Characterisation of TRAMP (Tyrosine Rich Acidic Matrix Protein) 
and its Role in Collagen Fibril Formation

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Doctor of Philosophy

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1992
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ABSTRACT OF THESIS

A protein (M_r 24 K) that co-purifies with porcine skin lysyl oxidase (M_r 34 K) has been characterised. Five variants of the 24 K protein were identified by Mono Q FPLC, as were 4 variants of lysyl oxidase. By amino acid analysis, the 24 K protein is particularly rich in tyrosine, and isoelectric focusing shows it to be acidic, so the name TRAMP (Tyrosine Rich Acidic Matrix Protein) is used to identify this protein. By amino acid sequence analysis and immunoblotting, TRAMP is unrelated to lysyl oxidase, though it is identical (in all but 4 residues) to a 22 K extracellular matrix protein from bovine skin that associates with dermatan sulphate proteoglycan. TRAMP is not a proteoglycan, however, as mass spectrometry indicates a molecular mass only 150 Da greater than that predicted from the amino acid sequence, and treatment with a number of deglycosylating enzymes does not alter the electrophoretic migration of the protein. Sequence analysis, mass spectrometry and susceptibility to sulphatase treatment indicates the presence of sulphated tyrosine residues in TRAMP. Although TRAMP occurs as a quantitatively minor component in skin, it shows a widespread tissue distribution. TRAMP does not appear to affect the activity of lysyl oxidase on an elastin substrate. However, turbidity time data shows that sub-equimolar concentrations of TRAMP accelerate the in vitro formation of fibrils from purified, lathyritic (i.e. non cross-linked) rat skin type I collagen. The fibrils which form are well ordered with a D-periodicity similar to those observed in vivo, though fibrils formed in the presence of TRAMP are significantly thinner. TRAMP binds to collagen fibrils as shown by cosedimentation, and once formed, fibrils are more resistant to low temperature solubilisation. TRAMP may have a important role in the early, nucleation stages of fibril formation. Since it is already known that lysyl oxidase activity on a collagen substrate is increased by prior assembly of the molecules into fibrils, TRAMP may influence lysyl oxidase activity indirectly via aggregation of the substrate, regulating subsequent cross-linking, and providing a possible mechanism for the regulation of
collagen metabolism, and ultimately connective tissue structural integrity. The further observation that TRAMP associates with dermatan sulphate proteoglycans, and that dermatan sulphate proteoglycans may inhibit collagen fibrillogenesis, may provide an additional level of control whereby the mechanical properties of tissues can be modulated.
Declaration

The work presented in this thesis was carried out between the 1st October 1988 and the 31st March 1992, under the supervision of Dr David J S Hulmes at the Department of Biochemistry, University of Edinburgh. All material presented in this thesis, unless stated otherwise, is the sole work of the author as is the composition.

Jonathan R E MacBeath
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First, I would like to thank Dr David Hulmes for giving me the opportunity to work in his laboratory, for his excellent supervision and valuable discussions during the last three years. I am also grateful to Dr Laurent Vuillard for having introduced me to many biochemical techniques, Mr Andrew Cronshaw for the amino acid analysis and sequencing, and Dr David Apps for assistance with the lectin blotting. Thanks are also due to staff from the Department's animal house for assistance with the immunisations, and to staff from the Dept of Pathology for ultra-microtomy. I also wish to thank Drs Tim and Linda Wess, Dr D Shackleton, Mr A Chogley, Mr I Purdom, Mr G Fiaux, Mr E Forbes and Mr M Marsden for their useful comments and advice.

Lastly I'm also largely indebted to my wife Alison for her support and her patience.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BAPN</td>
<td>β-Aminopropionitrile</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CEC</td>
<td>Critical electrolyte concentration</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate</td>
</tr>
<tr>
<td>CM</td>
<td>Carboxymethyl</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen bromide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>Endo F</td>
<td>Endoglycosidase F</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACITs</td>
<td>Fibril associated collagens with interrupted triple-helices</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GLcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>³H</td>
<td>Tritium</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HCN</td>
<td>Hydrogen cyanide</td>
</tr>
<tr>
<td>³HHO</td>
<td>Tritiated water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>Killodalton(s)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>kV</td>
<td>Killovolts</td>
</tr>
<tr>
<td>l</td>
<td>Litre(s)</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp(s)</td>
</tr>
<tr>
<td>MATRIXIN</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MBq</td>
<td>Megabecquerel(s)</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre(s)</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre(s)</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre(s)</td>
</tr>
<tr>
<td>ml/g</td>
<td>Millilitres per gram</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>Mr</td>
<td>Relative Molecular weight</td>
</tr>
<tr>
<td>msec</td>
<td>Millisecond(s)</td>
</tr>
<tr>
<td>mU</td>
<td>Milliunit</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NANA</td>
<td>5-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NC</td>
<td>Non-collageneous</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl malemide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre(s)</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered saline</td>
</tr>
<tr>
<td>PBU</td>
<td>Phosphate buffered urea</td>
</tr>
<tr>
<td>pC-collagen</td>
<td>Procollagen with N-propeptides removed</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan(s)</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>pN-collagen</td>
<td>Procollagen with C-propeptides removed</td>
</tr>
<tr>
<td>PQQ</td>
<td>Pyrroloquinoline quinone</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>Rf</td>
<td>Relative mobility</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SGP</td>
<td>Structural glycoprotein(s)</td>
</tr>
<tr>
<td>Stains-ALL</td>
<td>1-ethyl-2-[3-(1-ethynaphtho [1,2 d] thiazolin-2-ylidene)-2-methyl-propenyl]-naphtho [1,2d] thiazolium bromide</td>
</tr>
<tr>
<td>TBq</td>
<td>Terabecquerel(s)</td>
</tr>
<tr>
<td>TBU</td>
<td>Tris buffered urea</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TES</td>
<td>N-[tris(hydroxymethyl)methyl-2-amino]-ethanesulphonic acid</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-tosyl-L-phenylalanine chloromethylketone</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Tyrosine rich acidic matrix protein</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UA</td>
<td>Uranyl acetate</td>
</tr>
<tr>
<td>V</td>
<td>Volt(s)</td>
</tr>
<tr>
<td>V/V</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>W</td>
<td>Watt(s)</td>
</tr>
<tr>
<td>( \lambda_{max} )</td>
<td>Wavelength of maximal absorbance</td>
</tr>
<tr>
<td>W/W</td>
<td>Weight per volume</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 The Extracellular Matrix

In all vertebrates, structural integrity is maintained by connective tissues such as bone, tendon and skin. The properties of these tissues which allow it to withstand applied forces are conferred by the extracellular matrix (ECM). The ECM consists of collagens, elastin(s), proteoglycans (PGs) and structural glycoproteins (SGPs) that are secreted by cells and assembled locally into an organised network. The collagens and elastin(s) form the fibrous scaffolding of connective tissues, while the PGs and SGPs fill the interstices between cells and ECM. For an overview of recent results in ECM biochemistry, see Labat-Robert et al. (1990). The molecules of the matrix combine in many ways to produce a multiplicity of different structures, designed for a variety of mechanical roles, for example resistance to tensile, compressive and shear forces.

