat shocked at 42°C for 45 seconds and returned to ice for 2 minutes, before adding 900μl of LB-broth and shaking at 37°C for 1 hour. The cells were then spread on LB-agar plates supplemented with 0.005% (w/v) X-Gal, 0.003% (w/v) IPTG and 0.001% (w/v) ampicillin. White recombinant colonies were screened for the lysyl oxidase cDNA by making a mini-preprep of DNA, and then performing restriction digests (section 2.13.5) with *Bbr*P1 and *Hin*dIII, to check for insert DNA. Finally, partial DNA sequence analysis was obtained to confirm that the insert obtained coded for the mature lysyl oxidase cDNA (protocol 9).

2.16 ALO 1 production

2.16.1 Introduction

Two further primers were designed to produce a lysyl oxidase sequence from Asp 169 to the termination codon 418 (ALO 1). A polymerase chain reaction was set up (protocol 10) using 1μl of a midi-prep of VLO2 as template, 6μl of deoxynucleotide triphosphates (2 mM), and 0.5μl of Vent DNA polymerase (Promega; Vent DNA polymerase does not add extra adenosine bases to the amplified DNA).

DNA from this reaction was purified using a Geneclean kit (protocol 4) and ligated into the ampicillin resistant vector pUC18 (section 2.13.5), then transformed into JM109 competent cells (Promega), and the colonies produced were screened for insert DNA. Two colonies which contained inserts the correct size were subjected to restriction digests with *Sca*I and

*Kpn*I, which occur within the human lysyl oxidase coding region (Hamalainen *et al.,* 1991), *Hin*cII and *Sph*I, which are contained within the 5' and 3' primers respectively, and *Bam*HI, *Eco*RI and *Pvu*II, which occur in the polycloning site of pUC18.

2.17 ALO 2 production

2.17.1 Introduction

A further set of primers were designed with restriction sites incorporated that would allow cloning of ALO 2 directly into the yeast expression vector pDP315 (kindly donated by Dr. D. Pioli, Zeneca Pharmaceuticals, Macclesfield; Fig 4.19). The 5' primer contained a *Hin*cII site and the 3' primer contained a *Bam*HI site. A polymerase chain rection was carried out as before (protocol 10), and the DNA produced was purified using a Geneclean kit (protocol 4). Digestion of pDP315 with *Stu*I and the *Bam*HI restriction endonucleases was used to excise a segment of DNA from the vector. To prevent possible re-ligation of the restricted ends of pDP315, alkaline phosphatase was used. pDP315 (1μg) was incubated with 1 unit of alkaline phosphatase (Boehringer mannheim) and 1μl of phosphatase buffer at 37^{0}C for 30 minutes in a total volume of 10μl. The reaction temperature was then raised to 65^{0}C for 10 minutes. A phenol / chloroform extraction was performed to remove phosphatase and the DNA was concentrated by ethanol precipitation (protocol 5). ALO 2 was restricted with *Hin*cII and *Bam*HI, and ligated into the vector, and this was then used to transform JM 109 high efficiency competent cells (Promega).

The cells were plated onto LB-agar plates supplemented with 0.001% (w/v) ampicillin.

2.18 Transformation of yeast cells

2.18.1 Introduction

The construct (ALO 2 / pDP315) was used to transform the *S.cerevisiae* strain JRY188 (kindly donated by Dr. A Boyd; Table 4.1) by the method of Gietz *et al.* (1992) as follows. YEPD medium (1% (w/v) yeast extract, 2% bacto-peptone, 2% glucose; 5ml) was innoculated with JRY 188 and cultured overnight at 30^{0}C, 1ml of this was used to innoculate 50ml of YEPD, cultured at 30^{0}C until the optical density of the cells at 600nm reached 0.8. The cells were then centrifuged at 5000g for 5 minutes, and the cell pellet was washed in TE buffer (10 mM EDTA, 0.1M Tris-HCl, pH 7.5; 8 ml). The cells were centrifuged again at 5000g for 5 minutes and the cell pellet was re-suspended in 100mM lithium acetate. Approximately 0.5 μg (visually determined) of the construct (ALO 2 / pDP 315) was incubated with 300μl of the cells, 600μl of 50% (w/v) polyethylene glycol 400 and 50 μg of single stranded carrier DNA (herring sperm; donated by Dr. A Boyd) for 30 minutes at 30^{0}C. The cells were then heat shocked at 42^{0}C for 15 minutes, centrifuged at 6500g for 2 minutes and the pellet was re-suspended in 150μl TE buffer. The cells were plated on to HUW plates (2% agar, 2% glucose, 0.65% nitrogen base, 0.002% histidine, 0.002% uracil, 0.002% tryptophan) and incubated for 5 days at 30^{0}C.

2.19 Yeast lysyl oxidase cDNA expression

Colonies from the yeast transformation procedure were used to innoculate 100ml of SD-HUW medium (2% glucose, 0.65% nitrogen base, 0.002% histidine, 0.002% uracil, 0.002% tryptophan). The cells were grown to an optical density at 600nm of 2.0 and centrifuged at 3000g for 5 minutes. To the supernatant ammonium sulphate was added to 80% saturation, followed by stirring overnight at 4 ^{0}C. The precipitated protein was collected by centrifugation at 12000g for 10 minutes and re-suspended in 1 ml of 2% SDS, 125 mM Tris-HCl, pH 6.8. Approximately 20μg of protein was run on a 12% SDS-PAGE gel, under reducing conditions (protocol 2). To determine whether the protein contained lysyl oxidase activity, an assay using the ultrafiltration method was undertaken (section 2.5), with 100μl (0.2 mg / ml) of protein, 100μl (1 mg / ml) of [4,5-^{3}H] lysine labelled elastin in assay buffer (100mM sodium borate, 0.15 M NaCl, pH 8.0) in a total volume of 900μl.

Chapter 3

Immunological and enzymatic studies on TRAMP

3.1 Immunological studies of TRAMP

3.1.1 Introduction

Tyrosine rich acidic matrix protein (TRAMP) is a protein of molecular mass 22 kDa that co-purifies with lysyl oxidase (Cronshaw *et al.*, 1993) from 6 M urea extracts of porcine skin, after DEAE-Sephacel ion-exchange chromatography and selective interaction with Sephacryl S-400 (section 2.1). Under these conditions, TRAMP usually appears in great excess to lysyl oxidase (Fig. 3.1).

Several reports have documented the existence of proteins of molecular mass 22-24 kDa that are associated with lysyl oxidase. For example, Sullivan and Kagan, (1982) reported the gradual appearance of a 24 kDa protein in fractions of bovine aortic lysyl oxidase when stored for prolonged periods at 4°C, and peptide mapping with *S. aureus* V8 protease indicated that this was a likely degradation product of the functional enzyme. In addition, Burbelo *et al.* (1986) found that monoclonal antibodies raised against human umbilical cord lysyl oxidase cross-reacted with a 24 kDa protein that co-purified with lysyl oxidase. Kuivaniemi *et al.* (1984) also observed a contaminating 22 kDa protein on long term storage of human placental lysyl oxidase, though the nature of this protein was not explored. The same study revealed that a 23 kDa protein co-purified with lysyl oxidase on DEAE-cellulose ion-exchange chromatography. The amino acid composition of this 23 kDa contaminant

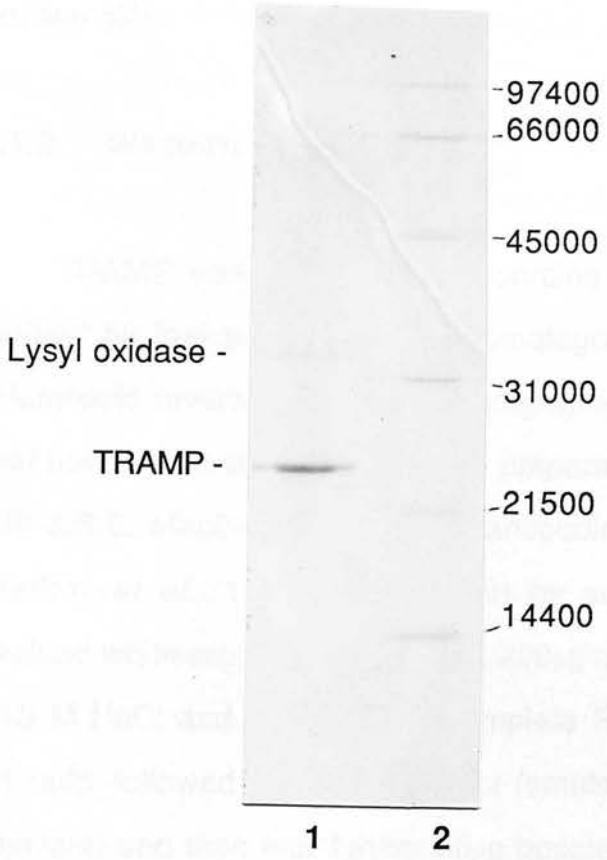


Figure 3.1. SDS-PAGE of partially purified TRAMP. 6M PBU (6M urea, 9 mM Na_2HPO_4 / 1 mM NaH_2PO_4, pH 7.8) extracts from porcine skin were purified by DEAE-Sepharose ion exchange chromatography (eluted in 6M PBU, 0.5 M NaCl) followed by exhaustive dialysis against PB (9mM Na_2HPO_4 ,1mM NaH_2PO_4, pH 7.8), and selective interaction with Sephacryl S-400. TRAMP and lysyl oxidase were then eluted with 6M PBU and analysed by discontinuous SDS-PAGE under reducing conditions with 12% acrylamide, 0.32% bis-acrylamide in the separating gel, followed by staining with Coomassie Blue.

was found to be distinct from lysyl oxidase. Finally, Wakasaki and Ooshima, (1990b) found a 24 kDa protein that co-purified with lysyl oxidase from human umbilical cord. Peptide mapping with *S. aureus* V8 protease and amino acid analysis showed this protein to closely resemble lysyl oxidase (Wakasaki and Ooshima, 1990b).

In order to explore the relationship between lysyl oxidase and TRAMP, polyclonal antibodies were raised to highly purified TRAMP from porcine skin.

3.1.2 Western blotting

TRAMP was prepared from porcine skin (section 2.1), and further purified by fast protein liquid chromatography on a Pharmacia Mono Q columnand reverse phase chromatography (Cronshaw *et al.*, 1993). This final purification step and antibody preparation were done in collaboration with J.R.E. MacBeath. Polyclonal antibodies were raised against TRAMP (Harlow *et al.*, 1988; section 2.6) by subcutaneous injection of New Zealand white rabbits with an initial 200μg dose of TRAMP diluted in 1 ml of 0.15 M NaCl and emulsified in complete Freund's adjuvant (Sigma), in a 1:1 ratio, followed by a 100μg boost (emulsified in 1:1Freund's incomplete adjuvant) and then four further 50μg boosts injected every six weeks. The rabbits were then sacrificed and exsanguinated.

A crude, 6 M urea extract was prepared from 500g of porcine skin, as described in the lysyl oxidase purification procedure (section 2.1). TRAMP was purified from this extract by the method of Cronshaw *et al.* (1993). Briefly, 6M PBU (6M urea, PB) extracts were pooled and filtered through Whatman No.3 filters, then diluted with PB to give a concentration of 2M

PBU (2M urea, PB) and passed through a CM-Sepharose fast flow column (2.6 cm x 40 cm) pre-equilibrated in 2M PBU. The flow through material was then loaded onto a DEAE-Sepharose fast flow column (2.6 cm x 40 cm), also pre-equilibrated in 2M PBU. After elution of the column in 3 M PBU, 0.3 M NaCl to remove lysyl oxidase and other contaminating proteins, TRAMP containing fractions were eluted in 6M PBU, 0.5 M NaCl. Urea and salt were removed from the eluent by gel permeation on a Sephadex G25 column (90cm x 5.6 cm), and finally the protein solution was loaded onto a Sephacryl S-400 column pre-equilibrated with PB, and TRAMP was eluted in 1.5 M PBU (1.5 M urea, PB).

Samples of the crude skin extract (50μg) and purified TRAMP (5μg) were run on a discontinuous 12 % SDS-PAGE gel, transferred to nitrocellulose and probed with the polyclonal antiserum using enhanced chemiluminscence detection (ECL) (section 2.9). As shown in Fig. 3.2, the polyclonal antiserum reacted strongly with TRAMP. No reactivity was observed between pre-immune serum and either the crude extract or partially purified TRAMP. There is some evidence that the antiserum recognises a protein of higher molecular weight than TRAMP.

Lysyl oxidase (prepared as described in section 2.1) did not cross-react with the TRAMP antiserum (or with the pre-immune rabbit serum) when run on SDS-PAGE and analysed by Western blotting (Fig. 3.3). Therefore lysyl oxidase does not contain epitopes that are recognized by the antiserum, which suggests that TRAMP and lysyl oxidase are unrelated.

The amino acid sequence of porcine TRAMP (A.D. Cronshaw, personal communication) is over 96 % identical to the bovine form (Neame

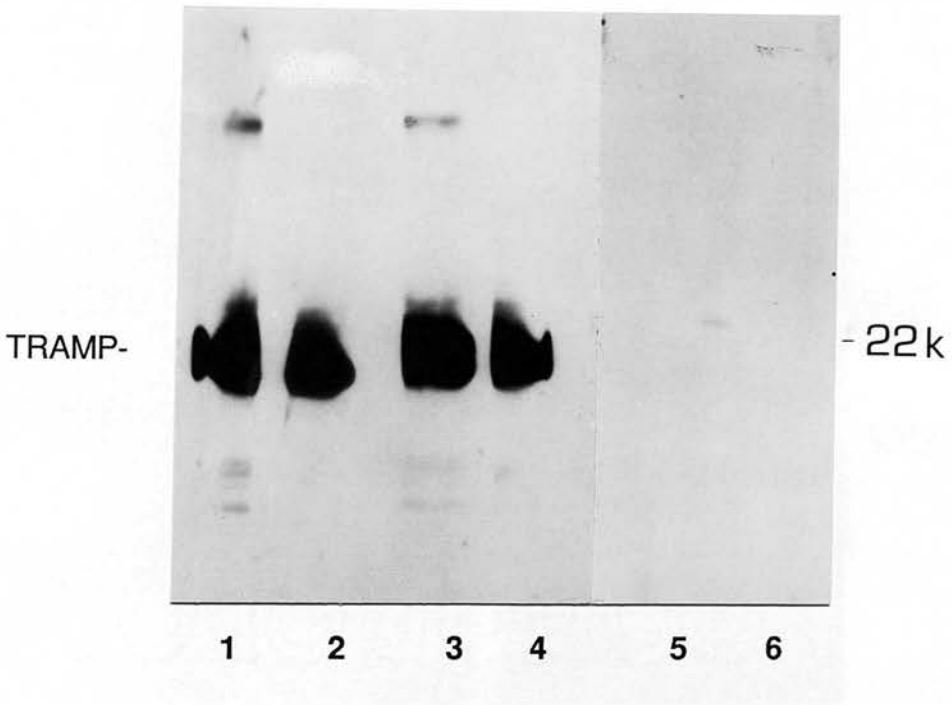


Figure 3.2. Reactivity of anti-TRAMP polyclonal antiserum and pre-immune serum with purified porcine TRAMP and a crude 6M urea extract of porcine skin. Samples were electrophoresed on a reducing 12 % SDS-polyacrylamide gel, Western blotted onto nitrocellulose and reacted with different concentrations of serum. Antibodies were detected by ECL, with a film exposure of 1 minute. Purified TRAMP (2 μg, lanes 1,3,5) and the crude urea extract (50 μg, lanes 2,4,6) were reacted against a 10,000-fold dilution of the anti-TRAMP antiserum (lanes 1 and 2), a 20,000-fold dilution of the anti-TRAMP antiseum (lanes 3 and 4) and a 10,000-fold dilution of pre-immune serum (lanes 5 and 6).

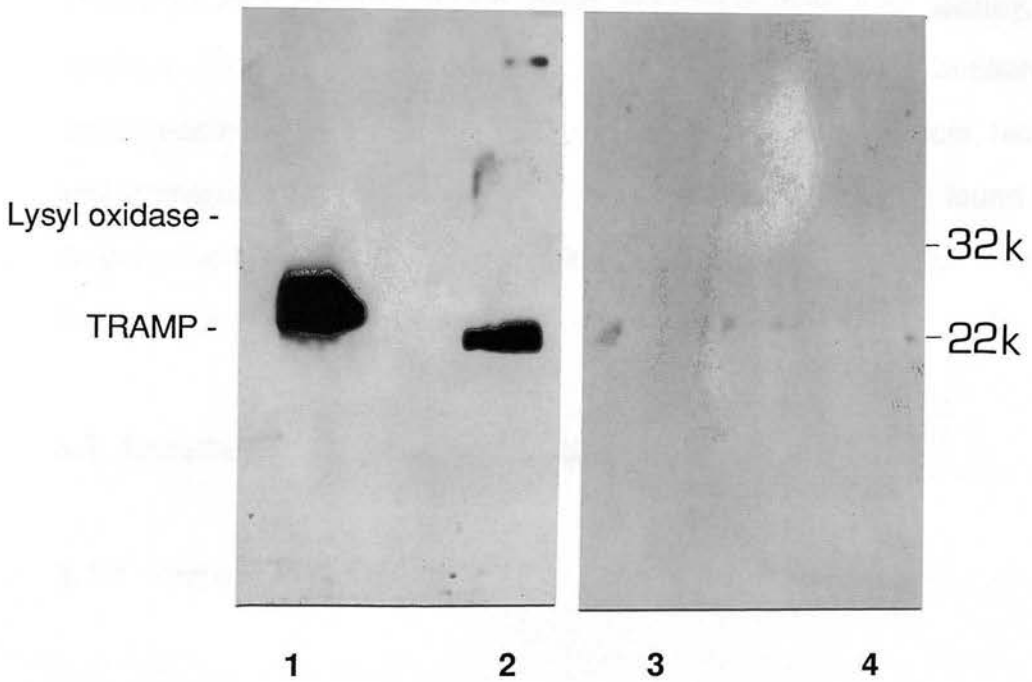


Figure 3.3. Lack of reactivity of anti-TRAMP antiserum against lysyl oxidase. Samples of partially purified lysyl oxidase (Fig 3.1)containing TRAMP (2μg ; lanes 1 and 2) and purified TRAMP (2μg ; lanes 2 and 4) were reacted with different concentrations of TRAMP antiserum and pre-immune serum. Antibodies were detected by ECL, with a film exposure of 1 minute. Lanes 1 and 2 : 20,000-fold dilution of anti-TRAMP antiserum. Lanes 3 and 4 : 10,000-fold dilution of pre-immune serum.

porcine TRAMP was used to screen skin extracts from human and other species. 6M PBU extracts of murine, avian and human skin were prepared (section 2.8) and blotted as above with the TRAMP antiserum (Fig. 3.4). Cross-reactivity occurred with extracts of both murine and human skin, at a single band co-migrating with porcine TRAMP. Extracts of avian skin did not cross-react with the antiserum indicating either an absence of TRAMP or (more likely) divergent primary structure.

To investigate the tissue distribution of TRAMP, 6M PBU extracts were prepared from several porcine tissues (section 2.8) and blotted with the anti-TRAMP antiserum at a dilution of 1:1000. The antiserum recognised TRAMP in PBU extracts of skin, heart, skeletal muscle, lung, kidney, spleen, cartilage, ulna and calvaria (Fig. 3.5). In a separate blot, the antiserum also cross-reacted with PBU extracts of murine skin, skeletal muscle, heart, lung and cartilage at a dilution of 1:2000 (Fig. 3.6). TRAMP is not found in brain or liver and therefore TRAMP distribution appears to correlate with tissues that are rich in connective tissue.

3.2 Enzymatic activity of TRAMP

3.2.1 Introduction

The wide distribution of TRAMP suggests that it may have a major structural or possibly catalytic role in tissue. The ability of TRAMP to accelerate collagen fibril formation *in vitro* has been demonstrated (MacBeath *et al.,* 1993) and possible associations with extracellular matrix

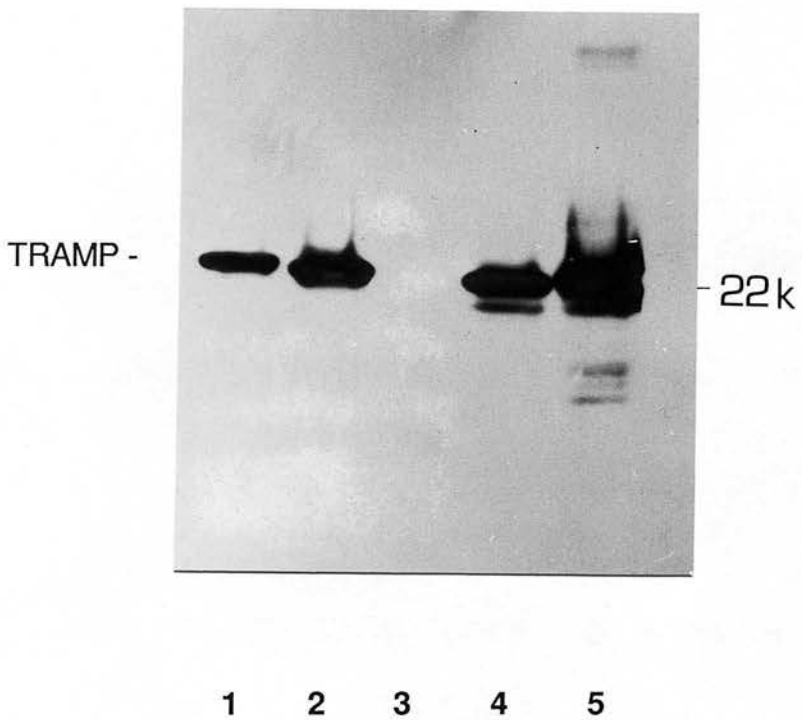


Figure 3.4. Reactivity of TRAMP polyclonal antiserum against 6 M PBU extracts from different species. Skins were extracted in 6M PBU, then 100 μg of each extract was electrophoresed, blotted onto nitrocellulose and exposed to the TRAMP polyclonal antiserum diluted 1:10000. Antibodies were detected by ECL with a film exposure time of 1 minute. Lane 1, porcine; 2, murine; 3, chicken; 4, human; 5, purified TRAMP (3 μg).