A large amount of information has emerged in recent years regarding the structure and biological functions of proteins in the ECM. New collagen types have been described and several glycoproteins and PGs have been isolated (Cunningham, 1987; van der Rest & Garrone, 1991). Cell-surface receptors mediating interactions between the ECM and different cell types have received much attention (Albelda & Buck, 1990). In some cases it has been shown that these components are involved in cell migration, adhesion and differentiation, these functions being exerted via multiple matrix-matrix and cell-matrix interactions.

1.2 The Collagens

The collagens constitute a family of related proteins that are assembled in a variety of supra-molecular structures in extracellular matrices. Fourteen different collagens are reported to date and there is no reason to believe this is the final number. Additionally there are proteins with collagen-like motifs, but are not called collagens (Hulmes, 1992).

The different collagens are referred to as collagen types and are designated by roman numerals I-XIV. All these molecules contain at least one triple-helical region.
composed of 3 polypeptide chains (α-chains) with a characteristic Gly-Xaa-Yaa repeat sequence, where Xaa and Yaa can be any amino acid but are often proline and hydroxyproline, respectively. Because the inflexible peptide bonds formed by proline and hydroxyproline are flanked by highly flexible bonds (formed by glycine), the individual α-chains must form a triple helix (Prockop, 1990). Although characterised by a triple helical structure along most of their length, the collagens exhibit a wide diversity in other structural and chemical features. Such diversity results from the different genes which synthesise the various collagen chains. Post-translational modifications extend this diversity.

Vertebrate collagens can be classified (Table 1.1) on the basis of their size and self assembly (van der Rest & Garrone, 1991). The fibrillar collagens (I, II, III, V and XI; Fig. 1.1) represent the largest collagen subclass and form fibrils composed of 67 nm (D) staggered molecules. Collagens IX, XII and XIV represent another subclass which do not form fibrils but instead appear to be associated with fibrils: for example collagen IX is cross-linked to type II collagen fibrils in cartilage (Vaughan et al., 1988). Collagens IX, XII and XIV are multidomain molecules containing triple helical domains interrupted by non-collageneous regions. The non-collageneous domain acts as a flexible hinge (Gordon et al., 1989) which permits, at least for type IX, a portion of the molecule to associate laterally with the fibril surface, leaving other domains projecting outward (Vaughan et al., 1988; Yada et al., 1992). This may mean that the projecting domain can interact with cells or other components of the ECM. Due to their molecular structure, the subclass containing collagens IX, XII and XIV are referred to as FACITs (Fibril Associated Collagens with Interrupted Triple-helices). The other non-fibrillar collagens (IV, VI, VII, VIII, X) are more difficult to classify, however distinct kinds of assembly occur allowing further subdivisions (Fig. 1.1). Collagens IV and VIII form sheets which constitute basement membranes and Descemet's membrane, respectively. Type VII collagen assembles into anchoring fibrils that bind epithelial basement membranes and entrap collagen fibrils from the underlying stroma.
<table>
<thead>
<tr>
<th>Type</th>
<th>α-chains</th>
<th>Most common molecular form</th>
<th>Representative tissues</th>
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<tr>
<td><strong>Fibrillar collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>α1(I), α2(I)</td>
<td>[α1(I)]₂ α2(I)</td>
<td>skin, tendon &amp; bone</td>
</tr>
<tr>
<td>II</td>
<td>α1(II)</td>
<td>[α1(II)]₃</td>
<td>cartilage &amp; vitreous humour</td>
</tr>
<tr>
<td>III</td>
<td>α1(III)</td>
<td>[α1(III)]₃</td>
<td>skin, lung &amp; vascular system</td>
</tr>
<tr>
<td>V</td>
<td>α1(V), α2(V), α3(V)</td>
<td>[α1(V)]₂ α2(V)</td>
<td>collagen I containing tissues</td>
</tr>
<tr>
<td>XI</td>
<td>α1(XI), α2(XI), α3(XI)</td>
<td>α1(XI) α2(XI) α3(XI)</td>
<td>collagen II containing tissues</td>
</tr>
<tr>
<td><strong>Nonfibrillar collagens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>α1(IV), α2(IV), α3(IV), α4(IV), α5(IV)</td>
<td>[α1(IV)]₂ α2(IV)</td>
<td>basement membranes</td>
</tr>
<tr>
<td>VI</td>
<td>α1(VI), α2(VI), α3(VI)</td>
<td>α1(VI) α2(VI) α3(VI)</td>
<td>skin &amp; cartilage</td>
</tr>
<tr>
<td>VII</td>
<td>α1(VII)</td>
<td>[α1(VII)]₃</td>
<td>dermal-epidermal junction</td>
</tr>
<tr>
<td>VIII</td>
<td>α1(VIII), α2(VIII)</td>
<td>[α1(VIII)]₃</td>
<td>Descemet’s membrane</td>
</tr>
<tr>
<td>IX</td>
<td>α1(IX), α2(IX), α3(IX)</td>
<td>α1(IX) α2(IX) α3(IX)</td>
<td>cartilage &amp; vitreous humour</td>
</tr>
<tr>
<td>X</td>
<td>α1(X)</td>
<td>[α1(X)]₃</td>
<td>hypertrophic zone of cartilage</td>
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<tr>
<td>XII</td>
<td>α1(XII)</td>
<td>[α1(XII)]₃</td>
<td>tendon &amp; skin</td>
</tr>
<tr>
<td>XIII</td>
<td>α1(XIII)</td>
<td>[α1(XIII)]₃</td>
<td>skin &amp; intestine</td>
</tr>
<tr>
<td>XIV</td>
<td>α1(XIV)</td>
<td>[α1(XIV)]₃</td>
<td>collagen I containing tissues</td>
</tr>
</tbody>
</table>
### Figure 1.1 Collagen molecules and supramolecular assemblies

Schematic representation of collagen molecules (left panel) and collagen aggregates. The molecules (not drawn to scale) are orientated with their N-termini to the left, triple helical domains are drawn as solid lines and globular domains by filled circles. Vertical arrows (drawn on the molecules) indicate the sites of action of the processing proteinases (adapted from van der Rest & Garrone, 1991).

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Aggregated forms</th>
</tr>
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<tr>
<td><strong>Collagens participating in quarter staggered fibrils</strong></td>
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</tr>
<tr>
<td>Collagens I, II, III, V, XI</td>
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<td>D (67 nm) staggered molecules</td>
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<td><strong>Fibril-associated collagens with interrupted triple helices (FACITs)</strong></td>
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<td>Collagens XII, XIV &amp; IX</td>
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<td>Collagen X (7) Collagen VIII</td>
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<td><strong>Collagen forming beaded filaments</strong></td>
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<td>Collagen VII</td>
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5
to “cement” the two structures together. Collagen VI forms thin, beaded filaments that may interact with fibrils and cells (van der Rest & Garrone, 1991).

The triple-helical conformation characteristic of all collagens is a unique structure among proteins (Prockop, 1990). This conformation results from the unique primary sequence of collagen α-chains. Three chains which may or may not be identical, combine via their (Gly-Xaa-Yaa)ₙ sequences to form a triple helix. The relatively large amount of hydroxyproline in the structure serves to stabilise the triple-helical conformation under physiological conditions. Steric restrictions require that the centre of the helix be occupied by glycyl residues. It is thought that the triple helical regions may serve as molecular rods that can physically separate globular domains in a protein. However the triple helix has another important property. From the structure, the amino acids in the Xaa and Yaa position have their side chains pointing out away from the helix, thus offering a high potential for lateral associations, particularly with other triple helices.

1.2.1 Fibrillar (Quarter-Staggered) Collagens (I, II, III, V, XI)

Collagens I, II, III, V, and XI were assigned to this group on the basis of their protein and gene structures. The molecules are composed of chains with a molecular weight of 95 kDa or greater and are characterised by the presence of a 300 nm uninterrupted helical domain. Short telopeptides at each end of the molecules serve as cross link-sites which stabilise the fibrillar assembly. Structural integrity in tissues is conferred by highly organised fibrils, the basic structure being provided by type I collagen in most tissues (e.g. tendon, skin, bone, lung, cornea) and by type II in tissues such as cartilage and vitreous humour. Collagens III and V represent, at least in mature tissues, quantitatively minor components which are found in collagen I containing tissues except in bone where type III is missing (Birk et al., 1988; Fleischmajer et al., 1990). In cartilage, type XI collagen is associated with type II (Mendler et al., 1989).
Electron microscopic studies of collagen fibrils indicate that the individual collagen molecules are arranged in a quarter stagger array (for review see Mayne and Burgeson, 1987). According to the Hodge-Petruska model of fibril axial structure (Hodge & Petruska, 1963), the collagen molecules overlap each other by integral multiples of 67 nm (D). Since each molecule is 300 nm (4.4 D) long, a gap of approximately 40 nm is left between the ends of non overlapping molecules (Fig. 1.1). This assembly is a direct consequence of the unique properties of the molecule.

Although fibril-forming collagens have been shown to form fibrils by themselves in vitro, they appear to participate in heterotypic fibrils in vivo, i.e. composite fibrils made from two or more collagen types (Keene et al., 1987; Mendler et al., 1989; Linsenmayer et al., 1990). Mixtures of collagen types are not required for the formation of the fibrils, but seem to be involved in the control of fibril lateral growth and diameter (Birk et al., 1990a; van der Rest et al., 1990). Immunoelectron microscopy has shown that fibrils of tendon, skin and amnion are copolymers of at least collagens I and III (Keene et al., 1987). Collagens I and V are codistributed in small diameter (20 nm) fibrils of adult corneal stroma, whose regular organisation are essential for corneal transparency (Birk et al., 1988). Cartilage fibrils are composed of collagens II, IX and XI (Mendler et al., 1989). It seems likely that other combinations may exist.

Collagens I, II, III, V and XI are very similar and are clearly derived from a single ancestral gene. However while collagens I and II are fully processed before their insertion into fibrils, collagens V and XI retain an N-terminal extension containing a short triple helical region and a globular domain. By allowing different collagens to bind and participate in fibrillar assemblies, probably via triple helix-triple helix interactions, the property of the fibril can be altered. The diverse nature of the globular domains located at the N-terminus in types V and XI probably plays a vital role in adding to or altering the nature of the fibril (van der Rest et al., 1990). For example globular domains projecting from the surface of a fibril may act to limit the lateral
1.2.1.1 Structure of Procollagen I

Procollagen I, the precursor of fibrillar type I collagen, represents the major intracellular form of collagen. The molecule is larger than the final collagen product by virtue of extension propeptides at the amino (N) and carboxy (C) termini bordering the central triple helix (Fig. 1.2). The fibrillar procollagens (II, III, V & XI) all show similar domain structures (Mayne & Burgeson, 1987). During the conversion of procollagen I to collagen I, the propeptides are enzymatically removed leaving short non-helical extensions called telopeptides. The C-terminal telopeptide is about 25 amino acids long and the N-terminal telopeptide is 16-18 amino acids in length. The procollagen molecule consists of two \( \alpha_1(I) \) and one \( \alpha_2(I) \) chains. Both chains are genetically distinct, although small amounts of homotrimeric type I collagen with chain composition \([\alpha_1(I)]_3\) have also been detected in skin (Grant & Ayad, 1988). The procollagen \( \alpha \)-chains (also called pro \( \alpha \)-chains) first associate by hydrophobic and electrostatic interactions among the C-propeptide which is then stabilised by interchain disulphide bonds (Prockop, 1990).

The central domain of the procollagen I molecule consists of three chains with the sequence \((\text{Gly-Xaa-Yaa})_n\) where each chain forms a left handed polyproline II helix. The three chains associate forming a right handed superhelix called the triple helix, apart from short extra-helical end regions of different conformation at the N- and C-termini (Bachinger & Davis, 1991). The conformation of the helix is such that the peptide bonds linking adjacent amino acids are buried within the interior of the molecule, the stucture is therefore highly resistant to general proteolysis. The helix, which is 300 nm long and 1.5 nm wide, is stabilised by hydrogen bonds between the amino groups of glycine and the carboxyl groups of the Xaa residue in an adjacent chain. The presence of 4-hydroxyproline serves to stabilise the collagen triple helix by forming extra hydrogen bonds, and contributes significantly to its thermal stability.

growth of the fibril.
Figure 1.2 Structure of the procollagen I molecule
Representation of the procollagen I molecule showing the central triple helix and the N- and C-terminal propeptide domains. Arrows indicate the cleavage sites of the processing enzymes (from Hulmes et al., 1989b, with permission).
Amino acid sequences derived from cDNA indicate that there are three distinct domains in the N-propeptide (Vuorio & de Crombrugghe, 1990). The N-propeptide consists of a large (65-71 residues), cys-rich domain (COL1) which is absent from the pro α2(I) chain, a short triple helical region and a small globular domain that links to the N-telopeptide. Both propeptides contain intrachain disulphide bonds, whereas interchain disulphides are only present between the C-terminal propeptides (Koivu & Myllyla, 1987). The cys residues which form these disulphides are highly conserved in the propeptides. The N-propeptide shows considerable variation among the different fibrillar procollagens and between the same procollagen in different species.

The function of the N-propeptide is not understood, although it appears to regulate collagen fibril diameter (Chapman, 1989). Another possible role has been suggested as a negative feedback inhibitor of collagen biosynthesis at the translational level (Fouser et al., 1991). The C-propeptide has also been implicated in regulating biosynthesis (Raghow & Thompson, 1989).