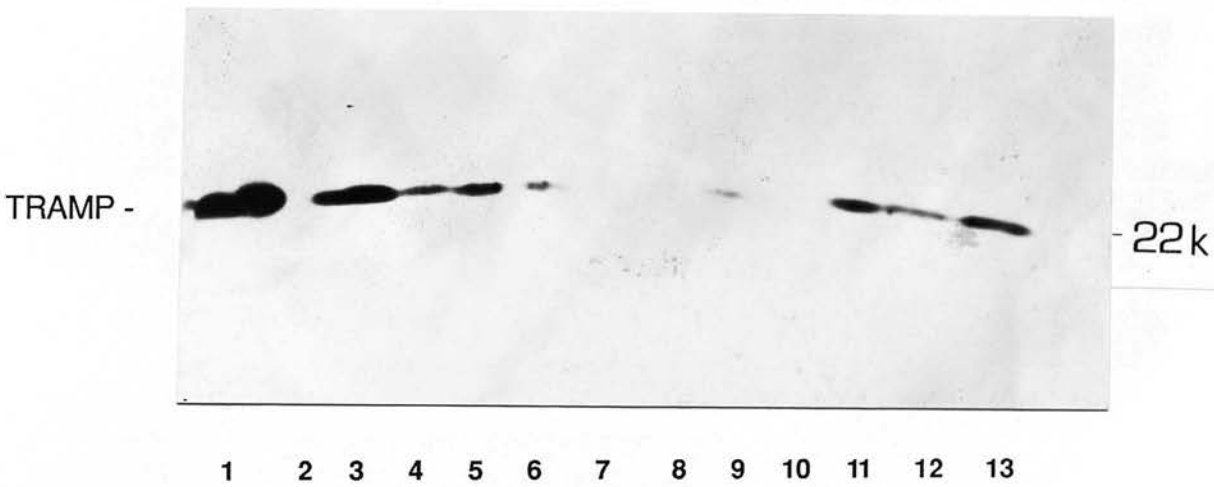


Figure 3.5. Reactivity of TRAMP polyclonal antiserum against 6M PBU extracts from different porcine tissues. Tissues were extracted in 6M PBU and 100μg of protein from each extract was run on a 12 % SDS-PAGE gel, then blotted and exposed to a 1,000 dilution of the polyclonal antiserum. Antibodies were detected by ECL with a film exposure of 1 minute. Lane 1, purified TRAMP; 2, blank; 3, skin; 4, heart; 5, skeletal muscle; 6, lung; 7, liver; 8, brain; 9, kidney; 10, spleen; 11, cartilage; 12, ulna; 13, calvaria.

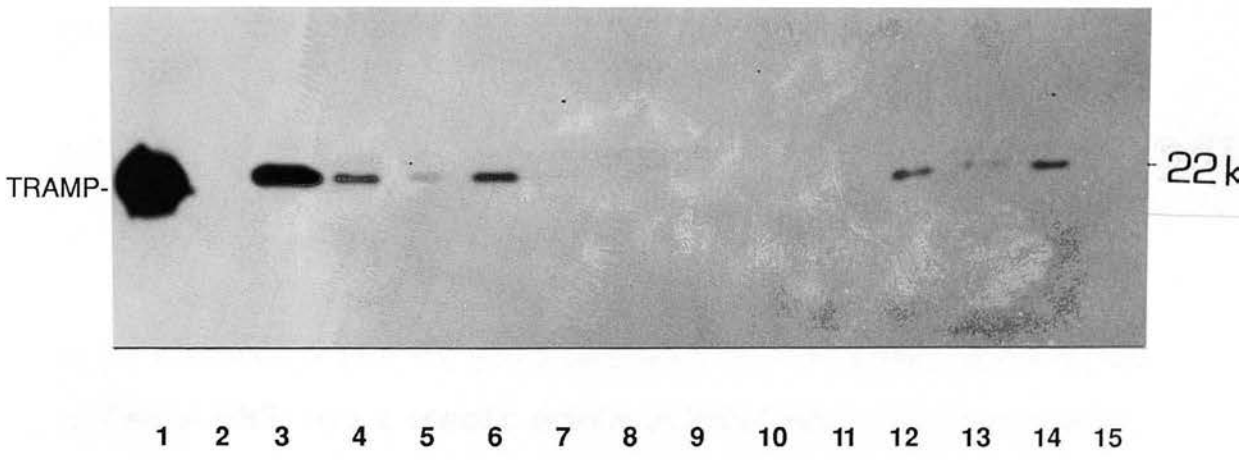


Figure 3.6. Reactivity of TRAMP polyclonal antiserum against PBU extracts from different murine tissues. Tissues were extracted in PBU and 100μg of each sample was run on a 12 % SDS-PAGE gel, then blotted and cross-reacted to a 2,000 dilution of the polyclonal antiserum. Antibodies were detected by ECL with a film exposure of 1 minute. Lane 1, TRAMP; 2, blank; 3, skin; 4, skeletal muscle; 5, heart; 6, lung; 7, liver; 8, kidney; 9, spleen; 10, brain; 11, eye; 12, tendon; 13; bone; 14, cartilage; 15, hair.

components may occur through a hexamer consensus sequence (Asp - Arg - Glx - Trp - Asn /Gln /Lys - Phe/ Tyr) that occurs three times in TRAMP (Neame *et al.*, 1989). Porcine, human and bovine TRAMP also include the sequence Tyr- Asn- Tyr- Asp- Tyr which is found in porcine kidney amine oxidase (Janes *et al.*, 1992). It has been proposed that the sequence Asn - Tyr - Asp / Glu is a consensus sequence for the incorporation of tri-hydroxy phenylalanine quinone (topaquinone) in a variety of copper dependent amine oxidases (Janes *et al.*, 1992 ; Table 3.1).

The involvement of TRAMP in collagen fibril formation *in vitro* and the presence of the putative topaquinone incorporation consensus sequence led to the following investigation of TRAMP as a possible collagen amine oxidase.

3.2.2. Determination of enzymatic activity

Collagen I was prepared from 17 day, chick embryo calvaria cultured in the presence of [4,5-^{3}H] lysine (section 2.3). The purifed collagen (120 μg / ml in PBS) had a specific activity of 28500 cpm / μg. Radiolabelled collagen (12 μg in 100μl of PBS), was incubated with 2μg TRAMP (in 100μl 6M PBU; Cronshaw *et al.*, 1993), in assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0) in a final volume of 900 μl at 37 °C overnight. Using the ultrafiltration method (Shackleton and Hulmes 1990b; section 2.4), preliminary results (Table 3.2) showed that tritium labelled low molecular weight (<10 kDa) material was released into the ultrafiltrate in the presence of TRAMP, when collagen was used as substrate. The presence of 0.2 mM β-aminopropionitrile (BAPN), which completely inhibits lysyl oxidase

Table 3.1. Comparison of the known topaquinone incorporation sequences of four amine oxidases with porcine, bovine and human TRAMP.

	1	2	3	4	5	6	7	8	9
Bovine Serum Ao	T	M	L	**N**	**X**	**D**	Y	V	_
Porcine Serum Ao	T	M	L	**N**	**X**	**D**	Y	V	_
Pea Seedling Ao	_	V	G	**N**	**X**	**D**	N	V	I
Pig Kidney Ao	T	V	Y	**N**	**X**	**D**	Y	I	_
Porcine TRAMP+	I	S	Y	**N**	**Y**	**D**	Y	Y	M
Bovine TRAMP^	I	S	Y	**N**	**Y**	**D**	Y	Y	M
Human TRAMP*	I	S	Y	**N**	**Y**	**D**	Y	Y	I

The following symbols are used: Ao= amine oxidase, X = observed site of topaquinone incorporation; _ denotes unknown amino acid. Bold letters represent the topaquinone incorporation consensus sequence as proposed by Janes et al., (1992). + Porcine TRAMP amino acid sequence (A.D.Cronshaw, personal communication). ^ Bovine TRAMP amino acid sequence (Neame *et al.*, 1989).

* Human TRAMP (dermatopontin) sequence deduced from cDNA (Superti-Furga *et al.*, 1993).

Table 3.2. Release of ^{3}H cpm by lysyl oxidase and TRAMP from radiolabelled collagen and elastin substrates.

Addition to assay	cpm ± S.E. (n=2)	activity % of control ± S.E. (n=2)
Collagen		
Control	1559 ± 15	100 ± 1.0
TRAMP	1735 ± 0.0	111 ± 0.0
Lysyl oxidase	1312 ± 42	84 ± 3.0
Collagen		
Control	1421 ± N/A	100 ± N/A
TRAMP	1602 ± 20	113 ± 1.2
TRAMP (200mM BAPN)	1382 ± 39	97 ± 2.8
Elastin		
Control	761 ± 26	100 ± 3.4
TRAMP	782 ± 14	103 ± 1.8
Lysyl oxidase	1152 ± 22	151 ± 1.9

TRAMP (2 µg) was incubated with 12µg [4,5-^{3}H] lysine labelled collagen (342,000 cpm) or 50µg [4,5- ^{3}H] lysine labelled elastin (300,000 dpm) in assay buffer (900µl total volume) overnight at 37^{0}C. The effect of 200mM BAPN on tritium release is also shown.

activity on elastin (Siegel *et al.*, 1970; Narayanan *et al.*, 1972), partially reduced the activity of TRAMP on collagen (not shown), while complete inhibition was observed at 200 mM BAPN (Table 3.2).

After incubation, a gel like structure was visible in the presence of TRAMP, in the presence and absence of 0.2mM BAPN, which was not observed in the absence of TRAMP. This observation is consistent with the known effect of TRAMP on collagen assembly (MacBeath *et al.,* 1993) and may reflect an increase in cross-linking between collagen molecules. Using the same assay, partially purified lysyl oxidase (2 μg; prepared by the method detailed in section 2.1), showed no activity on [4,5-^{3}H] lysine labeled collagen.

Further to the above, TRAMP (2 μg in 30μl PBS) was incubated with 12 μg [4,5-^{3}H] lysine labeled collagen (in 50μl PBS) in assay buffer (final volume150μl) at 37°C for 16 hours. The entire volume was then run on 6% discontinuous SDS-PAGE, under reducing conditions, and detected by fluorography, as described (section 2.11; Fig. 3.7). The fluorograms were then analysed on a Joyce-Loebl Chromoscan 3 scanning densitometer (Fig. 3.8). The β_{12} components (Piez, 1967) are increased almost 1.9 fold in the presence of TRAMP compared with the control samples, while the β_{11} components are increased almost 1.3 fold in the presence of TRAMP.

These preliminary results suggest that TRAMP may be involved in the cross-linking of collagen, and perhaps acts as a collagen specific 'lysyl oxidase'.

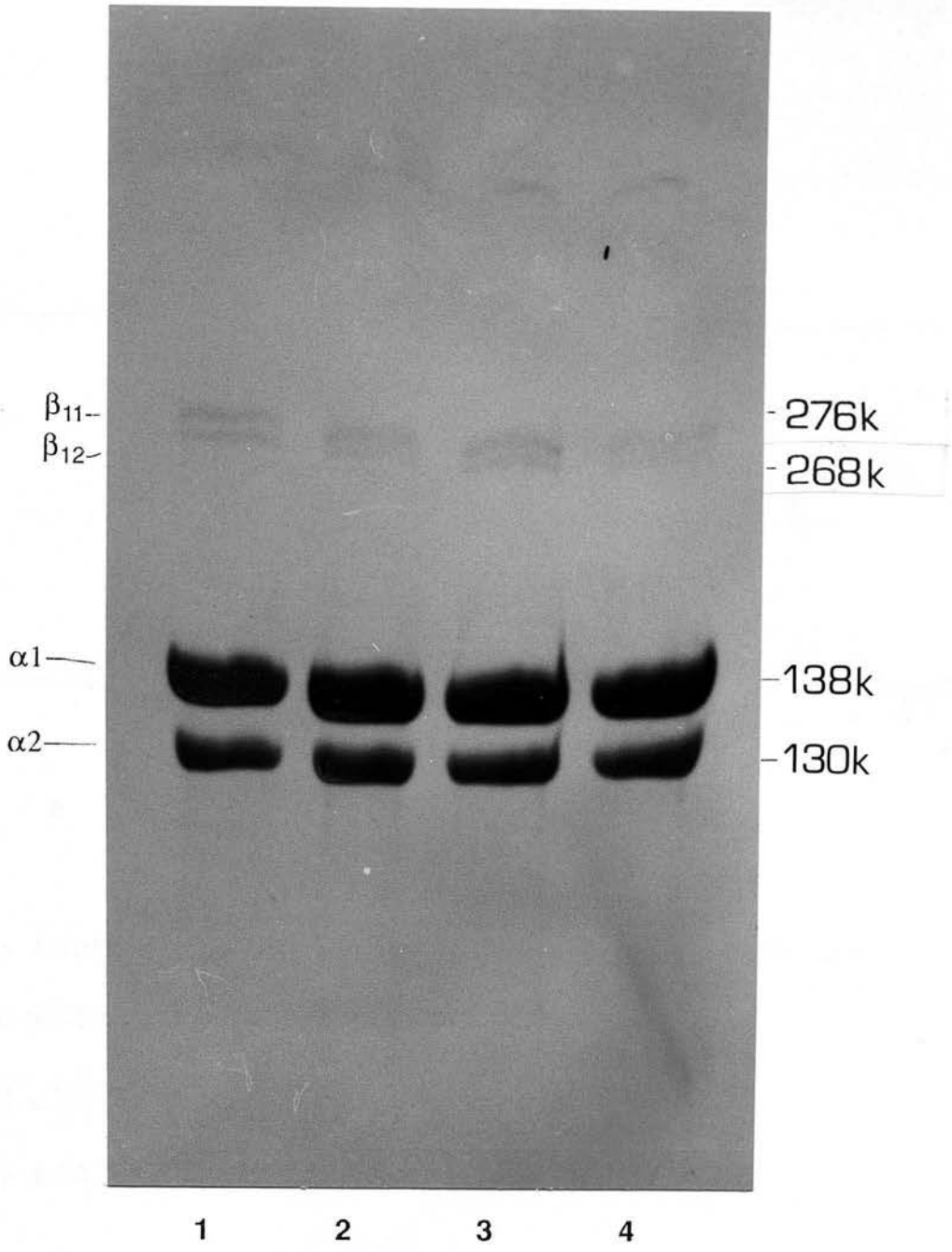
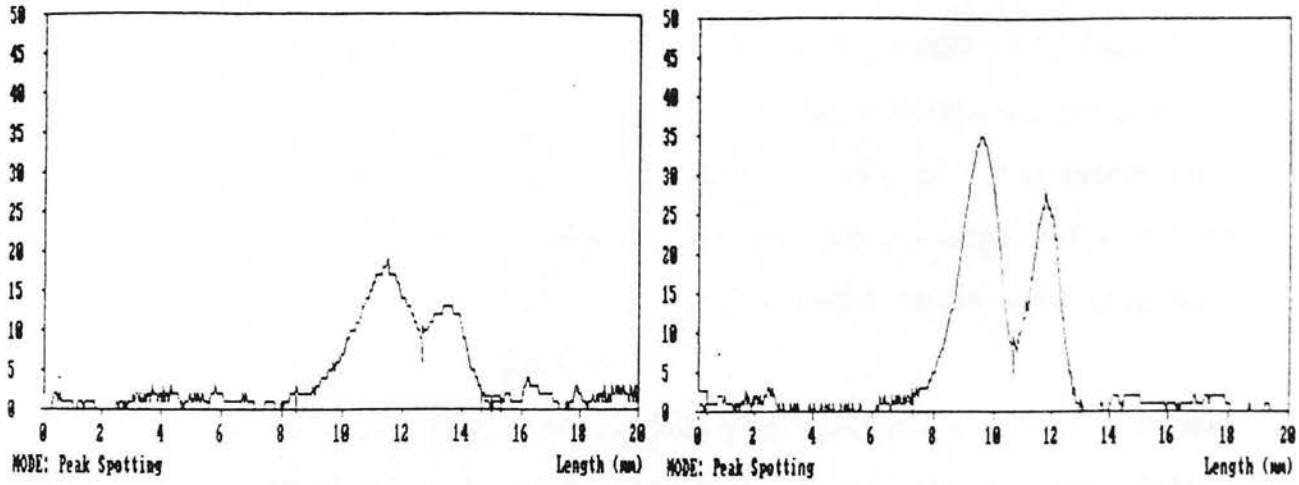


Figure 3.7. Effect of TRAMP on collagen cross-linking. Collagen labelled with [4,5-^{3}H] lysine (50μl of 120μg / ml) with a specific activity of 28500 cpm / μg, was incubated with TRAMP (2μg; 30μl) previously dialysed exhaustively against PBS (lanes 1 and 3), or with PBS (30μl) (lanes 2 and 4) and70μl of assay buffer at 37°C overnight. The 150μl samples were run on a 6 % SDS-PAGE gel and detected by fluorography.



a b

Collagen control (peak area)		**Collagen/TRAMP** (peak area)	
	S.E. (n=2)		S.E. (n=2)
β_{11}	989 ± 160	β_{11}	1252 ± 121
β_{12}	408 ± 14	β_{12}	766 ± 80

Figure 3.8. Densitometric scans of cross-linked collagen β components produced in the presence and absence of TRAMP. Collagen labelled with [4,5-^{3}H] lysine (50μl at 120μg / ml) with a specific activity of 382500 cpm / μg, was incubated with 70μl of assay buffer at 37°C overnight and either (a) PBS (30μl) or (b) TRAMP (30μl at 70μg / ml; section 2.1) and dialysed exhaustively against PBS. The 150μl samples were run on a 6 % SDS-PAGE gel and detected by fluorography. The β_{11} and β_{12} bands were then scanned in a Joyce-Loebl Chromoscan 3 densitometer.

3.3 Discussion

In the present study, polyclonal antibodies raised against purified porcine TRAMP were found not to cross-react with lysyl oxidase. TRAMP is therefore a distinct protein from lysyl oxidase. This result is consistent with the complete amino acid sequence of porcine TRAMP (A.D. Cronshaw, personal communication) which shows it to be unrelated to lysyl oxidase, but the same as a 22 kDa bovine skin extracellular matrix protein that purifies with dermatan sulphate proteoglycan (Neame *et al.*, 1989) and the same as recently discovered human equivalent called 'dermatopontin' (Superti-Furga *et al.*, 1993).

The polyclonal antiserum appeared to recognise a protein band with a higher molecular weight than TRAMP in Western blots of purified TRAMP (Fig 3.2). It is possible that this could represent a biosynthetic precursor of porcine TRAMP, although the cDNA of the human equivalent of TRAMP has been cloned and sequenced (Superti-Furga *et al.*, 1993), and appears to contain only a putative 18 amino acid signal peptide immediately upstream of the first residue present in porcine and bovine TRAMP (Cronshaw *et al.*, 1993 ; Neame *et al.*, 1989). It seems more likely therefore that this band is a contaminant of the purified TRAMP used to raise the rabbit anti-serum or a cross-linked multimer of TRAMP.

The Western blotting results show that TRAMP is a widely distributed protein, present in tissues that are rich in connective tissue. The role of TRAMP in the extracellular matrix is not yet understood. It is probable that the functions of lysyl oxidase and TRAMP are connected, since the formation of collagen fibrils *in vitro* is accelerated in the presence of TRAMP (MacBeath *et al.*, 1993) and collagen fibril cross-linking is initiated

by the action of lysyl oxidase on specific lysine residues. Preliminary studies here suggest that TRAMP may itself be involved in cross-link formation in type I collagen, but not in elastin. TRAMP also appears to influence the ratio of β_{11} and β_{12} components formed in collagen. These results raise the possibility that TRAMP is a collagen specific, lysyl oxidase-type enzyme. The activity demonstrated is not inhibitable by 0.2 mM BAPN, indicating that no contaminating lysyl oxidase is responsible for the activity observed.

Polyclonal antibodies raised against bovine lysyl oxidase (Kagan *et al.*, 1986) failed to recognise lysyl oxidase associated with collagen. However, the same antibodies did react with lysyl oxidase-collagen I complexes formed *in vitro* . Kagan *et al.* (1986) suggested that a collagen specific form of lysyl oxidase may exist *in vivo* that is not recognised by the antibodies raised to lysyl oxidase and which is capable of oxidizing both collagen and elastin substrates.

TRAMP contains the sequence Asn-Tyr-Asp, the consensus sequence for the modification of tyrosine to topaquinone in copper dependent amine oxidases (Janes *et al.,* 1992). This sequence is conserved in human, porcine and bovine TRAMP forms, however peptide sequencing of porcine TRAMP has indicated the presence of unmodified tyrosine at this position (Dr. A.D.Cronshaw, personal communication). Lysyl oxidase lacks the topaquinone incorporation consensus sequence. The incorporation site of the carbonyl cofactor in lysyl oxidase is still unknown, although Asn-Tyr-Tyr-Asp is present in the sequence.

The bovine form of TRAMP co-purifies with dermatan sulphate proteoglycans (Neame *et al.,* 1989). The dermatan sulphate proteoglycan

decorin has previously been found to associate with specific sites on the collagen fibril (Scott *et al.*, 1991) and has also been found to inhibit collagen fibrillogenesis *in vitro* (Vogel *et al.*, 1984). Bovine TRAMP has the ability to adhere to particular cell types, e.g. 3T3 cells and dermal fibroblasts, in the absence of dermatan sulphate proteoglycans, which suggest that bovine TRAMP may have an important role in mediating cell adhesion in the extracellular matrix (Lewandowska *et al.,* 1991). Inhibition of this adhesion activity by the integrin binding peptide Arg-Gly-Asp-Ser, and the presence of a potential integrin binding sequence Arg-Gly-Ala-Thr within the primary structure of bovine TRAMP, suggests that TRAMP binds to cell surface integrins. Dermatan sulphate chains have the ability to bind to bovine TRAMP and totally inhibit the binding activity (Lewandowska *et al.*, 1991).

It is possible that TRAMP is a multi-functional protein that serves in the communication between particular cells and their extracellular environment, and also performs a role in the development and maintenance of the matrix through interactions with forming collagen fibrils.

Chapter 4

Expression of lysyl oxidase cDNA

4.1 Introduction

In the original work on lysyl oxidase, enzyme activity was detected in a PBS extract of embryonic chick bone (Pinnell and Martin, 1968). Subsequently, it was discovered that much better yields of the enzyme could be obtained by extracting in 4-6M urea (Narayanan *et al,* 1974). Lysyl oxidase remains partially active in urea and is stable for several months. Urea also improves the recovery of the enzyme from ion exchange chromatography (Narayanan *et al.,* 1974). Subsequent removal of urea results in the formation of large aggregates (up to 1000 kDa) of enzyme (Jordan *et al.,* 1977). Solubilisation of lysyl oxidase in solutions of urea has remained as the primary step in most purification procedures (section 1.4.6).

A disadvantage of urea in enzyme purification is the spontaneous formation of cyanate ions, especially at low pH (Hagel *et al.,* 1971). Urea solutions containing cyanate can cause carbamylation of the α-amino group and of ε-amino groups on the side chains of lysyl residues in proteins, which then become differently charged, with possible effects on enzyme structure and function (Cole *et al.,* 1966). The presence of urea also causes proteins to unfold, and correct re-folding may not occur after the urea is removed.