The C-propeptide of fibrillar procollagens shows a high degree of sequence similarity among different types and species. The molecule has a globular structure which contains interchain disulphide bonds located close to the triple helix. The structure is stabilised by two intrachain disulphides formed by four C-terminal cys residues (Vuorio & de Crombrugghe, 1990). Carbohydrate groups (mannose and N-acetylglucosamine) are present in the C-propeptide; the function of these is unknown, but the sequence around the carbohydrate attachment site is highly conserved.

Several functional roles for the C-propeptide have been suggested and partially demonstrated. The folding of the propeptide and formation of the inter- and intrachain disulphides precede triple helix formation and allows for correct registration of the three α-chains. It is believed that chain association forming the triple helix occurs in a zipper-like fashion starting from the C-terminal end (Engel & Prockop, 1991). Mutations which alter the domain structure have confirmed that the C-propeptide is
important for correct chain alignment (Willing et al., 1990). It has further been suggested that the C-terminal domain determines whether the α-chains form homo or heterotrimeric molecules (Vuorio & de Crombrugghe, 1990). Following enzymatic cleavage, the C-propeptide of type II collagen has been found to accumulate in the matrix of mineralising cartilage and may play a role in the mineralisation process (Olsen, 1991).

1.2.2 Non-Fibrillar Collagens

While fibrillar collagens constitute a closely related group within the collagen superfamily, the non-fibril-forming collagen group is very heterogeneous both structurally and functionally (Vuorio and de Crombrugghe, 1990).

1.2.2.1 FACITs (IX, XII, XIV)

Collagens IX, XII and XIV are described as FACIT collagens (Fibril Associated Collagens with Interrupted Triple-helices; Shaw & Olsen, 1991). They do not form fibrils by themselves, but specifically associate with fibrillar collagen. Their structure strongly suggests that they represent molecular bridges between individual collagen fibrils and among collagen assemblies and other ECM components (Gordon & Olsen, 1990). A common, short triple helical domain (COL1), represents the major region of sequence similarity between collagens IX, XII and XIV (van der Rest et al., 1990). This structure conservation suggests the domain may play a similar function in each of the types.

Collagen IX is found in several tissues with type II collagen containing fibrils such as cartilage and the primary stroma of chick cornea (Shaw & Olsen, 1991; Yada et al., 1992). While the exact function of this disulphide bonded, heterotrimeric molecule is unknown, a considerable amount of structural information has been obtained. Collagen IX is 200 nm in length, and contains 3 short triple-helical domains interspersed between 4 non-collageneous (NC) globular domains (Fig. 1.1). Collagen IX is
synthesised in two forms, a collagen form and a proteoglycan form (Ayad et al., 1991; Yada et al., 1992). The molecule consists of three distinct polypeptide chains (\(\alpha 1(IX), \alpha 2(IX)\) and \(\alpha 3(IX)\)), of which the \(\alpha 2(IX)\) polypeptide bears the chondroitin sulphate side chain. The NC3 domain contains the attachment site for the single glycosaminoglycan (GAG) chain. A five residue loop, which gives rise to an observed kink on the NC3 domain, contains a serine residue to which the GAG is bound. The size of the GAG chain is highly variable among different tissues. For example, the molecular weight of the chondroitin sulphate chain in vitreous humour is about ten times larger than the cartilage form (Yada et al., 1992). Further variation is also produced by the use of two promoters in the gene encoding \(\alpha 1(IX)\), producing two forms of collagen IX with different amino terminal domains (Gordon & Olsen, 1990).

Collagen IX has been found to bind periodically along the surface of type II fibrils (Vaughan et al., 1988). The isolation of cross-links between the N-telopeptide of collagen II and the COL2 domain of collagen IX show the interaction to be highly specific (Fig. 1.1; van der Rest & Garrone, 1991). The GAG chain may mediate interactions between collagen II and proteoglycans. In addition the NC4 domain is basic (estimated pI 10) and could interact with acidic GAG chains. Fibril diameters in collagen II assemblies may also be regulated by the specific interaction with collagen IX (Ninomiya et al., 1990).

Collagen XII is a homotrimer consisting of three \(\alpha 1(XII)\) chains, with a molecular weight of approximately 220 K (Dublet et al., 1989). Immunolocalisation studies have indicated that collagen XII is present as a quantitatively minor component in dense connective tissues containing collagen I (Gordon et al., 1991), although direct fibril associations has not yet been observed. Each chain of the collagen XII molecule contains 2 triple helical (COL) domains, one of which is structurally very similar to collagen IX, other homologies also exist which may indicate a common ancestor. Such similarities suggest that collagen XII is fibril associated. However considerable differences between collagens IX and XII also exist. A large N-terminal globular
domain accounts for most of the protein, the collageneous domains comprising less than 10% of the molecule. Rotary shadowing visualises the collagen XII molecule as having a cross shape (Fig. 1.1).

Collagen XIV is the latest of the FACITs to be recognised. It is a homotrimeric molecule found in skin and tendon. It contains the characteristic triple helical (COL1) domain associated with all FACIT collagens. Primary indications are that, although very similar, collagen XIV is clearly distinct from collagen XII (Gordon et al., 1991).

1.2.2.2 Collagens Forming Sheets

Collagens IV and VIII are found in basement membrane and Descemet's membrane, respectively. These collagens constitute a group of molecules that are involved in the formation of protein sheets that surround tissues. In comparison to the fibrillar collagens there is no clear similarity between collagens IV and VIII. Collagen VII occurs in close proximity to basement membrane where it occurs in the form of anchoring fibrils, because of this I have included collagen VII in this part of the introduction.

1.2.2.2.1 Basement Membrane Collagens (IV, VII)

Basement membranes are thin (30-40 nm), specialised extracellular matrices found at the interface of epithelial and endothelial cell layers with their adjacent connective tissue, or surrounding muscle, nerve, and adipose cells (Yurchenco & Schittny, 1990). They play an important role in supporting epithelial tissue, modulating cellular activities and also serve as molecular sieves (e.g. in the glomerulus). A number of distinctive macromolecules constitute basement membranes including collagen IV, laminins, heparan sulphate and chondroitin sulphate proteoglycans, and nidogen (entactin). These major components provide the supramolecular lattice and the contact sites for cellular receptors. An important step in the formation of basement membranes is the self assembly of collagen IV, which together with laminin forms large and complex
networks to which other components may bind (Fox et al., 1991).