Recombinant DNA technology has been used to clone and express many proteins such as human insulin (Goeddal *et al.,* 1979a) and human growth hormone (Goeddal *et al.,* 1979b). At the start of the project, the cDNA for rat aorta lysyl oxidase had been cloned and sequenced (Trackman *et al.,* 1990; 1991). This enabled the possible detection of lysyl

oxidase cDNA in a human placental cDNA library using a fragment of the rat sequence as a probe. The human cDNA sequence could then be cloned and sequenced. In addition to this the cDNA of the human enzyme could then be expressed in yeast, to produce lysyl oxidase without the requirement for the presence of urea, or the time-consuming purification procedure that is currently necessary (section 2.1).

Cloning and expression of the human cDNA sequence will be useful for determining those regions of sequence that code for the active site of the enzyme, as well as cofactor binding sites, and inhibitor binding sites. Mutagenesis techniques will allow manipulation of the DNA to investigate how these sites function. These types of study may also be useful in probing the causes of genetic diseases such as Menkes' disease (Danks, 1983), X-linked cutis laxa (Byers *et al.,* 1976) and Ehlers-Danlos syndrome type IX (Kuivaniemi *et al.,* 1982; Peltonen *et al.,* 1983). Exploration of the link between lysyl oxidase and tumour supression may also be possible, since the rat cDNA sequence has been found to be 89% identical with murine ras-recision gene (Kenyon *et al.,* 1991). Studies probing the molecular basis of the lysyl oxidase variants (Kagan *et al.,* 1979a) could also be undertaken. The expression of a precursor form of the enzyme may also allow the ability to study proteolytic processing of the enzyme.

The following attempts were undertaken to produce a DNA construct from which expression of recombinant human lysyl oxidase in the yeast *S.cerevisiae* could be achieved.

4.2 Production of a (rat aorta lysyl oxidase) cDNA fragment (LOF 1) to probe a human placental cDNA library

4.2.1 Introduction

From the sequence of the cDNA encoding rat aorta lysyl oxidase precursor (Trackman *et al.*, 1990; 1991), two oligonucleotide primers were designed to produce a 532 bp fragment of rat lysyl oxidase cDNA using the polymerase chain reaction (section 2.13.3; protocol 3; Fig. 4.1). The cloned fragment could then be used to probe a human placental cDNA library. The primer design was aided by Gene Jockey software (Biosoft). A *Bam*HI restriction endonuclease site was incorporated into both primers to allow for non-directional cloning of the DNA produced into the polylinker site of pK19 (kindly donated by Dr. A Boyd; Fig. 4.2).

4.2.2 LOF 1 production using PCR

The method of Friedman *et al.*, (1988; section 2.13.3) was used for ampilfication of LOF 1 from a λgt11 library (Clontech), synthesised from Sprague-Dawley neonatal rat aorta cDNA. DNA was electrophoresed on a 0.8% agarose gel (section 2.13.4) and visualized under ultra-violet light (Fig. 4.3). A fragment of approximately 530 bp was observed.

4.2.3 LOF 1 ligation into pK19

The DNA fragment produced was excised from the agarose gel, solubilized with sodium iodide and purified using a Geneclean II kit (Bio

Figure 4.1. Sequence of rat aorta lysyl oxidase cDNA (Trackman *et al.*, 1990,1991).

```
    ATG CGT TTC GCC TGG ACC GTG CTC TTT CTG GGA CAG CTG CAG TTC TGT CCC CTT CTC CGC TGC
1   Met Arg Phe Ala Trp Thr Val Leu Phe Leu Gly Gln Leu Gln Phe Cys Pro Leu Leu Arg Cys

    GCC CCG CAG GCC CCG CGC GAG CCT CCC GCC GCC CCC GGT GCC TGG CGC CAG ACA ATC CAA TGG
22  Ala Pro Gln Ala Pro Arg Gln Pro Pro Ala Ala Pro Gly Ala Trp Arg Gln Thr Ile Gln Trp

    GAG AAC AAC GGG CAG GTG TTC AGT CTG TTG AGC CTG GGG GCG CAG TAC CAG CCT CAG CGA CGC
43  Glu Asn Asn Gly Gln Val Phe Ser Leu Leu Ser Leu Gly Ala Gln Tyr Gln Pro Gln Arg Arg

    CGC GAC TCC AGC GCC ACT GCC CCG AGA GCC GAC GGC AAC GCT GCA GCA CAG CCA CGC ACG CCC
64  Arg Asp Ser Ser Ala Thr Ala Pro Arg Ala Asp Gly Asn Ala Ala Ala Gln Pro Arg Thr Pro

    ATT CTG CTG CTG CGT GAC AAC CGC ACT GCC TCT GCC CGT GCG AGG ACT CCA AGC CCA TCT GGC
85  Ile Leu Leu Leu Arg Asp Asn Arg Thr Ala Ser Ala Arg Ala Arg Thr Pro Ser Pro Ser Gly

    GTC GCC GCG GGT CGT CCC CGG CCC GCA GCC CGC CAC TGG TTC CAA GTT GGT TTC TCG CCG TCC
106 Val Ala Ala Gly Arg Pro Arg Pro Ala Ala Arg His Trp Phe Gln Val Gly Phe Ser Pro Ser
                                        ↓
    GGG GCC GGC GAT GGA GCC TCA AGG CGC GCA GCG AAC CGG ACT GCG TCG CCA CAG CCT CCG CAG
127 Gly Ala Gly Asp Gln Ala Ser Arg Arg Ala Ala Asn Arg Thr Ala Ser Pro Gln Pro Pro Gln

    CTC AGT AAT CTG AGG CCA CCC AGC CAC GTA GAT CGC ATG GTG GGC GAC GAC CCC TAC AAT CCC
148 Leu Ser Asn Leu Arg Pro Pro Ser His Val Asp Arg Met Val Gly Asp Asp Pro Tyr Asn Pro

    TAC AAG TAC TCC GAC GAC AAC CCC TAT TAT AAC TAC TAT GAC ACT TAT GAG AGA CCC CGG TCC
169 Tyr Lys Tyr Ser Asp Asp Asn Pro Tyr Tyr Asn Tyr Tyr Asp Thr Tyr Glu Arg Pro Arg Ser

    GGG AGC AGG CAC CGA CCT GGA TAT GGC ACC GGT TAC TTC CAG TAC GGT CTC CCG GAC CTG GTA
190 Gly Ser Arg His Arg Pro Gly Tyr Gly Thr Gly Tyr Phe Gln Tyr Gly Leu Pro Asp Leu Val
      ____________Primer L1_________________
       Bam HI
       5'GCGGATCC TAC TAC ATC CAG GCA TCC ACG 3'
    CCC GAT CCC TAC TAC ATC CAG GCA TCC ACG TAC GTA CAA AAG ATG TCT ATG TAC AAC CTG AGA
211 Pro Asp Pro Tyr Tyr Ile Gln Ala Ser Thr Tyr Val Gln Lys Met Ser Met Tyr Asn Leu Arg

    TGC GCT GCG GAA GAA AAC TGC CTG GCC AGT TCA GCA TAT AGG GCG GAT GTC AGA GAC TAT GAC
23  Cys Ala Ala Glu Glu Asn Cys Leu Ala Ser Ser Ala Tyr Arg Ala Asp Val Arg Asp Tyr Asp

    CAC AGG GTA CTG CTA CGA TTT CCT CAG AGA GTG AAA AAC CAA GGG ACG TCT GAC TTC TTA CCA
253 His Arg Val Leu Leu Arg Phe Pro Gln Arg Val Lys Asn Gln Gly Thr Ser Asp Phe Leu Pro

    AGC CGC CCC CGC TAC TCC TGG GAG TGG CAC AGC TGC CAC CAA CAT TAC CAC AGC ATG GAT GAA
274 Ser Arg Pro Arg Tyr Ser Trp Glu Trp His Ser Cys His Gln His Tyr His Ser Met Asp Glu

    TTC AGC CAC TAC GAC CTG CTG GAT GCC AGC ACA CAG AGG AGA GTG GCC GAG GGC CAC AAA GCA
295 Phe Ser His Tyr Asp Leu Leu Asp Ala Ser Thr Gln Arg Arg Val Ala Glu Gly His Lys Ala

    AGC TTC TGT CTG GAG GAC ACT TCC TGT GAT TAT GGG TAC CAC AGA CGA TTT GCC TGT ACT GCA
316 Ser Phe Cys Leu Glu Asp Thr Ser Cys Asp Tyr Gly Tyr His Arg Arg Phe Ala Cys Thr Ala

   CAC ACA CAG GGG TTG AGT CCC GGA TGT TAT GAT ACT TAT GCA GCA GAC ATA GAC TGC CAG TGG
337 His Thr Gln Gly Leu Ser Pro Gly Cys Tyr Asp Thr Tyr Ala Ala Asp Ile Asp Cys Gln Trp
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    ATT GAT ATT ACA GAT GTA CAA CCC GGA AAT TAC ATT CTA AAG GTC AGC GTA AAC CCC AGC TAC
358 Ile Asp Ile Thr Asp Val Gln Pro Gly Asn Tyr Ile Leu Lys Val Ser Val Asn Pro Ser Tyr
                                                                    ____ Primer L2_
                                                          3' GCG ATG TGT CCT GTA
    CTG GTG CCT GAA TCA GAC TAC AGT AAC AAT GTC GTA CGC TGT GAA ATT CGC TAC ACA GGA CAT
379 Leu Val Pro Glu Ser Asp Tyr Ser Asn Asn Val Val Arg Cys Glu Ile Arg Tyr Thr Gly His

          Bam HI
    GTG CCTAGGCG 5'
    CAC GCC TAT GCC TCA GGC TGC ACC ATT TCA CCG TAT TAG
400 His Ala Tyr Ala Ser Gly Cys Thr Ile Ser Pro Tyr END
```

The position of the primers L1 and L2 used to amplify the LOF 1 cDNA (single underlined) are shown. ↓Denotes the putative proteolytic processing site suggested by Trackman *et al.* (1990).

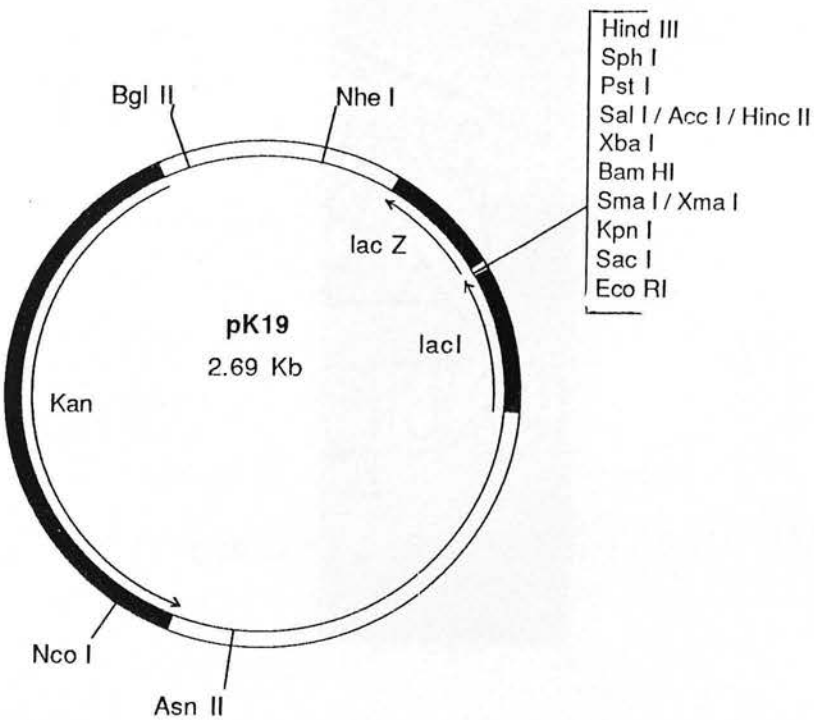


Figure 4.2. Plasmid pK19.

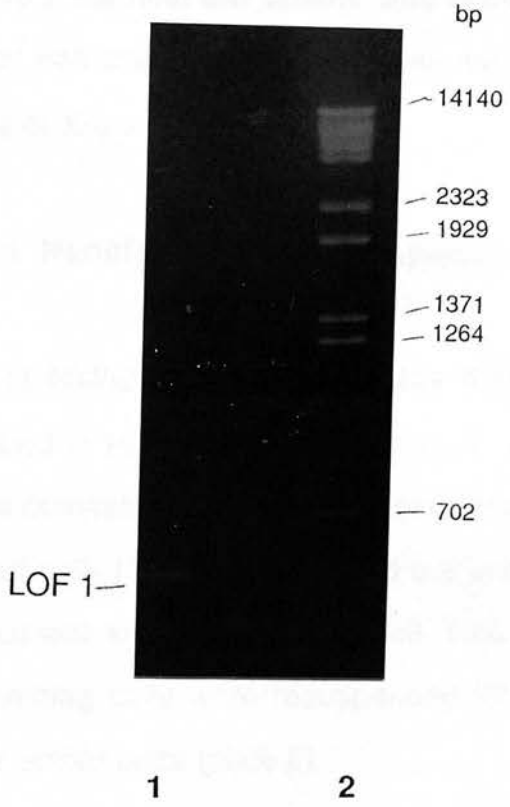


Figure 4.3. Fragment LOF 1 amplified from a rat aorta cDNA library by the polymerase chain reaction. Lane 1, LOF 1; lane 2, molecular weight markers (λ digested with *Bst*EII restriction endonuclease).

101 Inc.) as described in section 2.13.5 (protocol 4). This procedure removes ethidium bromide, DNA fragments smaller than 500 bp (e.g. oligonucleotide primers), and contaminating proteins. After digestion with *Bam*HI (section 2.13.5) a further Geneclean step was undertaken to remove this enzyme. LOF 1 was then ligated into the *Bam*HI site of pK19 (Pridmore, 1987) to produce pLOF 1. The plasmid pK19 is closely related to the ampicillin resistant pUC vectors, which have a very high copy number in bacterial cells (500 -700; Minton *et al.,* 1988), though pK19 encodes kanamycin resistance. These vectors encode the first 146 amino acids of the lac Z gene (β-galactosidase) which complements the partial lac Z gene in appropriate host cell strains, and allows blue / white screening for recombinant cell colonies when the cells are grown on LB-agar plates in the presence of X-gal and IPTG.

4.2.4 pLOF 1 transformation of competent cells

Competent NM522 *E.coli* cells (Table 4.1) were prepared by the method described in section 2.13.5 (protocol 6). pLOF 1 (10 μl) was then transfected into competent cells by the method described in section 2.13.6. The transformed cells (100 μl) were plated out onto an LB-agar plate (plate 1) supplemented with kanamycin (10 mg/ml), 10% X-gal and IPTG (200 mg / ml). The remaining cells were resuspended into 100μl of LB-broth, and plated out on a similar plate (plate 2).

Table 4.1. Bacterial and yeast cell strains

Type	Genotype	Reference
***E.coli* strains**		
NM522	Δ (lac-pro AB) his 5 (rk-,mk+) thi sup E F1(pro AB lac Iq) ZΔ M15	Gough and Murray (1983)
JM109	rec A1 supE44 endA1 hsdR17 gyrA96 F' [tra D36 proAB+ laclq lac Z M 15]	Gough and Murray (1983)
Y1090	sup F hsdR araD139 lon lac U16 rpsL trpC22 Tn*10*(tetr) pMC9	Young and Davis (1983)
***S.cerevisiae* strains**		
JRY188	Mat α *leu 2-3, 112 ura 3-52 trp1 his 4 sir 3-8 rme GAL*	Brake *et al.* (1984)

4.2.5 Recombinant colony screening

Colonies were screened for recombinant DNA after plasmid minipreps (section 2.13.7.2; protocol 7). Each colony was transferred into LB-broth, and grown overnight at 37^{0}C. The cells were then lysed with alkali, and the DNA was extracted in phenol / chloroform / isoamyl alcohol (25 : 24 : 1) and finally precipitated in ethanol. A restriction digest with *Bam*HI was performed on each miniprep as described in section 2.13.3.2 . Approximately 60 colonies appeared on plate 2, of which one colony was found to contain an insert of approximately 530 bp (Fig. 4.4). A larger scale preparation of plasmid DNA (section 2.13.7.2; protocol 8) was then performed to obtain a sufficient quantity of the recombinant plasmid DNA for further manipulation.

4.2.6 LOF1 DNA Sequencing

To confirm its identity, the DNA fragment present in the plasmid (excised by *Bam*HI) was sequenced using Sequenase version 2.0 DNA polymerase (USB) and 10 ng of PCR primer L1 (Fig. 4.1), according to the method of Sanger *et al.*, (1977) and Hsiao, (1991), as described in section 2.13.8 (protocol 9). The DNA sequence obtained (Fig. 4.5), allowed 97 bases to be read, and confirmed that the fragment amplified and cloned corresponded to partial rat aorta lysyl oxidase cDNA.

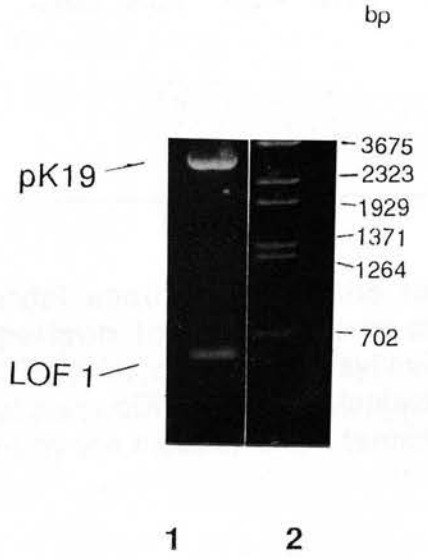


Figure 4.4. Screening minipreps of colonies for pK19 containing LOF1 with *Bam* HI. LOF 1 was sub-cloned into pK19, and a mini-prep of this DNA was cut with *Bam* HI restriction endonuclease. Lane 1, LOF 1; lane 2, molecular weight markers (λ DNA digested with *Bst* EII).

Rat Aorta	5'	1067	AGT	GGA	TTG	ATA	TTA	CAG	ATG	TAC	AAC
LOF 1	5'		AGT	GGA	TTG	ATA	TTA	CAG	ATG	TAC	AAC
Rat Aorta		1094	CCG	GAA	ATT	ACA	TTC	TAA	AGG	TCA	GTG
LOF 1			CCG	GAA	ATT	ACA	TTC	TAA	AGG	TCA	GTG
Rat Aorta		1121	TAA	ACC	CCA	GCT	ACC	TGG	TGC	CTG	AAT
LOF 1			TAA	ACC	CCA	GCT	ACC	TGG	TGC	CTG	AAT
Rat Aorta		1148	CAG	ACT	ACA	GTA	ACA	ATG	TC	3'	
LOF 1			CAG	ACT	ACA	GTA	ACA	ATG	TC	3'	

Figure 4.5. Partial sequencing of the rat lysyl oxidase fragment LOF1, and comparison to the rat aorta cDNA sequence (Trackman *et al.*, 1991). A 532bp fragment of rat lysyl oxidase cDNA (Fig 4.1) was amplified from a rat aorta cDNA library (Clontech), cloned into pK 19 and partially sequenced by the dideoxy-chain termination method (section 2.13.8; protocol 5).

4.2.7. LOF 1 labelling and use as a probe for human placental lysyl oxidase

LOF 1 was radiolabelled as described in section 2.13.9, and used to probe a human placental cDNA library (Clontech), plated out on large LB-agar plates (30cm x 30cm) as described in section 2.13.10. Nitrocellulose filters were used to lift DNA from the plates in duplicate, and these were developed using Hyperfilm MP X-ray film (Amersham). No coincident spots of radioactivity could be found on the duplicate filters indicating that the radiolabelled probe did not hybridize to the lysyl oxidase cDNA in the library. This failure may have been due to very few or no complete lysyl oxidase cDNA clones being present in the human placental cDNA library.

4.3 Cloning mature lysyl oxidase cDNA (VLO 1) from a human placental cDNA library.

4.3.1 Introduction

The cDNA sequence encoding the human placental cDNA lysyl oxidase precursor was published part way through the project (Hamalainen *et al.*, 1991). Hence an alternative strategy for cloning the cDNA encoding mature lysyl oxidase was undertaken.

The precise N-terminus of mature lysyl oxidase was unknown during this part of the project, although Trackman *et al.*, (1990) predicted that the site of proteolytic cleavage was likely to be between Arg 134 - Arg 135 in the rat aorta sequence, based on a calculation of the molecular mass C-terminal to this site (Fig. 4.1). The site is equivalent to Ser 140 - Arg 141 in

the human sequence (Hamalainen *et al.,* 1991; Fig. 4.6). In contrast, analysis of porcine skin lysyl oxidase by amino acid sequencing and mass spectrometry (Dr. A.D. Cronshaw, personal communication) has indicated that the N-terminal amino acid is one of three consecutive amino acid residues equivalent to valine 167, glycine 168 or aspartic acid 169 in the human sequence. In the work described below, primers were designed to amplify lysyl oxidase cDNA corresponding to an N-terminus in the region of Val 167.

4.3.2 PCR

Two PCR primers were designed (V1 and V2; Fig. 4.6) to amplify a 758 bp fragment of human lysyl oxidase cDNA from valine 167 to beyond the termination codon TAG at position 418. The 5' primer (V1) contained a *Sma*I restriction site and the 3' primer (V2) contained a *Bam*HI site. The use of different restriction sites allowed directional cloning of the DNA into a vector.