Collagen IV is a heterotrimer with chain composition \([\alpha 1(IV)]_2\alpha 2(IV)\). Clear evidence exists for the presence of additional chains in some basement membranes which have been designated \(\alpha 3(IV), \alpha 4(IV)\) and \(\alpha 5(IV)\), although there is no indication that they can form heterotrimers with either \(\alpha 1(IV)\) or \(\alpha 2(IV)\) (van der Rest & Garrone, 1991). A partial deletion in the gene which codes for the \(\alpha 5(IV)\) chain, has recently been shown to be responsible for Alport's syndrome, a heritable condition resulting in progressive loss of kidney function (Barker et al., 1990; Kuivaniemi et al., 1991). The collagen IV molecule contains a central triple helical region (approximately 400 nm in length) interrupted at several positions by short non-triple helical regions. The positions of the interruptions are conserved in several species, and correspond to areas of increased molecular flexibility, as observed by electron microscopy after rotary shadowing (Yurchenco & Schittny, 1990; Hulmes, 1992). The N-terminus of the molecule contains a further triple helical domain (called 7S) separated from the main helix by a kink. At the C-terminus there is a large globular (NC1) domain. Both domains are involved in intermolecular interactions mediating collagen IV assembly. The 7S domains associate to form a “spider” like tetramer, in which two pairs of anti-parallel molecules overlap by about 30 nm (Fig. 1.1). Interactions in the NC1 domains also take place, forming dimers which are stabilised by covalent linkages (van der Rest & Garrone, 1991; Reiser et al., 1992). Type IV collagen assembly in basement membranes has been reviewed by Yurchenco and Schittny (1990).

Collagen VII forms anchoring fibrils (about 800 nm long) which originate in the basement membrane and project into the underlying stroma. These fibrils often entrap interstitial collagen fibrils and appear to anchor the basement membrane to the matrix (Burgeson et al., 1990). The molecule is thought to be a homotrimer, it contains a large (420 nm long) triple helix containing non-helical discontinuities, with a small globular NC2 domain (probably at the C-terminus; Parente et al., 1991). A large (3 x 150 kDa)
non-helical domain (NC1), which shows a tridentate structure, is located at the other end. During assembly the molecules associate forming anti-parallel dimers overlapping at the C-terminus by 60 nm. Intermolecular disulphide bonds within the overlap stabilise the structure (Burgeson et al., 1990), and the NC2 domain appears to be cleaved during this process. The dimers then laterally aggregate in register to form the anchoring fibril (Fig. 1.1).

The importance of anchoring fibrils is clearly demonstrated in the phenotype of recessive dystrophic epidermolysis bullosa, characterised by blistering of the skin and external mucous membranes (Bruckner-Tuderman et al., 1989). The blisters result from separation of the basement membrane from the underlying matrix. In this disorder, histological and biochemical studies show that both collagen VII and anchoring fibrils are absent or abnormal.

1.2.2.2 Descemet's Membrane Collagen (VIII)

Collagen VIII was initially reported by Sage et al. (1983). The model proposed by Benya and Padilla (1986) predicted a homotrimeric molecule made up of 60 kDa chains. The polypeptide predicted from the cDNA agrees well with this structure (Gordon & Olsen, 1990). The molecule consists of a short (130 nm) triple helix containing several non-helical interruptions, bordered by two globular regions (Fig. 1.1). Descemet's membrane, produced by corneal endothelial cells, consists of a unique hexagonal network composed of collagen VIII (Fig. 1.1; Gordon & Olsen, 1990; Sawada et al., 1990). The function is not understood but it may be important in withstanding intraocular pressure (Hulmes, 1992).

1.2.2.3 Collagen VI

Collagen VI shows a widespread distribution in the ECM and appears to be important in maintaining the integrity of specific tissues (e.g. blood vessels, skin; Kielty et al., 1991). The three chains of collagen VI have a short (approximately 100
nm) triple helix which is extensively glycosylated having both N and O-linked carbohydrate. The helix is characterised by the presence of a large number of arginine-glycine-aspartate (RGD) sequences. Such sequences are required for the interaction of several ECM proteins (e.g. fibronectin, collagens, laminins, vitronectin) to cell surface receptors of the integrin type (Ruoslhti, 1991a). The large terminal globular domains which flank the triple helix, account for over two thirds of the molecular mass. These non-collageneous regions consist mainly of repetitive A domains of von Willebrand factor (Kielty et al., 1990) a widely occurring protein motif (Colombatti & Bonaldo, 1991). The functions of the A domain include adhesion between cells, between cells and ECM components, and between different ECM components. The presence of large numbers of RGD sequences together with ECM binding A modules suggests collagen VI plays a central role in cell-matrix interactions.

During the assembly of collagen VI, dimerisation occurs by anti-parallel association of two molecules, where the molecules overlap for most of their length, and then dimers aggregate laterally forming tetramers (Fig. 1.1). This assembly is stabilised by disulphide bonds rather than the lysine derived cross-links found in most other collagens. These tetramers associate end-to-end forming long thin "beaded" filaments with a 100 nm periodicity (Fig. 1.1). These filaments which are found in close association with the cell surface show a high degree of flexibility (Kielty et al., 1991).

1.2.2.4 Collagen X

Collagen X is localised to the hypertrophic zone of calcifying cartilage where it is produced in large amounts by chondrocytes (Schmid et al., 1990). The structure of the cDNA and gene for this short chain collagen is highly homologous over more than 75 % of the polypeptide chain to collagen VIII (Gordon & Olsen, 1990). The triple helical domain in collagen X contains 8 imperfections in the Gly-Xaa-Yaa repeat, similar imperfections are located in conserved regions in collagen VIII. The strong homology
between collagens X and VIII suggest that they may form similar supramolecular structures. Immunolocalisation studies have shown collagen X occurring as fine filamentous networks, showing some similarity to the hexagonal lattice of Descemet's membrane, there is also some co-localisation with collagen II fibrils (Schmid & Linsenmayer, 1990; van der Rest & Garrone, 1991). Possible functions for collagen X include participation in calcification of the cartilage matrix, and facilitation of matrix turnover (Schmid et al., 1990).

1.2.2.5 Collagen XIII

Collagen XIII has so far only been identified at the cDNA level (Tikka et al., 1991). The presumptive translation product for this unique short-chain collagen molecule shows three triple-helical and four non-collagenous domains (Vuorio & de Crombrugghe, 1990). The most striking feature of collagen XIII is the existence of alternative splicing, which affect both the collageneous and non-collageneous domains. The function of collagen XIII is not known and there is as yet no information on its molecular conformation.

1.3 Other Components of the Extracellular Matrix

1.3.1 Elastin

The elasticity and resilience of tissues such as skin, lung and blood vessels are conferred in part by a network of elastic fibres. Mature elastic fibres are composed of two distinct components, elastin and small (10-12 nm) microfibrils composed of several glycoproteins including fibrillin (Sakai et al., 1986; Kielty et al., 1991). There is no definite agreement on the number and proportion of the different glycoproteins present in these microfibrillar structures (Moczar et al., 1989). Elastin, the main component of elastic fibres, is initially synthesised as a soluble polypeptide of ~70 K (for a review on elastin see Rosenbloom et al., 1991). The newly synthesised
polypeptides undergo intracellular post-translational modifications, including hydroxylation of certain prolyl residues. The elastin polypeptides are then transported to the cell surface for cross-linking and fibre assembly. The fibrillogenesis of elastic fibres is initiated by deposition of microfibrils which form a scaffold onto which individual elastin molecules align (Uitto et al., 1991). The alignment is stabilised by lysyl-oxidase-mediated cross-linking of certain lysine residues. The formation of intermolecular cross-links called desmosines contribute to the insolubility of elastin (Reiser et al., 1992).

The deduced amino acid sequence from the human elastin cDNA depicts alternating regions of cross-link domains and hydrophobic domains rich in glycine, proline and valine residues. These domains are encoded by separate exons (Uitto et al., 1991). Although elastin is encoded by a single gene, alternative splicing generates several elastin isoforms. Therefore it can be presumed that significant variation in the function of the elastic fibres would occur. However the significance of alternative splicing has not been elucidated in detail.

1.3.2 Proteoglycans

Proteoglycans (PGs; reviewed by Gallagher, 1989; Oldberg et al., 1990; Kjellen & Lindahl, 1991) are ubiquitous components of extracellular matrices and cell surfaces which are necessary for stable assembly of the ECM and functional cell-matrix interactions.

Proteoglycans constitute a diverse class of complex macromolecules that contain a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. Glycosaminoglycans consist of hexosamine (D-glucosamine or D-galactosamine) and either hexuronic acid (D-glucuronic acid or L-iduronic acid) or galactose units (in keratan sulphate) that are arranged in alternating, unbranched sequence, and carry sulphate substituents in various positions. Several structurally distinct types of PGs have been described. The common GAGs include hyaluronan (HA) which does not
occur as PG but in the free form, the galactosaminoglycans, chondroitin sulphate (CS) and dermatan sulphate (DS), and the glucosaminoglycans heparan sulphate (HS), heparin, and keratan sulphate (KS). Each proteoglycan has a trivial name that refers either to biological activity (e.g. decorin, which binds to and decorates the surface of collagen fibrils; Fleischmajer et al., 1991a) or to sequence (e.g. serglycin, which contains a large alternating sequence of serine and glycine residues; Avraham et al., 1989). Proteoglycans may either be secreted and deposited in the ECM (e.g. aggrecan, versican, biglycan, decorin, fibromodulin; Esko, 1991), anchored to the plasma membrane (syndecan, betaglycan; Esko, 1991) or located intracellularly in secretory granules (e.g. serglycin; Avraham et al., 1989).

By amino acid sequence analysis, potentially important domains have been identified within core proteins from several PGs (Kjellen & Lindahl, 1991). Among the few PGs of known sequence, the large aggregating PGs of cartilage contain an HA binding domain and a lectin domain capable of binding simple sugars (Halberg et al., 1988). The large fibroblast PG versican (Zimmerman & Ruoslahti, 1989) contains domains similar to those of the cartilage PG, suggesting that both PGs are capable of the same interactions. The small PGs biglycan (chondroitin sulphate proteoglycan; also called PG-1) and decorin (dermatan sulphate proteoglycan; also called PG-II, DSPG-II and PG-S2) are also closely related through their amino acid sequence (Choi et al., 1989). As more sequence data becomes available, more detailed comparisons may be made. At present evidence exists that the cell surface PG syndecan represents a family of related PGs (Bernfield & Sanderson, 1990; Brauker et al., 1991) with high homology in their cytoplasmic amino terminal domains and variable extracellular domains. The specific GAG chains which are attached to the core proteins determine many biological activities of PGs. The binding of GAGs to various ECM molecules, cell-cell adhesion molecules and growth factors appears to be charge dependent because the strength of binding is largely determined by the degree of sulphation of the GAG (Jackson et al., 1991). For example, the more highly sulphated the GAG, the
better it will bind to fibronectin. Thus heparin binds most strongly, while HS, DS and CS bind less well (Klebe & Mock, 1982). The core protein appears to regulate the effects of GAGs by primarily acting as a scaffold for the immobilisation and appropriate spacing of GAG chains. For example, serglycin, produced by rat yolk sac tumor cells, contains closely spaced CS-chains (Bourdon et al., 1986) and inhibits the attachment of these cells to fibronectin (Brennan et al., 1983). It has been proposed that binding of the CS-chains to fibronectin sterically hinders the interaction of the protein with its cell-surface receptor (Hautanen et al., 1989). The effect is only seen with the intact PG but not with free CS-chains.

As well as their mechanical support functions, PGs are involved in highly diverse biological roles, including cell-cell and cell-matrix adhesion and regulation of cell growth (Kjellen & Lindahl, 1991). By virtue of their interactions with ECM molecules (e.g. collagens, fibronectin, thrombospondin, vironectin, laminin; Kjellen & Lindahl, 1991), cell adhesion is mediated in part through cell surface PGs (Akiyama et al., 1990; Esko, 1991). For example the membrane bound PG syndecan (Bernfield & Sanderson, 1990) can bind collagen and fibronectin through its HS chains and mediate cell attachment. It has been proposed that cell adhesion to the ECM through PGs is an auxiliary mechanism that complements integrin mediated adhesion (LeBaron et al., 1988). Soluble PGs can inhibit cell adhesion to fibronectin and collagen (Brennan et al., 1983; Rosenberg et al., 1986), and they appear to do this by preventing the binding of an integrin to the cell attachment site by steric hindrance (Hautanen et al., 1989). Several observations also indicate that PGs influence cell growth (Ruoslhti, 1989). For example, Yamaguchi and Ruoslhti (1988) found that expression of human decorin in Chinese hamster ovary (CHO) cells led to a decreased growth rate and altered morphology of the cells. It was further demonstrated that decorin exerted these effects by sequestering transforming growth factor (TGF)-β (Yamaguchi et al., 1990), a growth stimulatory factor for CHO cells.
1.3.3 Glycoproteins

Several multidomain ECM glycoproteins have been characterised. These include fibronectin, laminin, vitronectin, thrombospondin, tenascin and nidogen (entactin). For a brief overview on each of these molecules see Yamada (1991a). As far as their biological role is concerned, several of them have been shown to play a crucial role in cell-matrix interactions (Akiyama et al., 1990; Sage & Bornstein, 1991). Fibronectin is the most well understood example. This molecule helps mediate cell adhesion, embryonic cell migration and wound healing (Labat-Robert et al., 1990). Fibronectin was the first protein shown to interact with specific cell membrane receptors (integrins) through a arg-gly-asp or R-G-D sequence of amino acids. Recent studies on ECM glycoproteins, reveal that many contain common features. For example the molecules contain specialised polypeptide domains involved in binding to specific cell surface receptors as well as to other matrix constituents, such as collagen and heparin/heparan sulphate. Major parts of these molecules are also comprised of fibronectin or EGF like repeats (Albelda & Buck, 1990; Elices et al., 1991; Engel, 1991). Various forms of fibronectin (Schwarzbauer, 1991), laminin (Mercurio, 1990) and tenascin (Nies et al., 1991) are generated by gene duplication or alternative splicing to produce families of closely related proteins which have been shown to be developmentally regulated. A considerable amount of complexity exists between these cell interactive glycoproteins, and in their potential cell-matrix/matrix-matrix interactions. However it is important to remember that a cell often interacts with a complex mixture of matrix constituents, each of which may contribute in regulating cell behavior.