The PCR was carried out as before (section 2.13.1; protocol 3) using 5µl of a 34 week, human placental cDNA library (Clontech) as template. Initially very poor yields of amplified DNA were produced. In an attempt to improve the yield, more cycles and more template DNA were introduced, and the $MgCl_2$ concentration was altered from 1.5 mM to concentrations ranging from 0.5 mM to 2.5 mM, though without significant improvement. Instead, spurious DNA bands were observed (Fig. 4.7), so a 'hot start' variation to the procedure was introduced to allow more specific annealing of the primers to the template. This procedure (D'Aquila *et al.,* 1991)

Figure 4.6. Sequence of human placental lysyl oxidase cDNA (Hamalainen *et al.*, 1991).

```
1   ATG CGC TTC GCC TGG ACC GTG CTC CTG CTC GGG CCT TTG CAG CTC TGC GCG CTA GTG CAC
1   Met Arg Phe Ala Trp Thr Val Leu Leu Leu Gly Pro Leu Gln Leu Cys Ala Leu Val His

61  TGC GCC CCT CCC GCC GCC GGC CAA CAG CAG CCC CCG CGC GAG CCG CCG GCG GCT CCG GGC
21  Cys Ala Pro Pro Ala Ala Gly Gln Gln Gln Pro Pro Arg Glu Pro Pro Ala Ala Pro Gly

121 GCC TGG CGC CAG CAG ATC CAA TGG GAG AAC AAC GGG CAG GTG TTC AGC TTG CTG AGC CTG
41  Ala Trp Arg Gln Gln Ile Gln Trp Glu Asn Asn Gly Gln Val Phe Ser Leu Leu Ser Leu

181 GGC TCA CAG TAC CAG CCT CAG CGC CGC CGG GAC CCG GGC GCC GCC GTC CCT GGT GCA GCC
61  Gly Ser Gln Tyr Gln Pro Gln Arg Arg Arg Asp Pro Gly Ala Ala Val Pro Gly Ala Ala

241 AAC GCC TCC GCC CAG CAG CCC CGC ACT CCG ATC CTG CTG ATC CGC GAC AAC CGC ACC GCC
81  Gln Ala Ser Ala Gln Gln Pro Arg Thr Pro Ile Leu Leu Ile Arg Asp Asn Arg Thr Ala

301 GCG GGG CGA ACG CGG ACG GCC GGC TCA TCT GGA GTC ACC GCT GGC CGC CCC AGG CCC ACC
101 Ala Gly Arg Thr Arg Thr Ala Gly Ser Ser Gly Val Thr Ala Gly Arg Pro Arg Pro Thr
                                                                              ↓
361 GCC CGT CAC TGG TTC CAA GCT GGC TAC TCG ACA TCT AGA GCC CGC GAA GCT GGG CCC TCG
121 Ala Arg His Trp Phe Gln Ala Gly Tyr Ser Thr Ser Arg Ala Arg Glu Ala Gly Pro Ser

421 CGC GCG GAG AAC CAG ACA GCG CCG GGA GAA GTT CCT GCT CTC AGT AAC CTG CGG CCG CCC
141 Arg Ala Glu Asn Gln Thr Ala Pro Gly Glu Val Pro Ala Leu Ser Asn Leu Arg Pro Pro
                        _____Primer V1_____
                Sma I
            5' GTGCCCGGGTG GTG GGC GAC GAC CCT TAC AAC 3'      *Sca I*
481 AGC CGC GTG GAC GGC ATG GTG GGC GAC GAC CCT TAC AAC CCC TAC AAG TAC TCT GAC GAC
161 Ser Arg Val Asp Gly Met Val Gly Asp Asp Pro Tyr Asn Pro Tyr Lys Tyr Ser Asp Asp
                                                                    *Kpn I*
541 AAC CCT TAT TAC AAC TAC TAC GAT ACT TAT GAA AGG CCC AGA CCT GGG GGC AGG TAC CGG
181 Asn Pro Tyr Tyr Asn Tyr Tyr Asp Thr Tyr Glu Arg Pro Arg Pro Gly Gly Arg Tyr Arg

601 CCC GGA TAC GGC ACT GGC TAC TTC CAG TAC GGT CTC CCA GAC CTG GTG GCC GAC CCC TAC
201 Pro Gly Tyr Gly Thr Gly Tyr Phe Gln Tyr Gly Leu Pro Asp Leu Val Ala Asp Pro Tyr

661 TAC ATC CAG GCG TCC ACG TAC GTG CAG AAG ATG TCC ATG TAC AAC CTG AGA TGC GCG GCG
221 Tyr Ile Gln Ala Ser Thr Tyr Val Gln Lys Met Ser Met Tyr Asn Leu Arg Cys Ala Ala

721 GAG GAA AAC TGT CTG GCC AGT ACA GCA TAC AGG GCA GAT GTC AGA GAT TAT GAT CAC AGG
241 Glu Glu Asn Cys Leu Ala Ser Thr Ala Tyr Arg Ala Asp Val Arg Asp Tyr Asp His Arg

781 GTG CTG CTC AGA TTT CCC CAA AGA GTG AAA AAC CAA GGG ACA TCA GAT TTC TTA CCC AGC
261 Val Leu Leu Arg Phe Pro Gln Arg Val Lys Asn Gln Gly Thr Ser Asp Phe Leu Pro Ser

841 CGA CCA AGA TAT TCC TGG GAA TGG CAC AGT TGT CAT CAA CAT TAC CAC AGT ATG GAT GAG
281 Arg Pro Arg Tyr Ser Trp Glu Trp His Ser Cys His Gln His Tyr His Ser Met Asp Glu

901 TTT AGC CAC TTG TAC CTG CTT GAT GCC AAC ACC CAG AGG AGA TGG GCT GAA GGC CAC AAA
301 Phe Ser His Leu Tyr Leu Leu Asp Ala Asn Thr Gln Arg Arg Trp Ala Glu Gly His Lys

961 GCA AGT TTC TGT CTT GAA GAC ACA TCC TGT GAC TAT GGC TAC CAC AGG CGA TTT GCA TGT
321 Ala Ser Phe Cys Leu Glu Asp Thr Ser Cys Asp Tyr Gly Tyr His Arg Arg Phe Ala Cys
```

```
1021 ACT GCA CAC ACA CAG GGA TTG AGT CCT GGC TGT TAT GAT ACC TAT GGT GCA GAC ATA GAC
341  Thr Ala His Thr Gln Gly Leu Ser Pro Gly Cys Tyr Asp Thr Tyr Gly Ala Asp Ile Asp

1081 TGC CAG TGG ATT GAT ATT ACA GAT GTA AAA CCT GGA AAC TAT ATC CTA AAG GTC AGT GTA
361  Cys Gln Trp Ile Asp Ile Thr Asp Val Lys Pro Gly Asn Tyr Ile Leu Lys Val Ser Val


1141 AAC CCC AGC TAC CTG GTT CCT GAA TCT GAC TAT ACC AAC AAT GTT GTG CGC TGT GAC ATT
381  Asn Pro Ser Tyr Leu Val Pro Glu Ser Asp Tyr Thr Asn Asn Val Val Arg Cys Asp Ile

                                                  _____Primer V2_____
                                                                  Bam HI
                                                3' CG TGT TAA AGT GGC ATA ATCTTCCTAGGGC 5'
1201 CGC TAC ACA GGA CAT CAT GCG TAT GCC TCA GGC TGC ACA ATT TCA CCG TAT TAG AAGGCAAA
401  Arg Tyr Thr Gly His His Ala Tyr Ala Ser Gly Cys Thr Ile Ser Pro Tyr END
```

The position of the primers V1 and V2 used to amplify the VLO 1 cDNA (single underlined) are shown. ↓ Denotes the putative site of proteolytic processing as suggested by Trackman *et al.*, (1991).

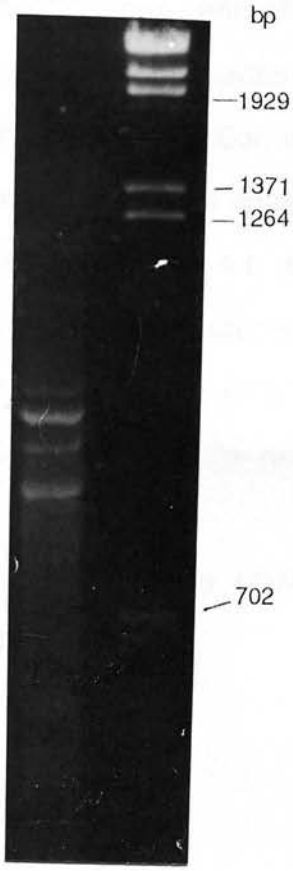


Figure 4.7. Amplified DNA produced by the polymerase chain reaction using the VLO 1 primers (V1 and V2). 'Cold start' PCR using a human placental cDNA library (Clontech) as template. Lane 1, DNA produced from a PCR reaction using primers V1 and V2; lane 2, molecular weight markers (λ DNA digested with *Bst*EII restriction endonuclease).

involves an initial denaturation of the DNA at 94-96^{0}C for four minutes before Taq DNA polymerase is added. At this temperature Taq polymerase has a half-life of about 6.5 hours. A reduction in spurious DNA bands was observed, though the DNA yields continued to be too low for further DNA manipulation (Fig. 4.8).

A DNA band of approximately 750 bp was found to have been primed at both ends by primer V1 alone (Fig. 4.9). In order to overcome the poor quality of the PCR amplifications, and the possibility of further single-primer induced amplifications, the reaction was completely re-designed (section 2.13.1; protocol 10). The reaction volume was reduced from 100μl to 30μl to promote uniform temperature changes within the heating block. A human placental cDNA Quickclone kit (Clontech) i.e. purified double stranded DNA synthesised from placental poly A RNA, was used as template cDNA. Quickclone cDNA contains no vector DNA, leading to fewer unwanted amplifications during the reaction.

4.4 Cloning mature lysyl oxidase cDNA (VLO 2) from a human placental cDNA Quickclone kit

4.4.1 Introduction

New PCR primers were obtained (V3 and V4; Fig. 4.10) to amplify a new 807 bp product VLO 2. The 5' primer (V3) was designed with a *Bbr*I restriction enzyme site at the 5' end. In this primer, the *Bbr*I site incorporates three bases that code for valine 167 and the DNA is cut blunt at the start of this codon. This allows *Bbr*I restricted VLO 2 to be cloned into an expression vector with immediate in-frame DNA coding for valine 167.

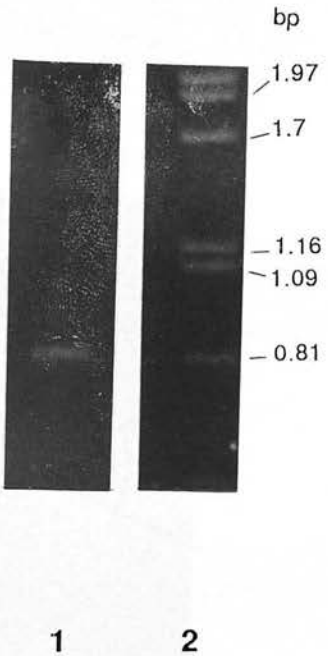


Figure 4.8. Amplified DNA produced by the polymerase chain reaction using the VLO 1 primers (V1 and V2). 'Hot start' PCR using a human placental cDNA library (Clontech) as template. Lane 1, DNA produced from a PCR reaction using primers V1 and V2,; lane 2, molecular weight markers (λ DNA digested with *Pst* I restriction endonuclease).

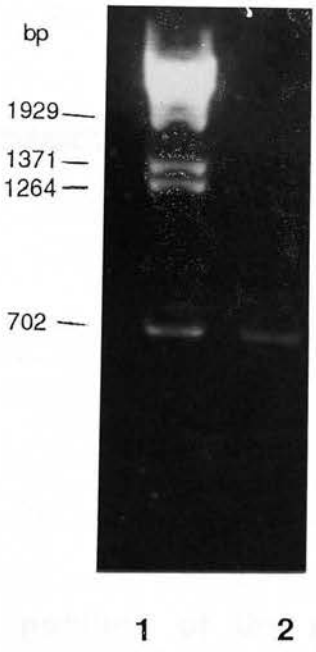


Figure 4.9. Amplified DNA produced by the polymerase chain reaction using the VLO 1 primer V1. 'Hot start' PCR using a human placental cDNA library (Clontech) as template. Lane 1, DNA produced from a PCR reaction using primer V1; Lane 2, molecular weight markers (λ DNA digested with *Bst* EII restriction endonuclease).

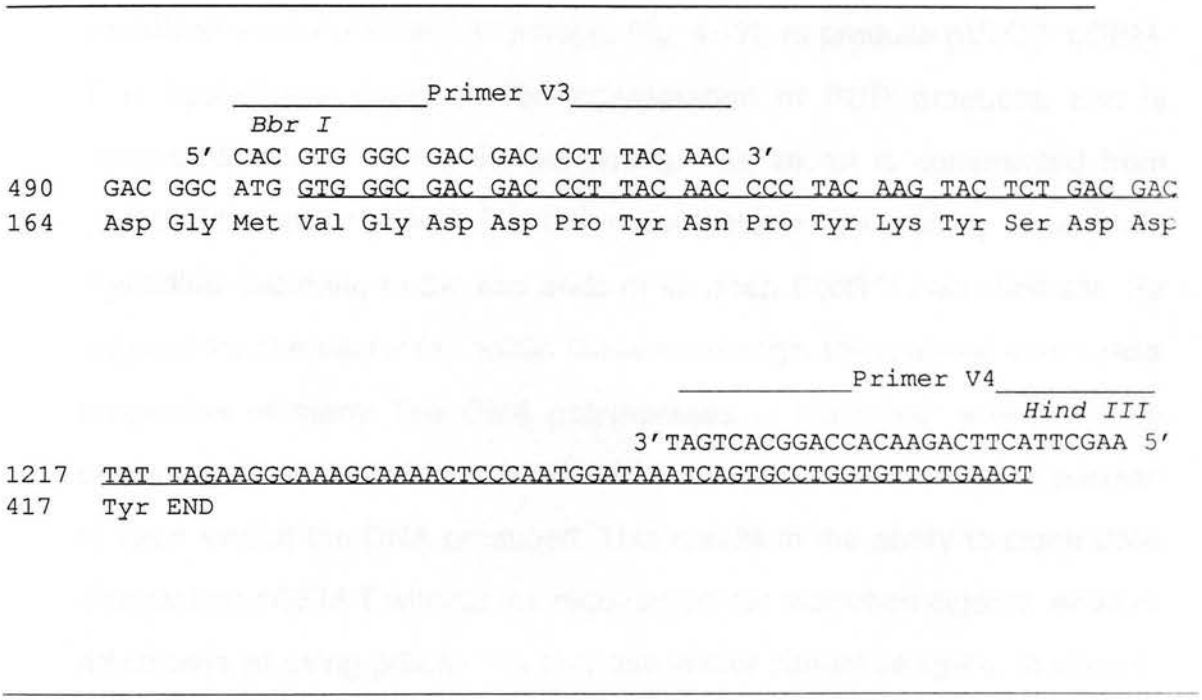


Figure 4.10. The position of the primers V3 and V4 on the human placental lysyl oxidase precursor sequence (Hamalainen *et al.*, 1991) used to amplify the VLO 2 cDNA.

Primer V4 contained a *Hin*dIII site to allow directional cloning. DNA of the expected size (approximately 800 bp) was generated following PCR (Fig. 4.11).

4.4.2 VLO 2 ligation into pGEM-T

Amplified DNA was excised from an agarose gel, solubilized and purified using a Geneclean II kit (protocol 4). This DNA was ligated into the linearised vector pGEM-T (Promega; Fig. 4.12), to produce pVLO2. pGEM-T is specifically designed for incorporation of PCR products, and is compatible with blue / white screening. The vector is constructed from pGEM-5Zf and modified (by the manufacturer) by adding a terminal thymidine overhang to the two ends of an open *Eco*R V restriction site. By engineering the vector to contain these overhangs, the terminal transferase properties of many Taq DNA polymerases is exploited, where a non-specified adenosine residue is added by the Taq enzyme in the 3' position to each end of the DNA produced. This results in the ability to clone DNA directly into pGEM-T without the requirement for restriction digests. Another advantage of using pGEM-T is that this vector cannot re-ligate, leading to more efficient ligation between vector and PCR product. Cloning PCR products into pGEM-T is non-directional, though because restriction enzyme sites were incorporated at each end of VLO 2, the DNA could be excised and directionally cloned into an expression vector.

High efficiency, competent JM109 cells (Promega; Table 4.1) were then transformed with pVLO 2. The transformed cells were plated onto LB-agar plates supplemented with ampicillin, X-Gal and IPTG, and these were incubated overnight at 37°C. As before, the colonies produced were

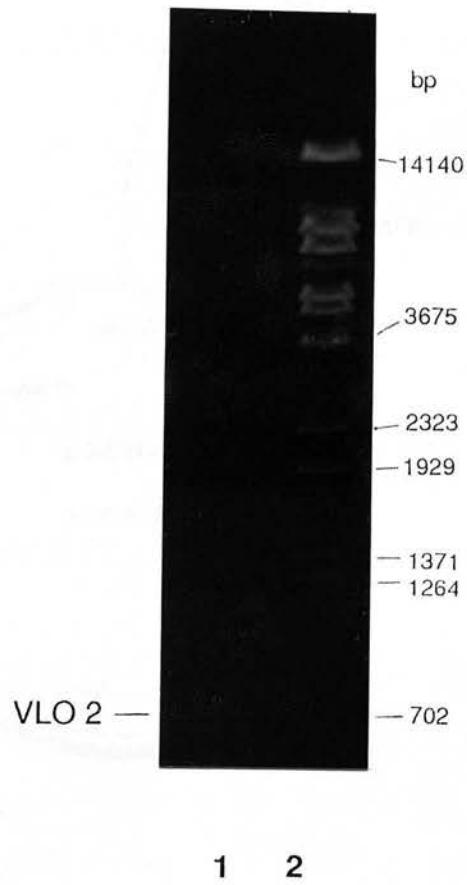


Figure 4.11 : VLO 2. 'Hot start' PCR was used to ampify the DNA from human placental quickclone cDNA (Clontech) using primers V3 and V4 (Fig 4.10). Lane 1, DNA produced from a PCR reaction using primers V3 and V4; lane 2, molecular weight markers (λ DNA, digested with *Bst* EII restriction endonuclease).

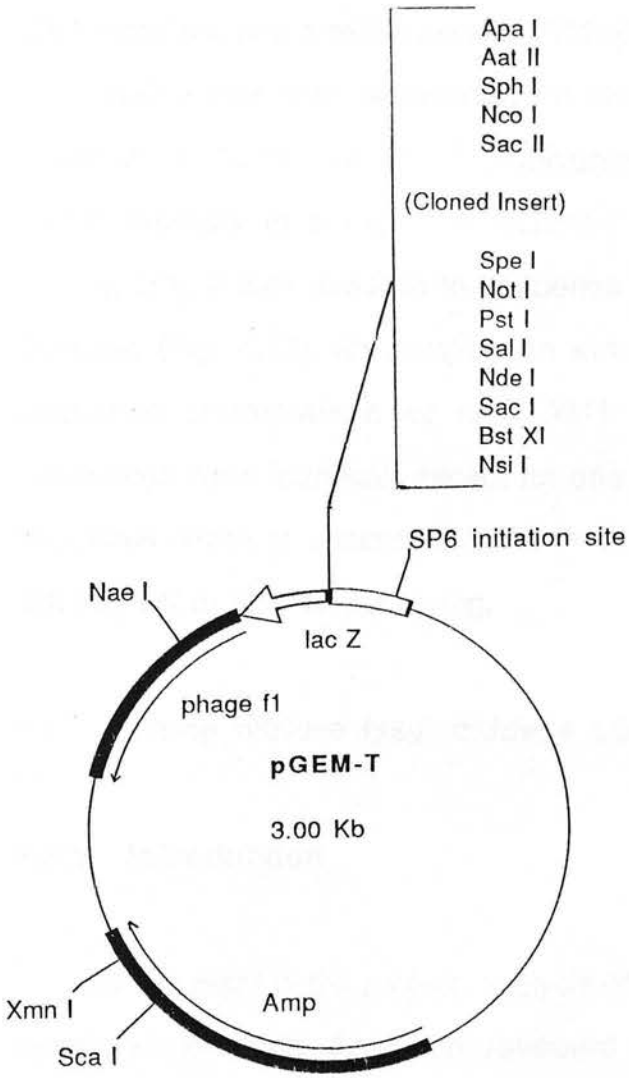


Figure 4.12. Plasmid pGEM-T.

screened for recombinant DNA by restriction analysis of plasmid minipreps. Sixteen colonies were produced and one colony contained a DNA fragment of the expected size (800bp).

VLO 2 was then sequenced (section 2.13.8 ; protocol 9) to further establish its identity. Using 17bp universal pUC / M13 sequencing primer, complementary to a region of pGEM-T starting 84bp upstream of the cloning site, it was possible to sequence 165 bp of VLO 2 in the forward direction (Fig. 4.13), for comparison with the human lysyl oxidase cDNA sequence (Hamalainen *et al.*, 1991). In the forward direction, the sequences were identical, except for one G at position 610 of the human sequence which is unaccounted for in VLO 2, probably due to poor gel / film contact or uneven gel drying.

4.5 Cloning mature lysyl oxidase cDNA (ALO 1) from VLO 2

4.5.1 Introduction

At this point in the project, analysis of mature porcine lysyl oxidase by cyanogen bromide digestion revealed that Asp 169 in the human sequence was likely to be the N-terminal amino acid of mature lysyl oxidase (Cronshaw *et al.,* submitted for publication). Hence new primers were designed to amplify lysyl oxidase cDNA begining at Asp 169.

Hamalainen	5'	499	GTG	GGC	GAC	GAC	CCT	TAC	AAC	CCC	TAC
VLO 2	5'	1	GTG	GGC	GAC	GAC	CCT	TAC	AAC	CCC	TAC
			V	G	D	D	P	Y	N	P	Y
Hamalainen		526	AAG	TAC	TCT	GAC	GAC	AAC	CCT	TAT	TAC
VLO 2		28	AAG	TAC	TCT	GAC	GAC	AAC	CCT	TAT	TAC
			K	Y	S	D	D	N	P	Y	Y
Hamalainen		553	AAC	TAC	TAC	GAT	ACT	TAT	GAA	AGG	CCC
VLO 2		55	AAC	TAC	TAC	GAT	ACT	TAT	GAA	AGG	CCC
			N	Y	Y	D	T	Y	E	R	P
Hamalainen		580	AGA	CCT	GGG	GGC	AGG	TAC	CGG	CCC	GGA
VLO 2		82	AGA	CCT	GGG	GGC	AGG	TAC	CGG	CCC	GGA
			R	P	G	G	R	Y	R	P	G
Hamalainen		607	TAC	GGC	ACT	GGC	TAC	TTC	CAG	TAC	GGT
VLO 2		109	TAC	G_C	ACT	GGC	TAC	TTC	CAG	TAC	GGT
			Y	G	T	G	Y	F	Q	Y	G
Hamalainen		634	CTC	CCA	GAC	CTG	GTG	GCC	GAC	CCC	TAC
VLO 2		136	CTC	CCA	GAC	CTG	GTG	GCC	GAC	CCC	TAC
			L	P	D	L	V	A	D	P	Y
Hamalainen		661	TAC	3'							
VLO 2		163	TAC	3'							
			Y								

Figure 4.13. Comparison of part of the VLO 2 sequence to bases 499-661 of human lysyl oxidase (Hamalainen *et al.*,1991). The DNA was sequenced using a universal M13 / pUC18 forward primer. The partial sequence of VLO 2 from the 5' end is shown. The corresponding positions of the human cDNA bases (Hamalainen *et al.*, 1991; Fig 4.6) are also shown. _ Denotes undetermined base. (Sequencing performed by Mr J Mackrill, NIMR, London).

4.5.2 PCR

Primers were designed to amplify cDNA from Asp169 in the human sequence to the termination codon TAG at residue 418 (A1 and A2; Fig. 4.14) in a PCR using VLO 2 as template (section 2.13.3; protocol 10). This amplification product was named ALO 1. The 5' primer (A1) contained a *Hin*cII restriction site and the 3' primer (A2) contained a *Sph*I site to allow directional sub-cloning into the polycloning site of the pUC 18 vector (Fig. 4.15).

Vent DNA polymerase (New England Biolabs) was used for the PCR. Taq DNA polymerase incorporates an incorrect base approximately every 3500 bases (Tindall *et al.*, 1988), whereas Vent DNA polymerase incorporates around five-fold fewer incorrect bases (Mattila *et al.*, 1991). Vent DNA polymerase also has 3' - 5' exonuclease activity, is more heat stable than Taq polymerase, and does not display terminal transferase activity.

ALO 1 was amplified (Fig. 4.16), and blunt end cloned into the *Sma*I restriction site in the polycloning site of pUC18. The DNA produced during Vent polymerase PCR reactions are blunt ended, and it is possible to ligate these products (without restriction digest) directly into a blunt ended restriction site such as *Sma*I. Analysis with the restriction enzyme *Sca*I revealed that the construct produced two DNA fragments of approximately 1825 bp and 1615 bp (Fig. 4.17). This is consistent with a *Sca*I site 22 bp downstream of the 5' end of ALO1, which is present in the human lysyl oxidase sequence at position 527-532 (Hamalainen *et al.,* 1991; Fig. 4.6). Further restriction digests revealed that the 5' terminal *Hin*cII site was not

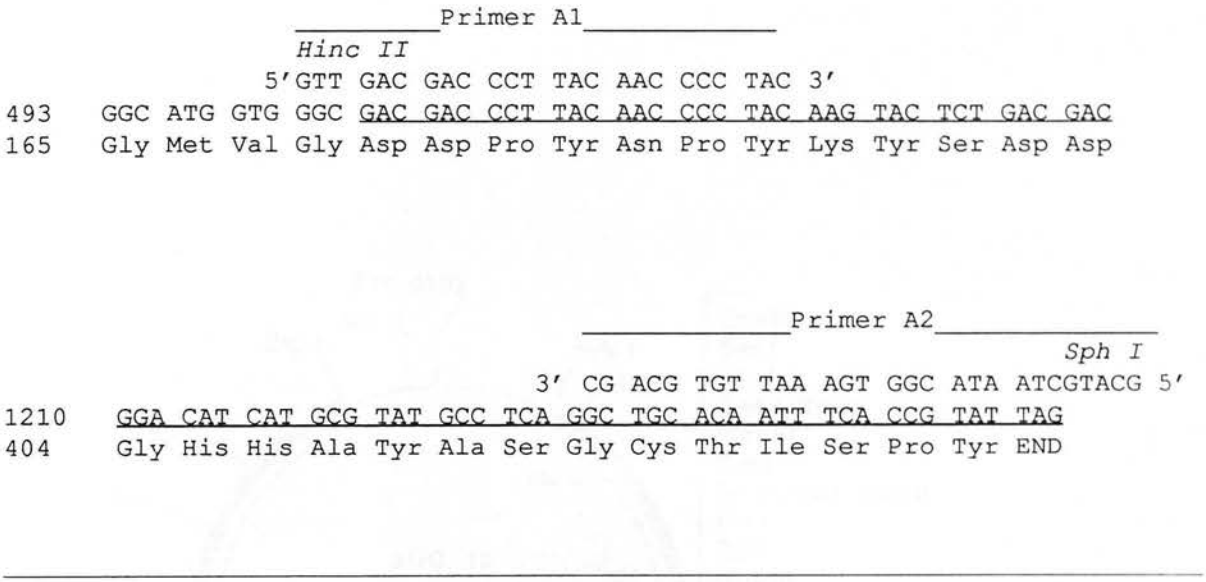


Figure 4.14. The position of the primers A1 and A2 on the human placental lysyl oxidase precursor sequence (Hamalainen *et al.*, 1991) used to amplify the ALO 1 cDNA.

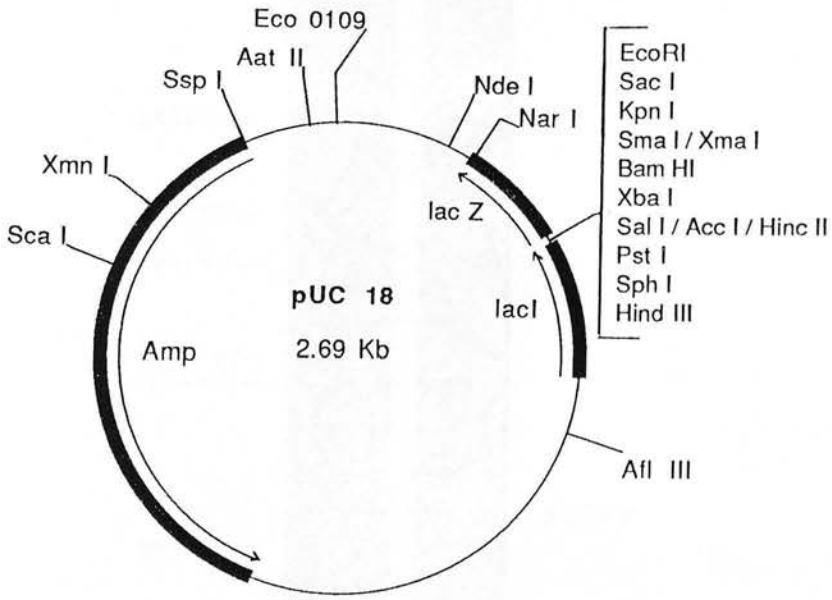


Figure 4.15. pUC 18

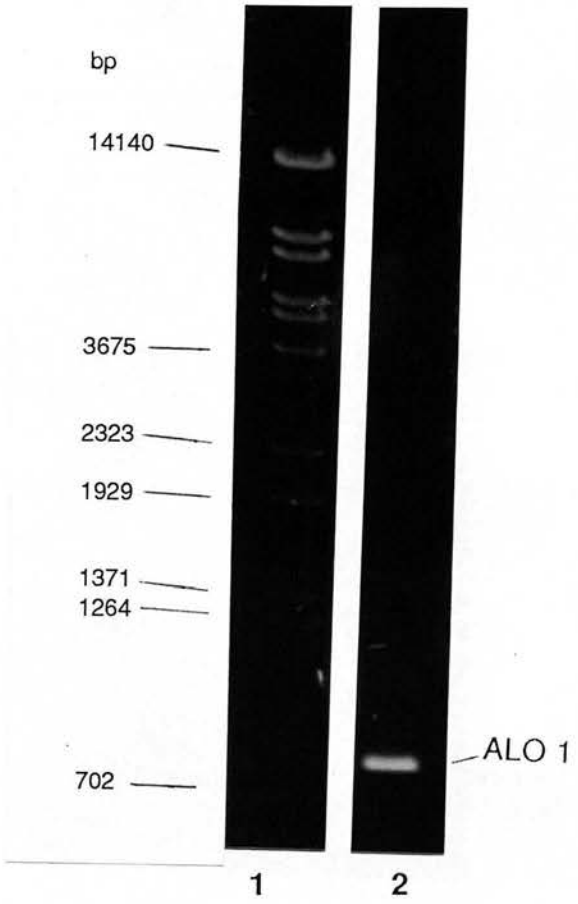


Figure 4.16. ALO 1. 'Hot start' PCR was used to amplify the DNA using VLO 2 as template and primers A1 and A2 (Fig. 4.14). Lane 1, molecular weight markers (λ DNA, digested with *Bst*EII restriction endonuclease); lane 2, DNA produced from a PCR reaction using primers A1 and A2.

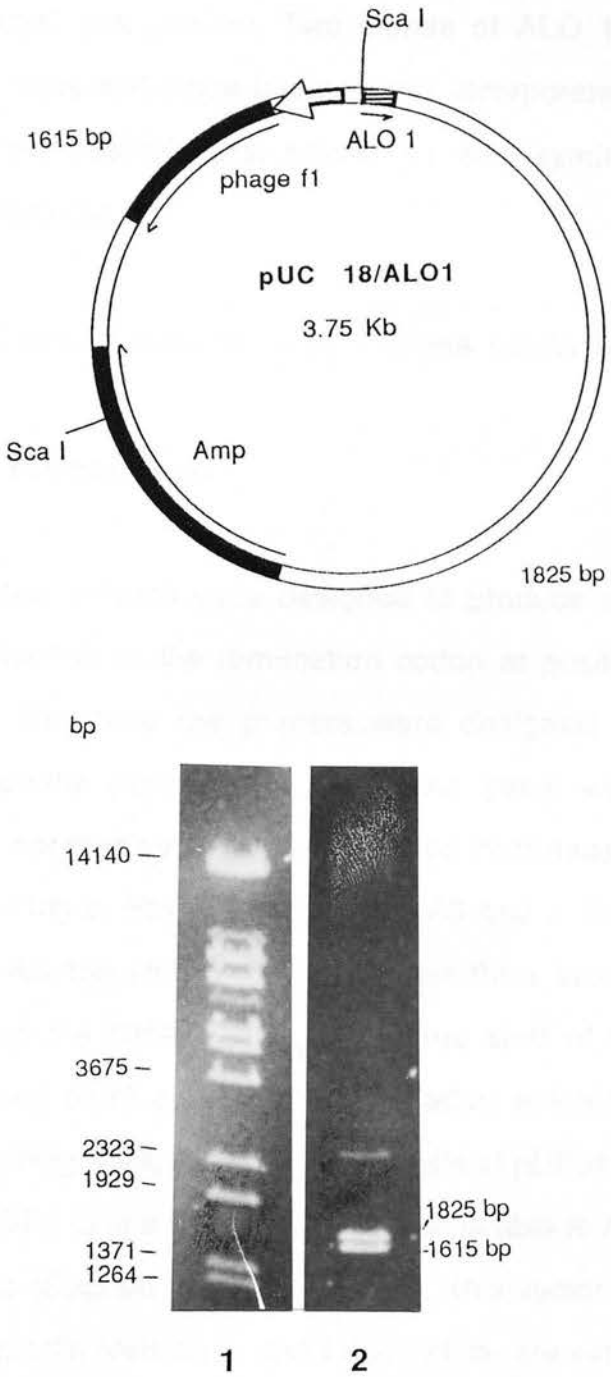


Figure 4.17. Restriction digest of the ALO 1 / pUC 18 construct with *Sca*I. (Above) Map of the ALO 1 / pUC 18 construct. (Below) Lane 1, molecular weight markers (λ DNA digested with *Bst*EII restriction endonuclease); lane 2, restriction digest of the construct with *Sca*I restriction endonuclease.

functional (not shown). Two clones of ALO 1 contained non-functional *Hin*cII sites and since this site was incorporated within the 5' primer, it is therefore possible that primer A1 was synthesised incorrectly by the manufacturer.

4.6 Cloning mature lysyl oxidase cDNA (ALO 2) from VLO 2

4.6.1 Introduction

New primers were designed to produce a partial VLO 2 sequence from Asp169 to the termination codon at position 418 (A3 and A4; Fig. 4.18). This time the primers were designed to allow the DNA to be directionally cloned directly into the yeast expression vector pDP315 (kindly donated by Dr D. Pioli, Zeneca Pharmaceuticals, Macclesfield ; Fig. 4.19) using a *Hin*cII site in primer A3 and a *Bam*HI site in primer A4. In primer A3, the *Hin*cII site incorporates three bases that code for aspartate 169 and the DNA is cut blunt at the start of this codon. Both primers contained blunt cutting restriction sites which allows ligation into other blunt cutting sites, such as the *Stu* I site of pDP315.

pDP315 is a shuttle vector which is able to replicate in both yeast and bacteria (Sikorski and Hiefer, 1989). This vector contains the gene coding for ampicillin resistance and *LEU2*, which are selectable markers for *E.coli* and yeast respectively. The *LEU2* gene encodes β-isopropylmalate dehydrogenase which allows yeast to synthesize leucine. Yeast *leu2* mutants require complementation with plasmids carrying the *LEU2* wild-type gene for growth in media lacking leucine.

Insertion of ALO 2 into pDP 315 at the polylinker site should result in

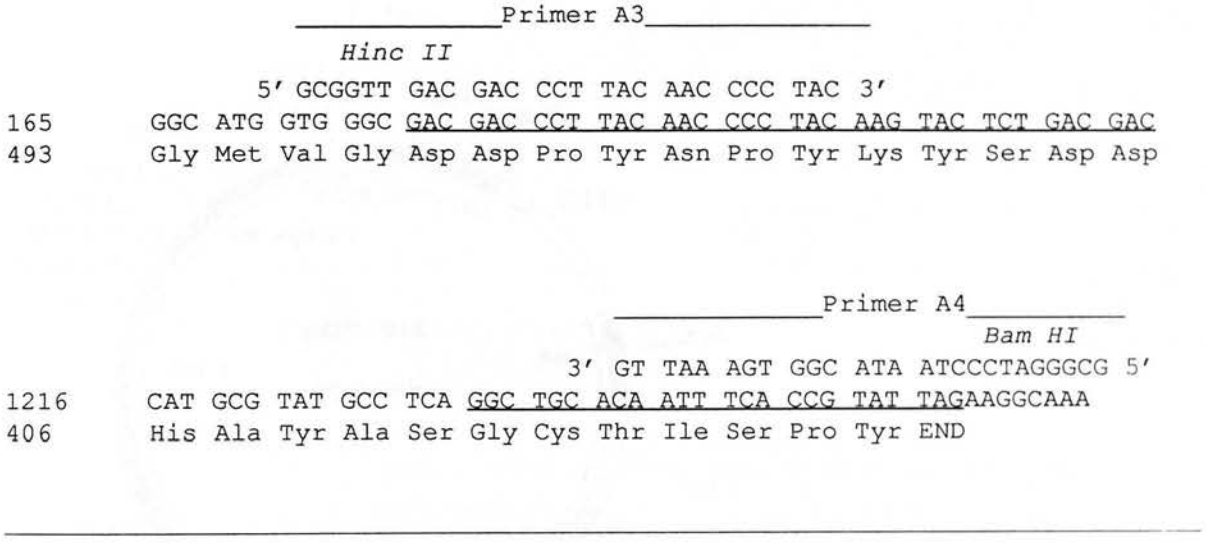


Figure 4.18. The position of the primers A3 and A4 on the human placental lysyl oxidase precursor sequence (Hamalainen *et al.*, 1991) used to amplify the ALO 2 cDNA.

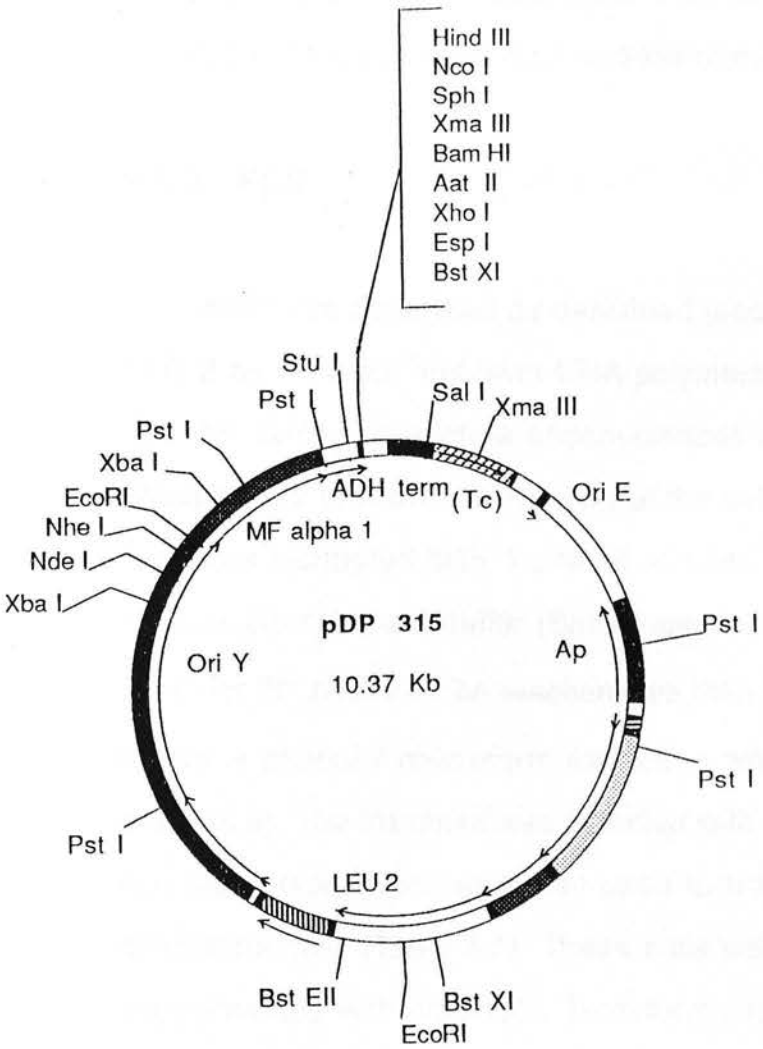


Figure 4.19. Plasmid pDP315.

a fusion between the gene coding for yeast pre pro-α-factor (80 amino acids) and ALO2, separated by DNA coding for the sequence Lys-Arg immediately upstream of ALO 2. When expressed, the Kex2 endopeptidase cleaves after this sequence (Julius *et al.,* 1984) in a late Golgi compartment of yeast cells (Graham and Emr, 1991) leading to secretion of the complete lysyl oxidase protein.

4.6.2 PCR

PCR was performed as described (section 2.13.3; protocol 10), using VLO 2 as template and Vent DNA polymerase. PDP315 was cut with *Stu*I and the *Bam*HI restriction endonucleases and then treated with alkaline phosphatase to prevent re-ligation of the cut ends. pDP315 (approximately 1μg) was incubated with 1 unit of alkaline phosphatase and 1μl of 10 x alkaline phosphatase buffer (Boehringer Mannheim) in a volume of 10μl at 37°C for 30 minutes. The reaction was then heated to 65°C for 10 minutes before a phenol / chloroform extraction was carried out (section 2.13.5; protocol 5). The fragment was digested with *Hin*cII and *Bam*HI, and ligated into the vector, which was then used to transform JM109 high efficiency competent cells (Table 4.1). These cells were plated onto LB-agar plates supplemented with ampicillin. Transformants were analysed by screening for the presence of plasmids which generated the pattern of fragment expected following digestion with *Eco*RI nad *Xho*I. Of approximately 100 colonies checked, 8 were found to contain the insert and were subjected to a plasmid midiprep. Reaction with *Sca*I and *Kpn*I (positions shown in Fig. 4.6) indicated that two of these colonies (C1 and C2) contained the mature

lysyl oxidase clone ALO 2 (Fig. 4.20). The construct from C1 was then sequenced (Fig. 4.21), and was found to contain a sequence that corresponded to human lysyl oxidase (Hamalainen *et al.*, 1991) from the codon for Asp 169 to the termination codon 418, except for the absence of one A at position 1207, which may have been caused by Vent DNA polymerase amplification of ALO2 or Taq DNA amplification of VLO2 .

4.6.3 Transformation of *S.cerevisiae* with the ALO 2 construct

The construct from C1 was used to transform the *S.cerevisiae* strain JRY 188 (kindly donated by Dr. A Boyd; Table 4.1). The method for transformation was adapted from Gietz *et al.* (1992) and is described in section 2.18.1. The transformed yeast were grown for 5 days on SD-HUW plates (0.65% (w/v) yeast nitrogen base, 2% (w/v) glucose, 2% (w/v) agar, 20 mg / ml histidine, 20 mg / ml uracil, 20 mg / ml tryptophan), which do not contain leucine to select for transformed yeast cells.

Approximately 80 colonies grew on the the plates which were then used to innoculate 400 ml of SD-HUW media. The cells were each grown to an optical density of 2.0 units at 600nm, and then centrifuged at 3000 g for 5 minutes. The volumes of the supernatants were measured and ammonium sulphate was added to 80% saturation, followed by mixing overnight at 4°C. The suspension was then centrifuged at 12000g for 10 minutes and the pellet was resuspended in 2% SDS, 0.5 M NaCl, 0.125 M Tris-HCl, pH 6.8. Approximately 40 μg of protein from each culture was run on a 12% discontinuous SDS-PAGE gel in reducing conditions. Only one very high

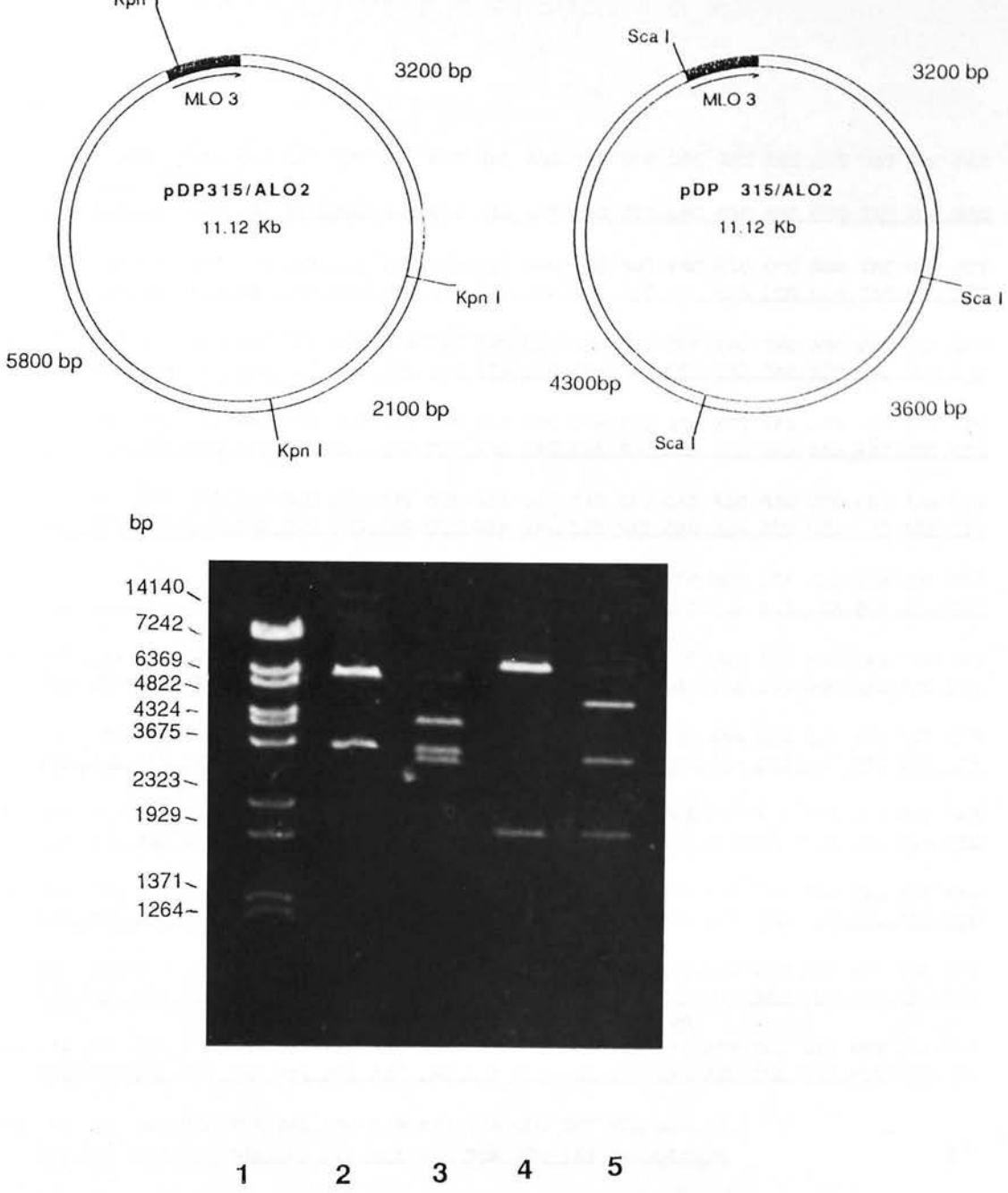


Figure 4.20: Restriction digests of the pDP 315 / ALO 2 construct. ALO 2 was amplified using VLO 2 as template, sub-cloned into the shuttle vector pDP 315 and digested with *Sca*I and *Kpn*I. (Above) Map of pDP 315 / ALO 2 construct, showing *Sca*I and *Kpn*I sites. (Below) Lane 1, molecular weight markers (λ DNA digested with *Bst*EII restriction endonuclease); lanes 2+4, pDP 315 digested with *Sca*I and *Kpn*I, respectively; lanes 3+5, pDP 315 / ALO 2 digested with *Sca*I and *Kpn*I, respectively.