1.3.4 Sulphated Proteins

Tyrosine sulphation is a widespread post-translational modification in ECM proteins, e.g. nidogen/entactin, fibronecin, dermatan sulphate proteoglycan (Huttner, 1988), procollagens III and V (Jukkola et al., 1990), bone sialoprotein II (Ecarot-ChARRIER et al., 1989; Midura et al., 1990) and fibromodulin (Antonsson et al., 1991).
The site of tyrosine sulphation in fibromodulin is an N-terminal, tyrosine-rich region in which tyrosine is every second or third residue (Antonsson et al., 1991). In types III and V procollagen, the anion is also located at the N-terminus and is the reason for the low isoelectric point of these proteins (Jukkola et al., 1990). Tyrosine sulphation occurs in the trans Golgi and is one of the last processing steps before proteins exit the Golgi complex. The reaction is catalysed by tyrosyl-sulpho-transferase which recognises specific tyrosine residues in exposed protein domains adjacent to acidic amino acids (Huttner, 1984; Hortin et al., 1986). The modification appears to be irreversible and therefore results in a permanent alteration in the structure of the protein (Huttner, 1988; Jukkola et al., 1990). Most tyrosine sulphated proteins are secretory (Antonsson et al., 1991). The role of tyrosine sulphation in ECM proteins has not been extensively investigated, though desulphation of fibronectin affects its binding affinities to fibrin, gelatin and heparin (Suiko & Liu, 1988). Functional studies should be facilitated by the use of chlorate, which is a potent non toxic inhibitor of sulphation in intact cells (Jukkola et al., 1990).

1.4 Cell/Matrix Interactions

Although many ECM proteins such as collagen and elastin contain inherent self assembly properties which foster their deposition and supramolecular assembly, cells ultimately dictate the composition and location of regional matrices. Cellular interactions with basement membranes and the ECM are vital in a number of biological processes which require specific cellular adhesion and migration of cells including embryonic development, tumour cell metastasis, wound healing and the maintenance of tissue integrity. However the interaction of a cell with its environment must be a dynamic one, since cells which ordinarily remain attached in vivo must be capable of diminishing the adhesive force to enable rounding and division, and cell movement also requires that interactions with the matrix are constantly being made and broken (Akiyama et al., 1990; Sage & Bornstein, 1991).
The major adhesive molecules for epithelial and mesenchymal cells include fibronectin, collagens I, II, III, IV, V and XI, laminin, vitronectin and nidogen/entactin, although it is recognised that some of these molecules bind via indirect linkages. For example laminin has been shown to bind heparan sulphate proteoglycan, promote cell attachment and bind to collagen IV generally via nidogen (Beck et al., 1990; Fox et al., 1991). Recent advances in the molecular basis of these interactions have converged on the concept that many cell interactions are dependent on specific adhesive ligand recognition sequences (Yamada, 1991b).

The complex interactions that occur between cell and cell and cell and matrix are mediated through several different families of receptor, which include peripheral and integral membrane glycoproteins and proteoglycans (Albelda & Buck, 1990). The integrins in particular play a pivotal role in mediating the adhesion of cells to fibronectin, collagen and laminin by recognising different specific peptide sequences in each (Yamada, 1991b). The observation that integrins are seen at regions of cell-to-cell contact further suggests that they also may be important in cell-cell interactions as well as cell-matrix interactions (Albelda & Buck, 1990).

Integrins (reviewed by Ruoslahti, 1991a; 1991b) are a family of transmembrane \( \alpha-\beta \) heterodimeric glycoproteins, which have been divided into three major subfamilies each containing a common \( \beta \)-subunit non-covalently associated with multiple \( \alpha \)-subunits. Because integrins with the same \( \beta \)-subunit but with a different \( \alpha \)-subunit often bind different ligands, it was suggested that the \( \alpha \)-domain dictates ligand specificity. However Cheresh et al. (1989) have shown that the association of a vitronectin receptor (which normally doesn’t bind fibronectin) \( \alpha \)-subunit, with a \( \beta \)-subunit expressed by carcinoma cells results in acquisition of fibronectin-binding activity. This implies that the ligand-binding site requires both subunits (Cheresh et al., 1989). The organisation of the integrins is complicated by the finding that \( \alpha \)-subunits may be associated with more than one \( \beta \)-subunit. Currently, at least 15 different \( \alpha \beta \) subunit combinations exist (Elices et al., 1991). Both subunits contain a large amino-
terminal extracellular domain, a single hydrophobic transmembrane sequence and short carboxy-terminal cytoplasmic domains. The interaction between integrins and cytoskeletal actin filaments has been shown to be via an indirect linkage involving talin and vinculin (Burridge et al., 1988) and requires the cytoplasmic domain of the β-subunit (Albelda & Buck, 1990). Ligand binding occurs through the amino domain and is a cation-dependent process involving the recognition of specific amino acid sequences within the ligand. The RGD sequence, originally described for fibronectin but found in a number of other matrix proteins which includes collagen, thrombospondin, vitronectin and laminin is the best described (Albelda & Buck, 1990). A single adhesive protein can also contain several different sequences, which are recognised by the same receptor or by other distinct receptors, and provides a single cell with a large repertoire of possible interactions. The regulation of integrins is still unclear, although existing data indicates that binding specificity and activity may be controlled at various stages, such as RNA splicing, post-translational modifications, calcium concentrations and by the membrane-lipid environment (Kirchhofer et al., 1991).

Integrins may play a central role in development by transmitting and transducing mechanical signals into a biochemical response, although other transmembrane receptors (e.g. syndecan, cadherin) may also mediate force transmission. During wound healing, fibroblasts produce wound contraction by exerting tension on their ECM adhesions. Control of wound reorganisation appears to depend on whether or not the α5β1 integrin receptors are expressed on the cell surface (Ingber, 1991). Cell-generated tension is transmitted over fibronectin-containing microfibrils (ligands for α5β1 integrins) which act as cables linking fibroblasts to each other and to the collageneous matrix, thus determining the spatial orientation and morphology of connective-tissue components. Hence integrins may function as mechanoreceptors, providing a gating function for signal transduction by either supporting or prohibiting force transmission between ECM and the cytoskeleton (Ingber, 1991).
Besides their importance in cellular processes, integrins are involved in platelet aggregation and immune functions. Some diseases are already known to be caused by mutation in integrin genes (Ruoslahti, 1991a). Knowledge of the targeted RGD amino acid sequences for many integrins can be exploited to design compounds controlling cell adhesion for therapeutic purposes. For example in the hereditary disorder Glanzmann's thrombasthenia, there is a deficiency in the group IIb/IIIa integrin receptor. Platelets from such individuals fail to aggregate in response to activation. Group IIb/IIIa is therefore an attractive target for therapeutic manipulation of platelet aggregation. RGD peptides that neutralise the activity of group IIb/IIIa provide a possible inhibition of platelet aggregation (Ruoslahti, 1991a). Highly active RGD peptides also exist in nature. Proteins called disintegrins are found in certain snake venoms that have an active RGD sequence in a highly conserved disulphide loop. Disintegrins inhibit group IIb/IIIa and other integrins and are potent inhibitors of platelet aggregation. However because of their apparent lack of specificity, these molecules seem to be less suitable as therapeutic agents than synthetic peptides (Gould et al., 1990).