5′

505 __pDp 315___GAC GAC CCT TAC AAC CCC TAC AAG TAC TCT GAC GAC AAC CCT TAT TAC AAC
__*Stu* I_
GAT AAA AGG GAC GAC CCT TAC AAC CCC TAC AAG TAC TCT GAC GAC AAC CCT TAT TAC AAC

565 TAC TAC GAT ACT TAT GAA AGG CCC AGA CCT GGG GGC AGG TAC CGG CCC GGA TAC GGC ACT
TAC TAC GAT ACT TAT GAA AGG CCC AGA CCT GGG GGC AGG TAC CGG CCC GGA TAC GGC ACT

625 GGC TAC TTC CAG TAC GGT CTC CCA GAC CTG GTG GCC GAC CCC TAC TAC ATC CAG GCG TCC
GGC TAC TTC CAG TAC GGT CTC CCA GAC CTG GTG GCC GAC CCC TAC TAC ATC CAG GCG TCC

685 ACG TAC GTG CAG AAG ATG TCC ATG TAC AAC CTG AGA TGC GCG GCG GAG GAA AAC TGT CTG
ACG TAC GTG CAG AAG ATG TCC ATG TAC AAC CTG AGA TGC GCG GCG GAG GAA AAC TGT CTG

745 GCC AGT ACA GCA TAC AGG GCA GAT GTC AGA GAT TAT GAT CAC AGG GTG CTG CTC AGA TTT
GCC AGT ACA GCA TAC AGG GCA GAT GTC AGA GAT TAT GAT CAC AGG GTG CTG CTC AGA TTT

805 CCC CAA AGA GTG AAA AAC CAA GGG ACA TCA GAT TTC TTA CCC AGC CGA CCA AGA TAT TCC
CCC CAA AGA GTG AAA AAC CAA GGG ACA TCA GAT TTC TTA CCC AGC CGA CCA AGA TAT TCC

865 TGG GAA TGG CAC AGT TGT CAT CAA CAT TAC CAC AGT ATG GAT GAG TTT AGC CAC TTG TAC
TGG GAA TGG CAC AGT TGT CAT CAA CAT TAC CAC AGT ATG GAT GAG TTT AGC CAC TTG TAC

925 CTG CTT GAT GCC AAC ACC CAG AGG AGA TGG GCT GAA GGC CAC AAA GCA AGT TTC TGT CTT
CTG CTT GAT GCC AAC ACC CAG AGG AGA TGG GCT GAA GGC CAC AAA GCA AGT TTC TGT CTT

985 GAA GAC ACA TCC TGT GAC TAT GGC TAC CAC AGG CGA TTT GCA TGT ACT GCA CAC ACA CAG
GAA GAC ACA TCC TGT GAC TAT GGC TAC CAC *GG CGA TTT GCA TGT ACT GCA CAC ACA CAG

1045 GGA TTG AGT CCT GGC TGT TAT GAT ACC TAT GGT GCA GAC ATA GAC TGC CAG TGG ATT GAT
GGA TTG AGT CCT GGC TGT TAT GAT ACC TAT GGT GCA GAC ATA GAC TGC CAG TGG ATT GAT

1105 ATT ACA GAT GTA AAA CCT GGA AAC TAT ATC CTA AAG GTC AGT GTA AAC CCC AGC TAC CTG
ATT ACA GAT GTA AAA CCT GGA AAC TAT ATC CTA AAG GTC AGT GTA AAC CCC AGC TAC CTG

1165 GTT CCT GAA TCT GAC TAT ACC AAC AAT GTT GTG CGC TGT GAC ATT CGC TAC ACA GGA CAT
GTT CCT GAA TCT GAC TAT ACC AAC AAT GTT GTG CGC TGT GAC ATT CGC TAC ACA GGA CAT

1225 CAT GCG TAT GCC TCA GGC TGC ACA ATT TCA CCG TAT TAG_*Bam* HI_
CAT GCG TAT GCC TCA GGC TGC ACA ATT TCA CCG TAT TAGGGATCCC 3′

Figure 4.21. Sequence of ALO 2 vs human placental lysyl oxidase cDNA (Hamalainen *et al.*, 1991). The sequence of Hamalainen *et al.*, 1991 is shown in comparison with ALO 2 (underlined).* Denotes missing base.

molecular weight band (90 kDa) was observed on the gels (not shown), and no lysyl oxidase activity was observed when 100µl (0.2 mg / ml) of protein was assayed with 100µl (1mg / ml) of [4,5-^{3}H] lysine labelled elastin in assay buffer (100mM sodium borate, 0.15 M NaCl, pH 8.0) in a total volume of 900µl (section 2.18.2; not shown).

4.7 Discussion

The position of the N-terminus of lysyl oxidase is likely to be Asp 169 (Cronshaw *et al.,* submitted for publication). Previously, Trackman *et al.* (1990) predicted the site of proteolytic cleavage to be Arg 134-Arg 135 in the rat aorta lysyl oxidase precursor. Indirect evidence also suggests that the N-terminus is downstream of this site. The sequence Arg-Arg is not conserved at the corresponding position in the human and chick sequences (Hamalainen *et al.*, 1991; Wu *et al.*, 1992), while the sequence Met-Gly-Asp-Asp-Pro-Tyr (166-172 human) is conserved in chick, human and rat lysyl oxidase. From cyanogen bromide and endoproteinase-Asp-N digests of porcine lysyl oxidase (Cronshaw *et al.,* submitted for publication), Asp-Asp-Pro-Tyr is also conserved at the N-terminus of the mature enzyme. There are two potential glycosylation sites in the rat precursor sequence, at positions 91-93 and 138-140. Glycosylation has been detected only in the precursor form and not the mature form (Trackman *et al.*,1992), which suggests that the enzyme is processed downstream of residue 140 in rat. In the chick lysyl oxidase sequence, the first 168 amino acids are poorly conserved with rat (37.5 %). After the sequence Met-Gly-Asp-Asp-Pro-Tyr, the extent of conservation becomes

very high (94 %), which suggests that the coding sequence for mature lysyl oxidase is contained within the well conserved region (Wu *et al.*, 1992). Finally, a restriction fragment length polymorphism (RFLP) that causes a G-A transition has been discovered within the first exon of the human lysyl oxidase gene (Csiszar *et al.*, 1993). This transition creates a novel *Pst* I restriction site which has been detected in 36% of the healthy patients screened during a study, and should result in a glutamine being encoded instead of arginine at position 158 (arginine at this position is conserved in the chick, rat and human sequence). If the processing site of lysyl oxidase was upstream of this amino acid substitution as suggested by Trackman *et al.* (1991), the effect (if any) of this substitution would be more likely to cause disruption to the enzyme function than if it was within the precursor region.

A yeast expression vector construct has been prepared that contains the cDNA sequence of human placental lysyl oxidase from the codon for Asp 169 to the termination codon at position 418 (Hamalainen *et al.*, 1991). The vector has been used to transform JRY 188 *S. cerevisiae*, and the medium precipitated with ammonium sulphate. No lysyl oxidase activity could be detected in the medium and only one high molecular weight protein band (90 kDa) was present when a fraction of concentrated medium was run on 12% SDS-PAGE in reducing conditions. This result is consistent with the base omission at position 1207, which would cause a frame shift, leading to incorrect translation of the last 16 codons in the sequence and incorrect termination. To overcome the problem of the missing base in the sequence, site-directed mutagenesis may be used to insert an adenine at position 1207.

Successful expression and secretion of lysyl oxidase may depend on

optimizing cell density, medium composition and temperature. Alternative yeast strains may also be tried to optimize secretion. The extraction procedure for the secreted enzyme from the medium may require manipulation since the SD-HUW medium is highly viscous after concentration in ammonium sulphate and centrifugation, leading to difficulty in re-suspension of the pellet. A less viscous medium may be useful to ensure a high recovery of protein.

It is unknown whether yeast cells are capable of producing active lysyl oxidase. Lysyl oxidase contains an unidentified carbonyl containing co-factor (topaquinone or PQQ; Kagan *et al.,* 1986). The yeast amine oxidase gene from *Hansenula polymorpha* contains topaquinone (Mu *et al.,* 1992), however it is unknown whether *S.cerevisiae* is capable of modifying such proteins with topaquinone. The *E.coli* strain K-12 has been shown to produce an amine oxidase which contains topaquinone and copper (Cooper *et al.,* 1992). Since lysyl oxidase contains no detectable glycosylation, it may be possible to produce active lysyl oxidase from this K-12 strain of *E.coli.*

Chapter 5

Studies on an enhancing protein of lysyl oxidase

5.1 Introduction

Lysyl oxidase acts extracellularly to initiate cross-linking in collagens and elastin. To date, the regulation of lysyl oxidase activity has only been studied at the hormonal level (1.4.8). Other control mechanisms such as processing of the 50kDa precursor (Trackman *et al.*, 1992) are yet to be studied.

Stimulation of lysyl oxidase by cationic amphiphiles such as dodecylamine hydrochloride has been demonstrated by Kagan *et al.* (1981). Lysyl oxidase activity can be stimulated up to four times in the presence of these molecules. Lysyl oxidase is a highly acidic protein with an isoelectric point of pI = 5 (J.R.E MacBeath, personal communication), whereas elastin is largely hydrophobic. It has been suggested that cationic amphiphiles help provide favourable electrostatic interactions between lysyl oxidase and elastin (Kagan *et al.,* 1981).

Prior to the present study, partially purified urea extracts of porcine skin (with little or no endogenous lysyl oxidase activity) were found to stimulate lysyl oxidase up to four fold using an elastin substrate (Dr D.R. Shackleton, personal communication). These extracts were not found to stimulate activity when [4,5-^{3}H] lysine labelled collagen was used as substrate. Attempts were made to isolate the activating factor, which is referred to as 'Eclefin': Elastin Cross-Linking Enhancer Factor.

5.2 Purification of lysyl oxidase

Lysyl oxidase was prepared from stillborn piglet skin (section 2.1) using

the method of Shackleton and Hulmes (1990a). The skins were minced and homogenised in PBS (9 mM Na_2HPO_4, 1mM NaH_2PO_4, 0.15M NaCl, pH 7.8), and then washed in PB (9 mM Na_2HPO_4, 1mM NaH_2PO_4, pH 7.8) before extraction in 6M PBU (PB, 6 M urea, pH 7.8). (To reduce the accumulation of cyanate, all urea solutions were passed through a mixed bed deionizing resin before use, then stored at 0°C and used within 2-3 days of preparation.) The filtered extract was loaded onto a DEAE - Sephacel fast flow ion exchange column (2.6 x 40 cm), pre-equilibrated with 6M PBU, pH 7.8. The lysyl oxidase fraction was eluted in 6M PBU, 0.5 M NaCl and dialysed exhaustively against PB, pH 7.8. The protein was then loaded onto a Sephacryl S-400 column, pre-equilibrated with PB pH 7.8. In the absence of urea, lysyl oxidase binds selectively to Sephacryl S-400 and can be eluted with PBU, pH 7.8, providing an almost 140 fold purification (Shackleton and Hulmes, 1990a). Fractions from the S-400 column were analysed by SDS-PAGE (Fig. 3.1), and showed two major protein bands, lysyl oxidase (32-33 kDa) and TRAMP (Tyrosine Rich Acidic Matrix Protein; 24 kDa). The amount of TRAMP was approximately 2-4 fold greater than lysyl oxidase.

To improve the separation of lysyl oxidase and TRAMP, the procedure was modified as described by Cronshaw *et al.* (1993). In brief, the initial 6M PBU extract was diluted with PB, pH 7.8 to a final urea concentration of 2 M. The diluted extract was then loaded onto a DEAE-Sepharose column, pre-equilibrated with 2M PBU, pH 7.8 (2 M urea, PB, pH 7.8), and the column was washed in 2M PBU followed by PB. The column was eluted with PB, 0.3 M NaCl, pH 7.8 to remove the bulk of the protein, followed by 3M PBU, 0.3 M NaCl, pH 7.8, which removes lysyl oxidase and a small

quantity of TRAMP. Finally, TRAMP was eluted from the column with 6M PBU, 0.5 M NaCl, pH 7.8. The lysyl oxidase fraction was then loaded onto a Sephadex G-25 gel filtration column (5.6cm x 100cm), pre-equilibrated with PB, pH 7.8 to remove urea and salt. Finally, this desalted lysyl oxidase fraction was loaded as before onto an Sephacryl S-400 column (2.6cm x 40 cm), pre-equilibrated with PB pH 7.8, then washed with PB and finally eluted in 6M PBU, pH 7.8.

5.3 Assay of lysyl oxidase activity

Lysyl oxidase activity was assayed using the trituim release assay. Tritium was collected by ultrafiltration (protocol 1a) or microdistillation (protocol 1b) using 100μl of [4,5-^{3}H] lysine labelled elastin (3000 dpm / μl), as substrate (Shackleton and Hulmes 1990b; section 2.4). Assays were regularly perfomed in duplicate, together with control assays in the presence of 0.2 mM β-aminopropionitrile, a specific and irreversible inhibitor of lysyl oxidase activity (Siegel *et al.,* 1970; Narayanan *et al.,* 1972).

5.4 Definition of eclefin activity

Control samples containing 100μl of [4,5-^{3}H] lysine labelled elastin, 100μl of 6M PBU and 700μl of assay buffer were assayed to calculate background counts, and this was subtracted from all other assay results. The enhancement of lysyl oxidase activity produced by eclefin is defined as follows. Counts from assays containing lysyl oxidase in the absence of

eclefin, and from assays containing eclefin only (to correct for any endogenous lysyl oxidase activity) are subtracted from counts of assays containing lysyl oxidase in the presence of eclefin, to give counts attributable to enhancement only. To calculate the enhancement factor, enhancement counts were divided by counts from the assay of lysyl oxidase only.

i.e. $$F = \frac{(A - B) - (C - B) - (D - B)}{(C - B)}$$

A= Lysyl oxidase + Eclefin

B= Background

C= Lysyl oxidase

D= Eclefin

5.5 Eclefin purification

Eclefin was prepared from 6M PBU extracts of porcine skin by cation-exchange chromatography using a CM-Sepharose column (2.6 x 40 cm) pre-equilibrated with 6M PBU, and then eluted with 6M PBU, 1 M NaCl, pH 7.8. No eclefin activity was observed in fractions that bind to DEAE-Sepharose at pH 7.8. After exhaustive dialysis of the pooled, protein containing fractions against PB, pH 7.8, the partially purified eclefin was loaded onto a Sephacryl S-400 column (2.6 x 40 cm) pre-equilibrated with PB, pH 7.8, and the eclefin containing fractions were eluted in 6M PBU, pH 7.8 (Fig. 5.1).

Analysis of the eclefin containing fractions on 12% SDS-PAGE under reducing conditions, followed by staining with Coomassie blue (Fig. 5.2), showed bands of approximately 14 kDa, 15 kDa, 22kDa, 32-33 kDa, 47

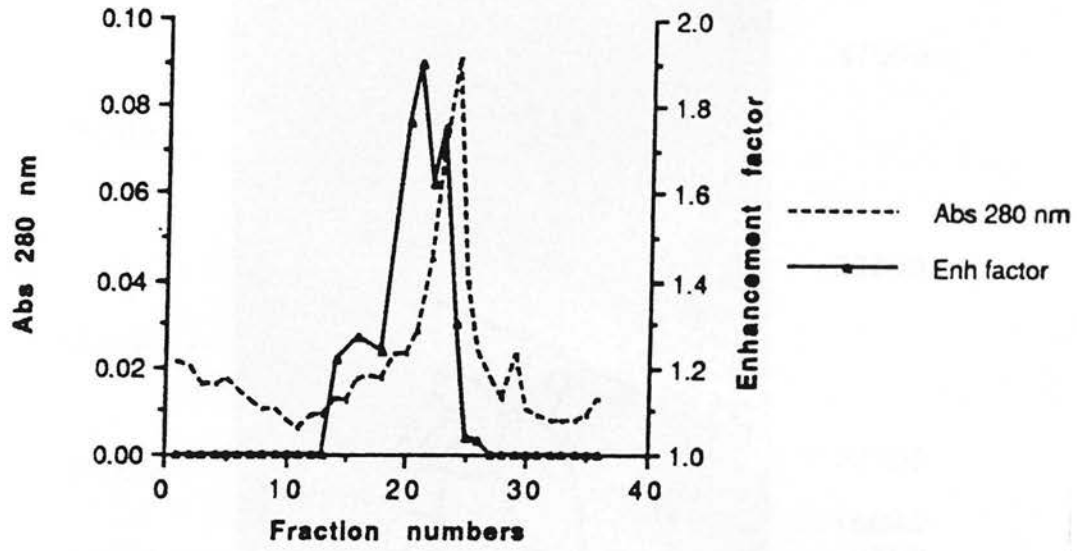


Figure 5.1. Enhancing activity of eclefin fractions from Sephacryl S-400. Urea extracts of porcine skin were were loaded on to a CM-Sepharose ion exchange column and protein fractions were eluted with 6M PBU / 1 M NaCl. The fractions were dialysed against PB, pH 7.8 and then loaded on to Sephacryl S-400 in the absence of urea. Eclefin was eluted in 6M PBU, and the fractions were measured for protein content at 280nm and assayed for enhancement of lysyl oxidase activity using [4,5-^{3}H] lysine labelled elastin as substrate, with the ultrafiltration assay.

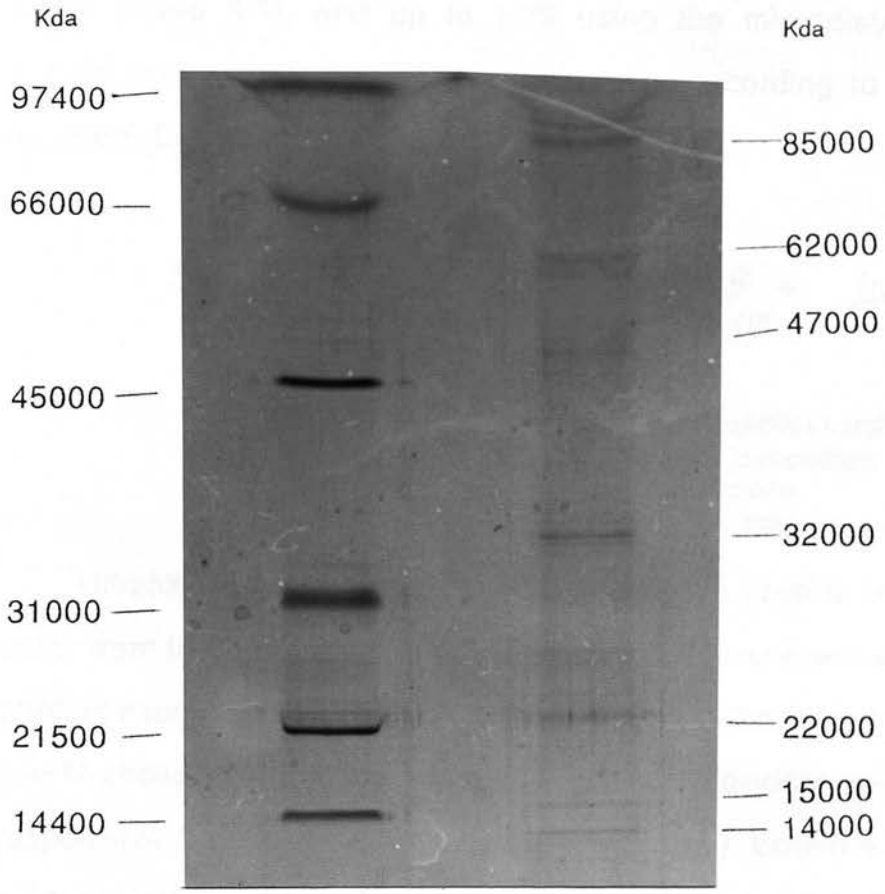


Figure 5.2. Discontinuous SDS-PAGE of lysyl oxidase enhancing extracts eluting from Sephacryl S-400. The fraction showing the greatest enhancement factor from Fig. 5.1 was analysed on SDS-PAGE with a 12% separating gel, followed by Coomassie blue staining (right). Molecular mass was determined by a plot of migration distance vs log kDa, using protein standards as shown (left).

kDa, 62 kDa and 85 kDa.

Inclusion of 1μg of the eclefin fraction in the tritium release assay with 50μl (2μg) of lysyl oxidase and 100μl of [4,5-^{3}H] lysine labelled elastin produced an enhancement factor of up to 2.00 using the ultrafiltration assay (Table 5.1), and up to 1.79 using the microdistillation assay. Enhancement factor errors were calculated according to the following equation (D.J.S.Hulmes, personal communication) :

$$\delta Y^2 = \frac{\delta a^2}{(b-d)^2} + \frac{(c-a)^2\,\delta b^2}{(b-d)^4} + \frac{\delta c^2}{(b-d)^2} + \frac{(c-a)^2\,\delta d^2}{(b-d)^4}$$

a = Eclefin + Lysyl oxidase
b = Lysyl oxidase
c = Eclefin
d = Control

Ultrafiltration and microdistillation were both used to isolate tritiated water from the reaction to determine whether enhancement was due to an artefact caused by the water isolation technique. The presence of 0.2 mM BAPN abolished all activity, indicating that lysyl oxidase was specifically responsible for all the activity observed (Table 5.2). Eclefin enhanced lysyl oxidase activity after exhaustive dialysis of both against PB, pH 7.8 to remove urea (not shown)

5.6 Fast protein liquid chromatography

Attempts were made to further purify and identify the molecular mass of eclefin using fast protein liquid chromatography (FPLC). An enhancing fraction (1 ml) containing 20μg of protein obtained from Sephacryl S-400 was dialysed exhaustively against PBS, pH 7.8 and concentrated 10x

Table 5.1. Effect of eclefin on lysyl oxidase activity using [4,5-^{3}H] lysine labelled elastin as substrate.

Addition to assay	cpm ± S.E. (n=3)	activity % of control ± S.E. (n=3)	Enhancement factor
Ultrafiltration			
Control	2256 ± 152	100 ± 6.7	----
Lysyl oxidase	3711 ± 187	164 ± 5.0	1
Eclefin	2223 ± 39	100 ± 1.7	----
Eclefin + Lysyl oxidase	5170 ± 216	229 ± 4.2	2.00 ± 0.4
Microdistillation			
Control	2537 ± 26	100 ± 1.0	----
Lysyl oxidase	3706 ± 159	146 ± 4.3	1
Eclefin	2631 ± 56	104 ± 2.1	----
Eclefin + Lysyl oxidase	4797 ± 135	189 ± 2.8	1.79 ± 0.28

Lysyl oxidase (2μg), eclefin (1μg; section 5.5) and 100μl [4,5-^{3}H] lysine labelled elastin were incubated in assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0) in a total volume of 900μl overnight at 37°C. Control samples contained substrate alone with no lysyl oxidase or eclefin.

Table 5.2. Effect of 2 mM β-aminopropionitrile on the enhancement of lysyl oxidase activity by eclefin.

Addition to assay	dpm ± S.E.	activity % of control ± S.E.	Enhancement factor
Experiment 1	(n=3)	(n=3)	
Control	2279 ± 47	100 ± 2.1	----
Lysyl oxidase	4099 ± 163	180 ± 4.0	1.00
Eclefin	2387 ± 112	105 ± 4.7	----
Eclefin + Lysyl oxidase	4602 ± 120	202 ± 2.6	1.28 ± 0.14
Eclefin + Lysyl oxidase (BAPN)	1950 ± 66	86 ± 3.4	<1.00
Experiment 2	(n=2)	(n=2)	
Control	334 ± 12.5	100 ± 3.7	----
Lysyl oxidase	773 ± 5	231 ± 0.6	1.00
Eclefin	432 ± 39	129 ± 9.0	----
Eclefin + Lysyl oxidase	1026 ± 24	307 ± 2.3	1.58 ± 0.11
Eclefin + Lysyl oxidase (BAPN)	426 ± 38	127 ± 8.9	----

Lysyl oxidase (2μg) was assayed with eclefin (1μg; section 5.5) and 100μl [4,5-^{3}H] lysine labelled elastin overnight at 37°C in assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0) using the ultrafiltration method.

using an Amicon centricon filter unit with a molecular mass cut off point of 10 kDa. A 100µl sample of the concentrate was then loaded onto a Pharmacia Superose 6 gel filtration column (1.6cm x 30 cm), pre-equilibrated in PBS, pH 7.8 , and eluted at 500µl / minute. Superose 6 gives optimal separation of proteins in the range 5 - 5000 kDa. Fractions from the column were then assayed for lysyl oxidase enhancing activity using the ultrafiltration assay (section 2.4). Two fractions (8 and 19), eluting separately from the Superose 6 column, showed enhancing activity (Fig. 5.3). When analysed by SDS-PAGE, followed by silver staining (section 2.10.3; Fig. 5.4), a band at 14kDa was observed in fractions 17,18 and 19; and a 15kDa band was also observed in fraction 19.

5.7 Peroxidase release-coupled fluorometric assay

The activity of lysyl oxidase can be measured continuously by the hydrogen peroxide produced using artificial substrates such as 1,5-diaminopentane (Trackman *et al.*, 1981; section 2.5). The reaction is carried out at an optimal temperature of 55°C in the presence of horse radish peroxidase and a chromophore such as homovanillate. The peroxidase catalysed oxidation of the chromophore by H_2O_2 produces a fluorescent molecule that can be measured using excitation and emisson wavelengths of 315 nm and 425 nm respectively.

The reaction was calibrated by adding known amounts of hydrogen peroxide to the reactants in the absence of lysyl oxidase, to give a standard curve (Fig. 5.5). The use of nonpeptidyl diamine substrates causes lysyl oxidase to become irreversibly inactivated after 300 catalytic turnovers

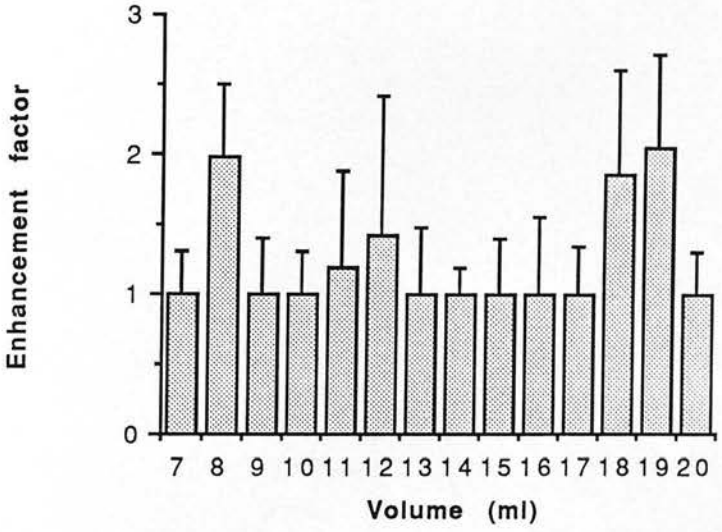


Figure 5.3. Gel permeation of concentrated eclefin fractions in the absence of urea on FPLC. An eclefin fraction (20μg ; section 5.5) was dialysed against PBS and concentrated 10 x using an Amicon Centricon filter unit (10kDa) and loaded on to a Superose 6 gel permeation column pre-equilibrated in PBS, pH 7.8. Fractions (1ml) were eluted at 500μl / minute, and 50μl of each fraction was assayed with lysyl oxidase (2μg) and [4,5-^{3}H] lysine labelled elastin (100μl) using the ultrafiltration method.

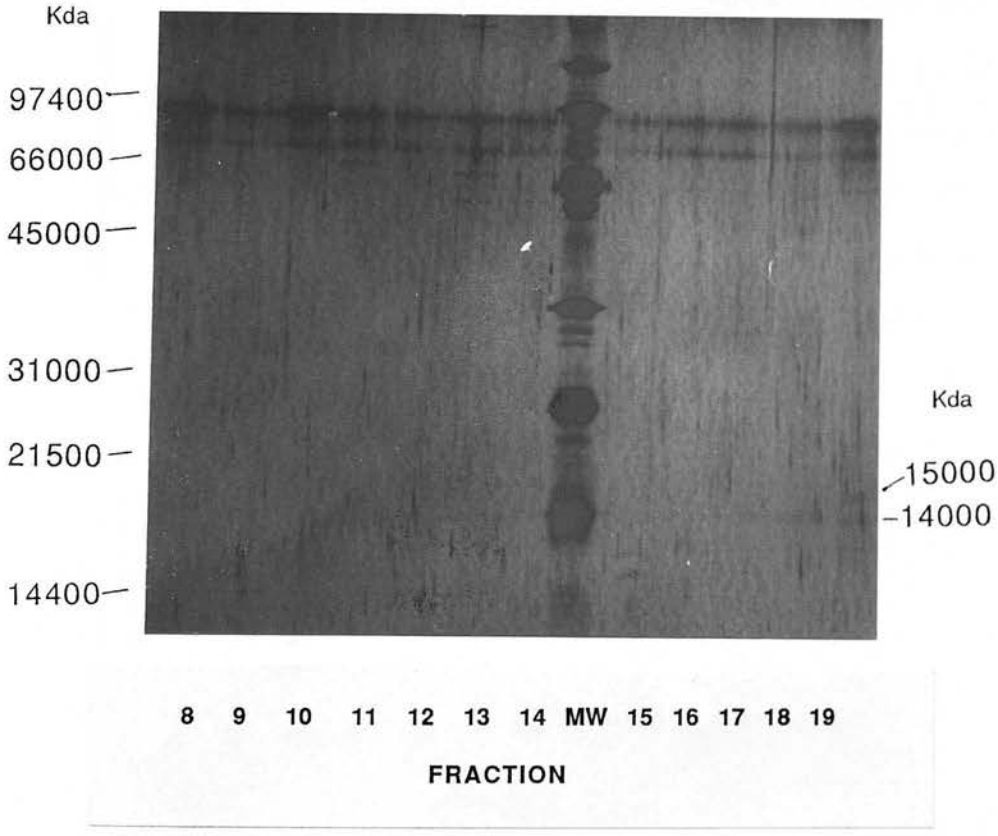


Figure 5.4. Discontinuous SDS-PAGE of concentrated eclefin fractions eluted from an FPLC gel permeation column in the absence of urea. An eclefin fraction (20μg ; section 5.5) was dialysed against PBS, concentrated 10x using an Amicon Centricon filter unit (10 kDa) and loaded on to a Superose 6 gel permeation column pre-equilibrated in PBS, pH 7.8. Fractions were eluted at 500μl/ minute, and 100μl of each fraction were analysed on SDS-PAGE with a 12% separating gel, followed by silver staining (section 2.10.3).

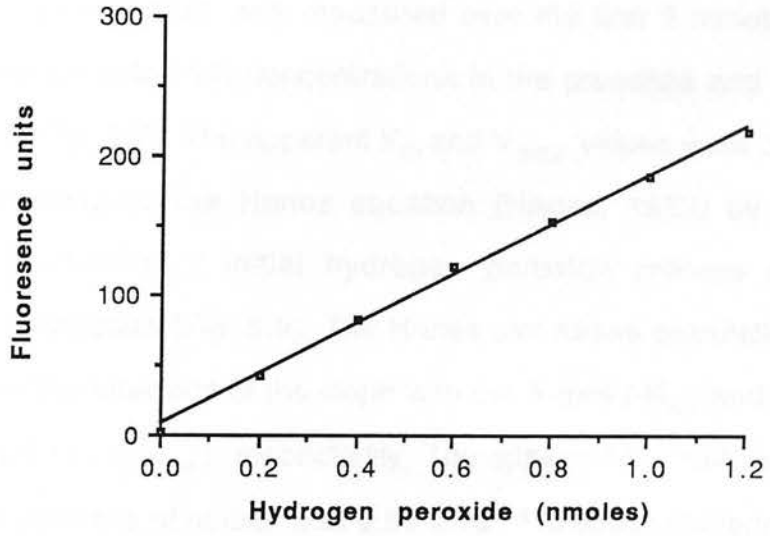


Figure 5.5. Standard curve of fluorescence versus hydrogen peroxide concentration in the peroxidase catalysed oxidation of homovanillate. Hydrogen peroxide was added in 200 pmole aliquots to 125μg homovanillate, and 20μg horse radish peroxidase in a total volume of 1 ml of assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0), and incubated at 55°C. Fluoresence was measured with an excitation wavelength of 315nm and an emission wavelength of 425nm.

(Trackman *et al.,* 1981). As a result, the rate of release of H_2O_2 fell to zero due to enzyme inactivation before substrate was exhausted. Subsequent addition of new lysyl oxidase restored hydrogen peroxide release to initial rates (Fig. 5.6).

Inclusion of eclefin (1μg) in the reaction caused a slight reduction in release of hydrogen peroxide over a 45 minute period (Fig. 5.7). Hydrogen peroxide release was measured over the first 3 minutes of the assay at different substrate concentrations in the presence and absence of eclefin (1μg; Fig. 5.8). The apparent K_m and V_{max} values were calculated for these according to the Hanes equation (Hanes, 1932) by plotting substrate concentration / initial hydrogen peroxide release against substrate concentration (Fig. 5.9). The Hanes plot allows calculation of K_m and V_{max} from the intercept of the slope with the X-axis ($-K_m$) and the gradient of the slope ($1 / V_{max}$), respectively. The apparent K_m with 2μg lysyl oxidase in the absence of eclefin was 9.93×10^{-4} M which compares with the results of Trackman *et al.* (1981) who observed an apparent K_m of 5×10^{-4} M using 4μg of purified lysyl oxidase. In the presence of eclefin (1μg) the apparent K_m was 7.27×10^{-4} M. The apparent V_{max} in the absence of eclefin was 69.4 pmoles min^{-1} and 65.4 pmoles min^{-1} in the presence of eclefin. These results indicate that eclefin probably has no enhancing activity on lysyl oxidase using 1,5-diaminopentane as substrate.

5.8 Stability of eclefin

Exposure to 80°C for 90 minutes slightly reduced the ability of eclefin

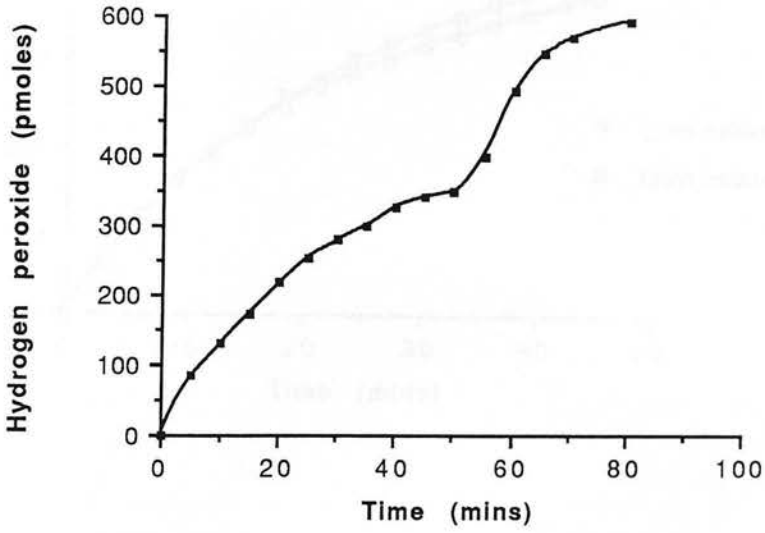


Figure 5.6. Lysyl oxidase inactivation using 1,5-diaminopentane as substrate. Lysyl oxidase (2μg) was added at t=0 and t=52 minutes to the reaction containing 1,5-diaminopentane (175μg), horse radish peroxidase (20μg), homovanillate (125μg) and assay buffer (0.1M sodium borate, 0.15 M NaCl, pH 8.0), total volume 1ml. The reaction was carried out at 55°C.

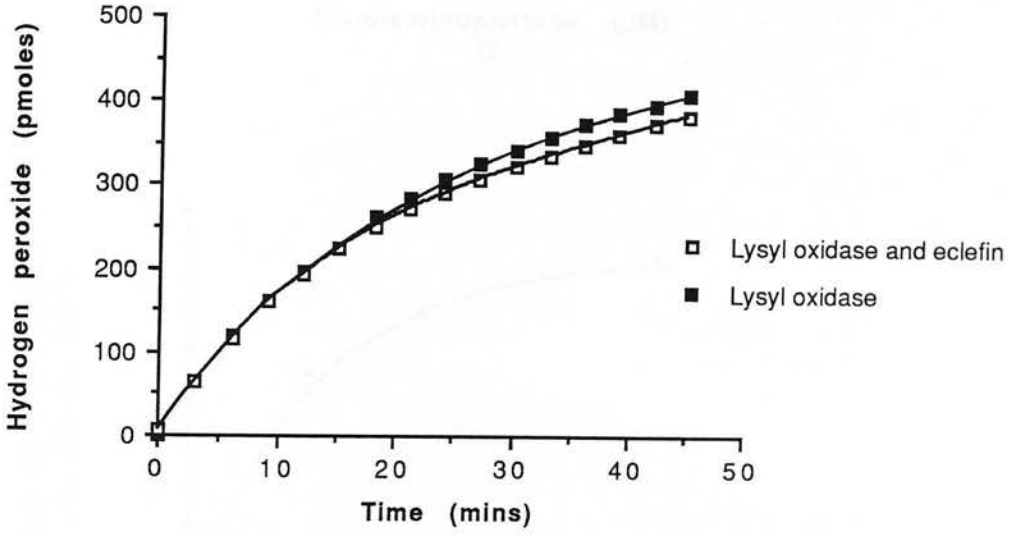
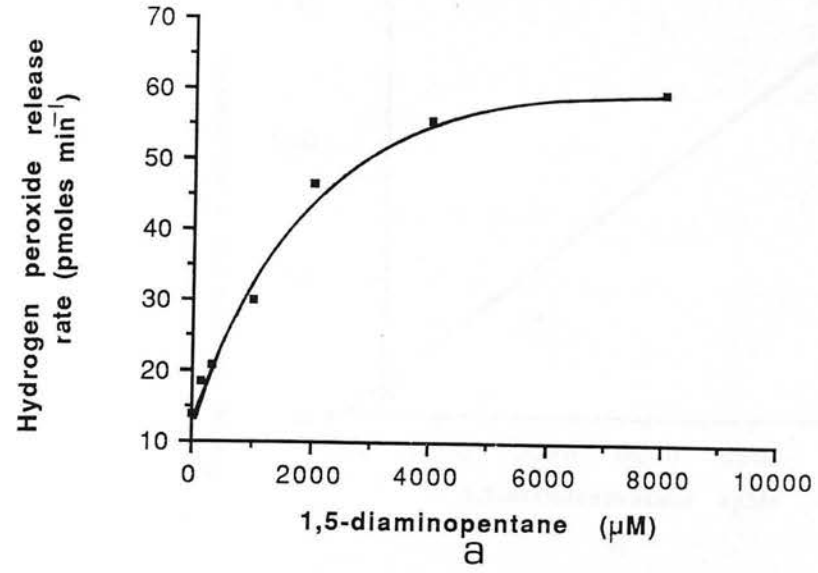


Figure 5.7. Hydrogen peroxide (pmoles) released from the fluoresence coupled lysyl oxidase assay in the presence and absence of eclefin. Lysyl oxidase (2μg) was assayed for activity in the presence and absence of eclefin (1μg; section 5.5). Hydrogen peroxide was measured by the fluorescence produced as a result of the reaction between hydrogen peroxide and homovanillate (125μg) in the presence of horse radish peroxidase (20μg), and 175 μg 1,5-diaminopentane as substrate at 55°C. Fluoresence was measured with an excitation wavelength of 315 nm and an emission wavelength of 425 nm.



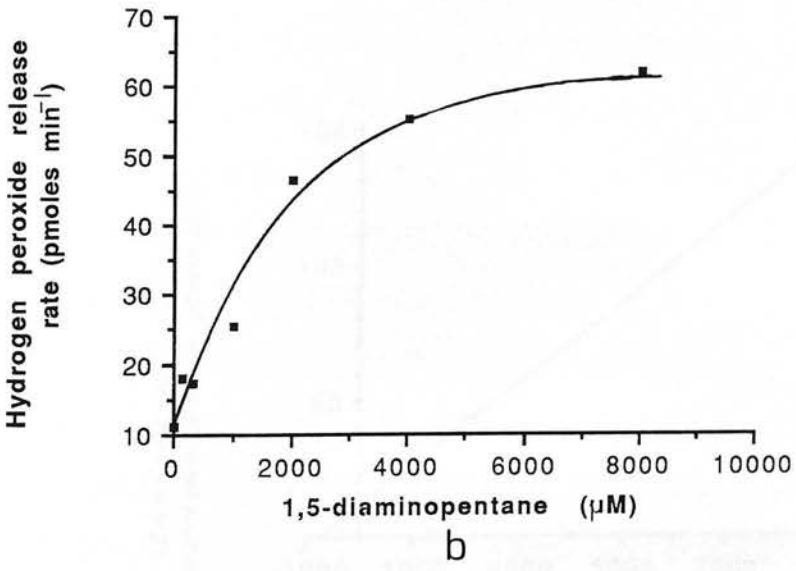
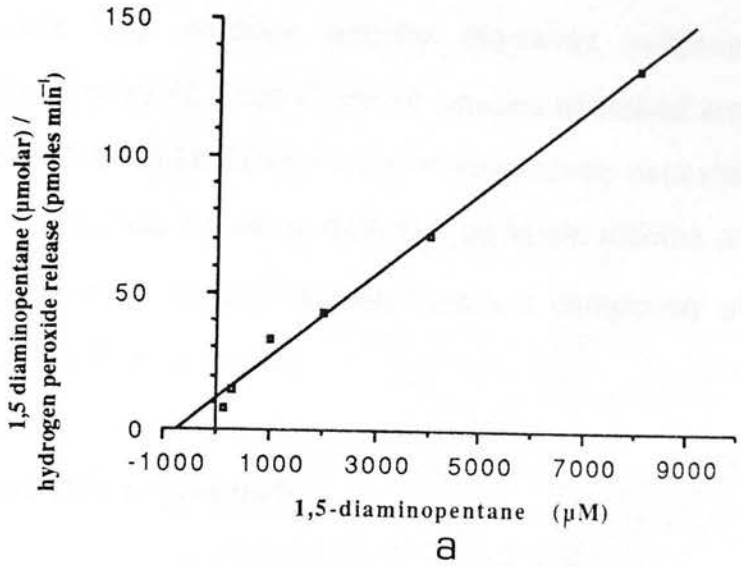


Figure 5.8. Dependence of the initial rate of hydrogen peroxide release on the concentration of 1,5-diaminopentane in the peroxidase-coupled assay. Lysyl oxidase (2 μg) was assayed using the peroxidase coupled assay (section 2.5). Hydrogen peroxide release was measured over 3 minutes, and the mean rate of release per minute was calculated in the presence (a) and absence (b) of eclefin (1μg; section 5.5).



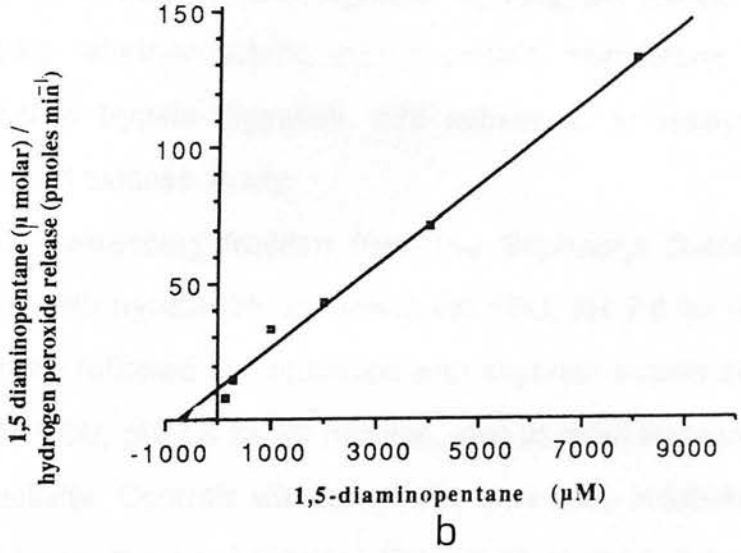


Figure 5.9. Hanes plots of the peroxidase-coupled lysyl oxidase assay. Concentration of 1,5-diaminopentane (µM) divided by the initial rate of release of hydrogen peroxide over three minutes (pmoles min $^{-1}$) in a peroxidase coupled assay (section 2.5) using 2µg lysyl oxidase vs concentration of 1,5-diaminopentane (µM) in the presence (a) and absence (b) of eclefin (1µg).

to enhance lysyl oxidase activity. However, subjecting eclefin to temperatures of 95 ^{0}C - 100 ^{0}C for 10 minutes abolished enhancing activity completely (Table 5.3). Eclefin is therefore relatively heat stable.

Eclefin activity could be detected up to six months after purification when stored at 4 ^{0}C, but activity was lost completely after short term storage at -10 ^{0}C (not shown).

5.9 Is eclefin a protein?

Trypsin (E.C.3.4.21.4) is a mammalian serine protease that cleaves polypeptide chains at Lys / Arg-X sites, where X is non-specific. Trypsin is enzymatically fully active in urea solutions up to 6.5 M (Delaage and Lazdunski, 1968), and its optimal working pH range is 8.5-8.8. To investigate whether eclefin was a protein, stimulating fractions were subjected to trypsin digestion, and subsequently assayed in a tritium release lysyl oxidase assay.

An enhancing fraction from the Sephacryl S-400 column was incubated with trypsin (25 μg / ml) in 6M PBU, pH 7.8 for 5 hours at room temperature followed by incubation with soybean trypsin inhibitor (2 mg / ml) in 6M PBU, pH 7.8 for 20 minutes, also at room temperature, to inhibit trypsin activity. Controls without eclefin were also incubated with trypsin and soybean trypsin inhibitor. Eclefin (1μg) was then assayed for enhancing activity on lysyl oxidase by microdistillation, using [4,5-^{3}H] lysine labelled elastin (100μl) as substrate. A control, containing inactivated trypsin incubated with lysyl oxidase alone showed that trypsin was totally inactivated by the soybean trypsin inhibitor, since lysyl oxidase remains

Table 5.3. Effect of prior heat treatment of eclefin on enhancement activity.

Addition to assay	cpm ± S.E. (n=2)	activity % of control ± S.E. (n=2)	Enhancement factor
Unheated eclefin			
Control	2537 ± 261	100 ± 1.0	----
Lysyl oxidase	3706 ± 159	146 ± 4.3	1
Ecleptin	2631 ± 56	104 ± 2.1	----
Ecleptin + Lysyl oxidase	4797 ± 135	189 ± 2.8	1.79 ± 0.28
Eclefin heated at 80^0C			
Control	3048 ± 88.5	100 ± 2.9	----
Lysyl oxidase	4332 ± 10.5	142 ± 0.2	1.00
Ecleptin	3104 ± 22.5	102 ± 0.7	----
Ecleptin + Lysyl oxidase	5190 ± 0.5	170 ± 0.0	1.59 ± 0.18
Eclefin heated at 100^0C			
Control	3004 ± 156	100 ± 5.2	----
Lysyl oxidase	5400 ± 72	180 ± 1.3	1.00
Ecleptin	3011 ± 149	100 ± 4.9	----
Ecleptin + Lysyl oxidase	5458 ± 56	181 ± 1.0	1.02 ± 0.1

(Top) Untreated eclefin (1μg; section 5.5) assayed for activity with lysyl oxidase (2μg) and 100μl [4,5-^{3}H] lysine labelled elastin using the ultrafiltration method. (Middle) Eclefin (1μg) heated to 80°C for 90 minutes before cooling and being assayed with lysyl oxidase (2μg). (Bottom) Eclefin (1μg) heated to 100°C for 10 minutes and then cooled before assaying with lysyl oxidase.

fully active. No stimulation was observed from eclefin fractions that had been pre-treated with trypsin followed by soybean trypsin inhibitor (Table 5.4).This indicates that eclefin is probably a protein.

5.10 Effect of substrate concentration

Enhancement of tritiated water release was measured at different concentrations of [4,5-^{3}H] lysine labelled elastin, with eclefin (1μg) and lysyl oxidase (2μg). The results (Fig 5.10) show that enhancement was greater at low concentrations of radiolabelled elastin. This indicates that eclefin may increase the affinity between lysyl oxidase and elastin, or increase the number of lysine residues accessible to lysyl oxidase.

5.11 Discussion

Background radioactivity in the lysyl oxidase assay, using 100μl of [4,5-^{3}H] lysine labelled elastin can give a high signal-to-noise ratio, as observed in the assays shown in Table 5.1, where control samples show greater than 2000 dpm. During the lysyl oxidase assay using ultrafiltration, it is possible that small radiolabelled peptides generated from the elastin may filter through the 10kDa cut-off membrane used. Another contributing factor to the high background may be endogenous lysyl oxidase activity in the elastin substrate, which is not inhibited by the addition of BAPN (0.2 mM) during preparation of the elastin (section 2.3).

There is evidence from Superose-6 gel permeation FPLC that eclefin

Table 5.4. Effect of prior trypsin treatment on eclefin.

Addition to assay	cpm ± S.E. (n=3)	activity % of control ± S.E. (n=3)	Enhancement factor
Control	1253 ± 14	100 ± 1.1	----
Lysyl oxidase	1946 ± 4.5	155 ± 0.2	1.00
Eclefin	1275 ± 144	102 ± 11.3	----
Eclefin + Lysyl oxidase	2228 ± 67	178 ± 3.0	1.41± 0.23
Eclefin (trypsin) +Lysyl oxidase	1961 ± 3	156 ± 0.2	1.02± 0.21
Control (trypsin) + Lysyl oxidase	2059 ± 59	164 ± 2.9	----

Eclefin (1μg; section 5.5) was incubated with 25μg / ml trypsin for 5 hours followed by inactivation with 2mg / ml soybean trypsin inhibitor type I-S. Trypsin treated eclefin (1μg; 50μl) was then incubated with lysyl oxidase (2μg; 50μl) and [4,5-^{3}H] lysine labelled elastin (100μl). Radiolabelled water produced in the reaction was isolated using the microdistillation technique.

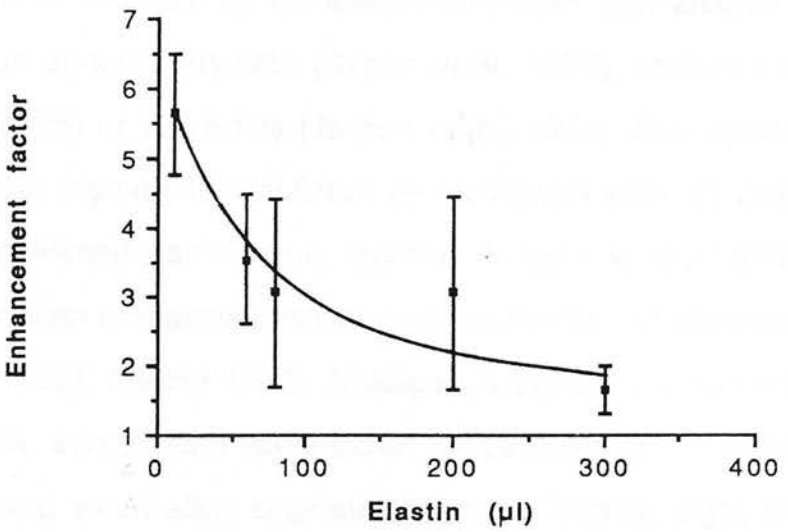


Figure 5.10. Effect of [4,5-^{3}H] lysine labelled elastin concentration on the enhancement of lysyl oxidase activity by eclefin. Lysyl oxidase (2µg) was incubated with increasing amounts of radiolabelled elastin in the presence of eclefin (1µg) and assay buffer, at 37^{0}C overnight in a total volume of 900µl.

may aggregate in the absence of urea, since eclefin dialysed exhaustively against PBS and then run on a gel permeation column eluted in two separate fractions (8 and 19).

The mechanism of lysyl oxidase enhancement by eclefin is unknown. However, studies carried out with cationic and anionic amphiphiles suggest that the affinity of pancreatic elastase and lysyl oxidase for elastin may be influenced by charge and hydrophobicity of the substrate. Susceptibility of elastin to digestion by pancreatic elastase has been shown to increase by incubation of elastin with anionic detergents e.g. sodium dodecyl sulphate (Kagan *et al.,* 1972), sodium linolate (Kagan *et al.,* 1979b) or bile acids (Jordan *et al.,* 1974). Susceptibility of elastin to elastase digestion is inhibited by incubation with the cationic detergent, dodecyltrimethylammonium chloride (Kagan *et al.,* 1979b) and also by maleylation of elastase, which stops adsorption of elastase to elastin (Hall *et al.,* 1961; Gertler 1971). Changes in the circular dichroism spectrum of soluble elastin and stimulation of pancreatic elastase activity were observed when alkyl sulphate chains containing eight or more carbons were incubated with elastin (Jordan *et al.,* 1974). This suggests that ligand-induced conformational changes are responsible for stimulating effects. An anionic polar group was found to be necessary for stimulation, whereas neutral group or non-ionic detergents had no effect and cationic groups inhibited activity.

The effects of cationic amphiphiles have also been studied on lysyl oxidase (Kagan *et al.,* 1981). It was found that sodium dodecyl sulphate, bile salts and fatty acid salts are able to inhibit elastin oxidation by lysyl oxidase, whereas neutral detergents and non-hydrophobic molecules had no effect. Cationic amphiphiles such as dodecylamine hydrochloride could

stimulate lysyl oxidase activity up to five fold (Kagan *et al.,* 1981). No effects on the oxidation of n-butylamine were observed when this substrate was treated with dodecylamine hydrochloride, which indicates that there is no direct effect on lysyl oxidase by these charged molecules.

Studies on the effects of cationic amphiphiles may provide a model for the effect of eclefin on the oxidation of elastin by lysyl oxidase. Eclefin does not seem to enhance activity when radiolabelled collagen or 1,5-diaminopentane is used as substrate, consistent with an elastin specific interaction. Eclefin is extracted from porcine tissue in the presence of 6M urea, which indicates that it is probably hydrophobic, and requires the disruption of hydrogen bonds for solubilization. Also, unlike lysyl oxidase, eclefin binds to CM-Sepharose in the presence of 6M PBU, pH 7.8.

It is possible that eclefin binds to elastin by hydrophobic interactions, and disrupts the structure of the substrate in such a way as to increase accessibility of potential lysine cross-linking sites to lysyl oxidase. Alternatively, eclefin may produce favourable electrostatic interactions between lysyl oxidase and localised areas of the elastin molecule, allowing lysyl oxidase to oxidise lysine residues in these areas.

Evidence supporting a mechanism whereby more lysine residues become available for oxidation by the action of eclefin comes from observing enhancement at different elastin concentrations (section 5.10). At low elastin concentrations, stimulation appears to be greatest, which could indicate that eclefin is able to increase the number of potential cross-linkable lysine residues, thereby increasing the effective substrate concentration and increasing the reaction velocity towards V_{max}. At higher elastin concentrations where the reaction velocity tends towards V_{max}, the reaction appears to be stimulated less. Data regarding the K_m and V_{max} of

the lysyl oxidase reaction with elastin are difficult to obtain using the discontinuous trituim release assay because initial reaction velocities cannot be easily measured. Studies using the fluoresence-coupled assay (Trackman *et al.,* 1983) will be necessary to establish the effect of eclefin on K_m and V_{max} in the lysyl oxidase reaction with elastin, although this substrate is poor in comparison to artificial substrates such as 1,5-diaminopentane when used in this assay.

It is possible that eclefin is a regulatory enzyme of elastin turnover. Since cationic amphiphiles are capable of inhibiting the action of pancreatic elastase, and are able to stimulate lysyl oxidase activity, this is consistent with a system in which elastin degradation can be down-regulated and elastin synthesis increased when required. Eclefin, which is isolated from the same porcine tissue as lysyl oxidase and similarly hydrophobic, may be a naturally occurring lysyl oxidase enhancing enzyme involved in such an elastin regulating mechanism *in vivo* . Work is required to find out if eclefin is able to inhibit elastase activity, just as cationic amphiphiles are able to stimulate lysyl oxidase activity and abolish elastase activity.

Chapter 6

Final Discussion

6.1 TRAMP

Part of the present study has been concerned with distribution of TRAMP in murine tissue, and its possible enzymatic role in tissue. It has been found previously that TRAMP is able to accelerate collagen fibril formation *in vitro* (MacBeath *et al.,* 1993), and work here suggests that TRAMP may be a collagen specific lysyl oxidase. Further information on interactions between TRAMP and collagen may be obtained using the polyclonal antiserum raised against TRAMP, and immunogold localisation in the electron microscope. The studies presented here focus on collagen I, and therefore future work may be directed to demonstrate the effect of TRAMP on other collagen types, especially the fibrillar collagens.

The interaction of TRAMP with other extracellular matrix components may also be determined using electron microscopy. It has been suggested that dermatan sulphate proteoglycans may bind to TRAMP, through the dermatan sulphate chain (Lewandowska *et al.,* 1991). The same study reported TRAMP / cell surface binding to human and bovine dermal fibroblasts as well as Balb/c 3T3 cells *in vitro*, possibily mediated through the sequence Arg-Gly-Ala-Thr in TRAMP (Neame *et al.,* 1989), which has potential integrin binding ability (Lewandowska *et al.,* 1991).

To determine more accurately the lysyl oxidase activity of TRAMP on collagen, it may be necessary to label collagen with [6-^{3}H] lysine instead of [4,5-^{3}H] lysine, because during the conversion of lysine to hydroxylysine, one tritium atom is lost from the 5-position (Popenoe *et al.,* 1965), which means that there is less potential radioactivity in the exchangeable 5-position in highly hydroxylated collagen (Miller, 1972).

TRAMP carries the sequence Asn / Tyr / Asp which has been reported

as a consensus sequence for the incorporation of topaquinone in copper containing amine oxidases. To determine whether TRAMP actually contains topaquinone, this can be detected by derivatizing the protein with phenylhydrazone and examining the spectrophotometric properties of the derivatives. At neutral pH, the derivatives of other copper dependent amine oxidases have a λ_{max} of 457 - 463 nm. When these are exposed to 2 M KOH the λ_{max} shifts to 578 - 585nm. These maxima closely resemble those displayed by the *p*-nitrophenylhydrazone derivative of topaquinone hydantoin, which has a λ_{max} of 455nm and 575nm in neutral and basic solutions respectively (Janes et al., 1992). These spectral properties are thought to be unique to topaquinone, and can be utilised for detection in TRAMP.

The publication of the human TRAMP cDNA sequence (Superti-Furga *et al.,* 1993) allows the possibility of sequencing porcine TRAMP cDNA, and then expressing this in a yeast expression vector. Production of TRAMP will then be possible in the absence of urea, which denatures proteins and may cause carbamylation of α–amino and ε–amino groups on the side chains of lysine residues. Expression of recombinant TRAMP cDNA altered by site directed mutagenesis will allow investigation of regions that are necessary for catalysis.

6.2 Lysyl oxidase

A construct for the expression of mature human lysyl oxidase in *S. cerevisiae* has been synthesised during this study from human placental cDNA amplified using the polymerase chain reaction. This construct has

been found to lack one adenine base at position 1205 compared with the human lysyl oxidase cDNA sequence published by Hamalainen *et al.* (1991), probably due to an error made by either the Taq DNA polymerase used to amplify VLO 2 or the vent DNA polymerase used to amplify ALO 2 .

The missing base can be inserted using an oligonucleotide which will facilitate mutagenesis. In this method (Wallace *et al.,* 1981; Zoller and Smith, 1982), an oligonucleotide is synthesised complementary to the DNA flanking the missing base. The oligonucleotide carries the extra base at the correct position for insertion and acts as a primer for a single stranded ALO2 / vector template which is copied using DNA polymerase and then ligated at the ends, forming heteroduplex DNA. When transformed into *E. coli,* the heteroduplex denatures and both strands replicate, leading to double stranded wild-type and mutant DNA (i.e. with the extra base). There are ways to identify a mutant clone i.e. the mutant oligonucleotide used to introduce the mutation can be radiolabelled and hybridized to *E.coli* colony DNA. The temperature of the hybridization can be raised to a point at which the oligonucleotide will only bind mutant DNA, allowing its identification.

Successful expression of the protein from this construct in yeast will depend on several factors which are currently being addressed. To detect the presence of the protein in the yeast medium, [^{14}C] labelled BAPN is being prepared. This compound should be capable of irreversibly binding to and labelling lysyl oxidase secreted by yeast cells, therefore allowing identification of the enzyme in large volumes of medium. [^{14}C] BAPN however requires the presence of the enzyme carbonyl containing cofactor before irreversible binding takes place (Tang *et al.,* 1983) and currently it is not known whether *S. cerevisiae* will incorporate the correct cofactor into

the enzyme.

To overcome problems of expression of active lysyl oxidase in yeast, it may be necessary to use a bacterial expression system. *E.coli* K-12 produces an amine oxidase which catalyses oxidation of 2-phenylethylamine and contains copper and topaquinone cofactors (Cooper *et al.,* 1992). Since there is no detectable glycosylation in lysyl oxidase, a bacterial cell may be able to produce active enzyme.

Manipulation of the lysyl oxidase DNA by reverse genetic techniques may be used to study many aspects of the enzyme structure and functions such as the active site, cofactor binding domains, substrate binding domains. The nature of lysyl oxidase variants may also be elucidated by such techniques.

Information regarding the effects of mutations in lysyl oxidase can then be used to address the causes of genetic diseases within connective tissue, and also may prove valuable in cancer therapies, since the rat lysyl oxidase gene has been found to be a homologue of the mouse ras recision gene Kenyon *et al.*, (1991). In turn, mutation studies may also shed light on the possible link between an enzyme that initiates collagen and elastin cross-linking and the apparent ability to suppress tumours.

Lysyl oxidase expression is down-regulated in H-ras transformed NIH 3T3 cells (Contente *et al.*, 1990) and immortilized rat 208F fibroblasts (Hajnal *et al.,* 1993), but can be restored upon chemically induced reversion of these cell lines. Contente *et al.,* (1990) retransformed cell lines after transfecting NIH cells with an antisense rrg expression vector. This evidence suggests that lysyl oxidase is important in maintaining a non-transformed phenotype within certain cells. Genetic manipulation of lysyl oxidase cDNA is therefore also of great importance in identifying

causes of tumour susceptibility, as well as being able to express and study mutant enzymes unable to support the non-transformed phenotype.

6.3 Eclefin

Eclefin has been found to enhance activity of lysyl oxidase when [4,5-^{3}H] lysine labelled elastin is used as substrate. All activity is abolished in the presence of 0.2 mM BAPN. Eclefin activity is susceptible to trypsin activity, which indicates that it is probably a protein. Eclefin appears to enhance lysyl oxidase more in the presence of low elastin concentrations. The heat stability of eclefin may allow for the introduction of an 80^{0}C heating step during its preparation since most other contaminating proteins are likely to denature at this temperature. Studies to determine the molecular weight of eclefin may be continued using FPLC gel permeation chromatogaphy, although the low yields of eclefin activity obtained usually result in the loss of all activity after further purification steps.

Work should be carried out to determine whether eclefin will influence the binding of enzymes to elastin. Pancreatic elastase activity is inhibited by cationic amphiphiles such as dodecyltrimethylammonium chloride (Kagan *et al.,* 1979b), whereas dodecyltrimethylammonium bromide stimulates the activity of lysyl oxidase (Kagan *et al.,* 1981). The possible effect of eclefin on pancreatic elastase should be investigated.

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Publications

Forbes, E.G., Cronshaw A.D., MacBeath, J.R.E., Hulmes, D.J.S. (1994). Tyrosine Rich Acidic Matrix Protein (TRAMP) is a widely distributed protein of the extracellular matrix. (In Press)

Abstracts

Poster presented at the XII meeting of the Federation of European Connective Tissue Societies, Bialystok, Poland, July 1990. Shackleton D.R., Cronshaw A.D., Forbes E.G., Hulmes D.J.S. An enhancer of lysyl oxidase

Poster presentation at the British Connective Research Society meeting, Manchester, England. Forbes, E. G., Cronshaw A. D., MacBeath J.R.E., Kerr I.E., Coutts, S.R.J., Genscel, U. H., Simpson, F., Hulmes, D.J.S. March 1993. Tissue distribution and synthesis of TRAMP (Tyrosine Rich Acidic Matrix Protein) in fibroblast culture. Abstract : J. Exp. Pathol (1993) (in press).

Poster and oral presentation at the Gordon Conference on Elastin, Kimball Union Academy, New Hampshire, United States, August 1990. Forbes, E.G., Hulmes, D.J.S. Elastin cross-linking: Stimulation of lysyl oxidase activity by an extract of porcine skin.

Poster presented by D.J.S. Hulmes at the Gordon Conference on Collagen, Colby - Sawyer College, New Hampshire, United states, July 1993. Cronshaw A.D., Forbes E.G., MacBeath J.R.E., Kerr I.E., Coutts S.R.J., Genschel U.H., Simpson F, Hulmes D.J.S. TRAMP (Tyrosine Rich Acidic Matrix Protein) tyrosine sulphation and tissue distribution of an extracellular matrix protein that accelerates collagen fibril formation.

Poster and oral presentation at the XIV meeting of the Federation of European Connective Tissue Societies, Lyon, France, August 1994. Forbes E.G., Bear H.M. and Hulmes D.J.S. TRAMP (Tyrosine Rich Acidic Matrix Protein): Tissue distribution and biological functions.