In addition to the integrins, other receptors for the ECM components exist (reviewed in McDonald, 1989; Albelda & Buck, 1990). The mechanisms by which cells coordinate the activity of these various receptors is unknown, however a huge diversity in receptor functions exists and provides cells with varied capabilities to recognise adhesive substrates.

Recently, evidence has been accumulating that the inhibition of cell attachment and spreading may be equally important for tissue morphogenesis and development. Several anti-adhesive proteins have been described which include SPARC (Secreted Protein Acidic and Rich in Cysteine) and the large disulphide linked glycoprotein tenascin, although the biological importance of these molecules still remains to be fully realised (Chiquet-Ehrismann, 1991).
1.5 Collagen I Biosynthesis

Collagen synthesis is a complex process involving the regulated expression of members of the large collagen gene superfamily as well as many co-translational and post-translational modifications (Table 1.2) catalysed by at least nine specific enzymes and several non-specific ones. This section, unless stated otherwise, deals only with collagen I and is intended as a brief overview. For a more detailed treatment of this subject, the reader is referred to Olsen (1991) and references therein.

1.5.1 Synthesis of Preprocollagen

At least 25 genes code for the constituent chains of the various collagen types (for reviews on collagen gene structure and expression, see Raghow & Thompson, 1989; Byers, 1990; Ramirez et al., 1990; Vuorio & de Crombrugghe, 1990; de Crombrugghe et al., 1991). A minimum of nine distinct genes code for the fibrillar collagens, the genes being dispersed in the human genome, the exception being α1(III) and α2(V) which are co-localised on chromosome 2. The genes are large (15-50 kb) with complex exon structures (>50 exons). The exons must be appropriately transcribed and spliced to produce a mature mRNA. The overall organisation of the fibrillar collagen genes is highly similar. A small but variable number of exons (<10) codes for the signal peptide and N-propeptide. The C-propeptide is encoded by 4 exons and the major triple helix by around 44 exons. The beginning and end of the triple helix are coded for by so-called joining exons, which also code for telopeptide and propeptide regions. All the triple helical exons start with a complete codon for Gly, and end with a complete codon for the amino acid in the Yaa position, thus the exons are a multiple of 9 bp encoding a single collagen triplet. Because the majority of the triple helical exons are 54 bp (or multiples of 54), it was proposed that the fibrillar collagen genes evolved through duplication, recombination and point mutations in a primordial collagen gene. The introns show no such conservation in length, and vary between 86 and 2000 bp in humans. Invertebrate collagens show a different gene organisation (Vuorio & de
Table 1.2. Co-translational and post-translational modifications in the synthesis of collagen I

<table>
<thead>
<tr>
<th>Biosynthetic Modification</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intracellular</strong></td>
<td></td>
</tr>
<tr>
<td>Removal of signal peptide</td>
<td>Membrane translocation</td>
</tr>
<tr>
<td>Proline 3-hydroxylation</td>
<td>Unknown</td>
</tr>
<tr>
<td>Proline 4-hydroxylation</td>
<td>Stable triple helix</td>
</tr>
<tr>
<td>Lysine hydroxylation</td>
<td>Cross link formation; O-glycosylation of hydroxylysine residues</td>
</tr>
<tr>
<td>O-glycosylation of hydroxylysine residues</td>
<td>Possible role in fibrillogenesis</td>
</tr>
<tr>
<td>N-glycosylation of asparagine residues</td>
<td>Not known</td>
</tr>
<tr>
<td>Disulphide bonding and chain association</td>
<td>Triple helix formation</td>
</tr>
<tr>
<td>Triple helix formation</td>
<td>Functional protein</td>
</tr>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
</tr>
<tr>
<td>Removal of N-propeptide</td>
<td>Normal fibril morphology</td>
</tr>
<tr>
<td>Removal of C-propeptide</td>
<td>Fibril assembly</td>
</tr>
<tr>
<td>Ordered aggregation</td>
<td>Fibril formation</td>
</tr>
<tr>
<td>Cross linking</td>
<td>Formation of stable fibrils</td>
</tr>
</tbody>
</table>

It should be remembered that in the other collagens not all the extracellular modifications occur, and that other collagens may undergo additional modifications, for example sulphation at tyrosine residues in procollagens III and V. (Adapted from Olsen, 1991).
Regulation of synthesis can be exerted through control of mRNA levels by regulation of gene transcription and RNA maturation or the stability of mRNA. In collagen there is a good correlation between the rate of synthesis and the level of mRNA. Therefore regulation of collagen production appears to be influenced at the transcriptional level rather than by control of mRNA translation. There are two broad categories of gene regulation. The first is in response to demands in the developmental program and leads to unique tissue-specific collagen types. For example collagen X is transiently expressed in the zone of calcification in hypertrophic cartilage. The second category of regulation involves the modulation of collagen genes by biological and pharmacological agents. For example cell-cell interactions, cytokines and growth factors all profoundly influence collagen biosynthesis. Most of the attention to regulatory elements have been focussed on the 5' end of the gene, and several DNA binding proteins have been found which exert positive and negative effects on transcription. For collagens I and II there is evidence that sequences located in the first intron are important in transcriptional regulation.

The α1 and α2 chains of procollagen I are coordinated products of the COL1A1 and COL1A2 genes located on chromosomes 17 and 7 respectively. There is a large difference in size between the 2 genes, but the relative size and location of the exons are similar. Collagen polypeptides are translated from mature mRNA on membrane bound polysomes. The procollagen is synthesised as a high molecular weight precursor called preprocollagen (Peltonen et al., 1985). The preprocollagen contains a short amino terminal hydrophobic signal sequence typical of secretory proteins, which is rapidly processed by a membrane-bound signal peptidase activity as the molecule enters the lumen of the ER. The signal peptide serves as a vector to ensure translocation across the RER membrane, which occurs via interactions with cytoplasmic and membrane bound receptors (Palmiter et al., 1979; Walter & Lingappa, 1986; Olsen, 1991). During and shortly after chain elongation and translocation into
the RER several lysine residues and virtually all prolyl residues in the Yaa position of the triple helix are hydroxylated.

1.5.2 Co-translational and Post-translational Modification of Procollagen

1.5.2.1 Hydroxylation of Proline and Lysine Residues

Three enzymes belonging to a group called the 2-oxoglutarate dioxygenases are responsible for the hydroxylating activity associated with the ER (Table 1.2). Two of the enzymes convert some prolyl residues to 4-hydroxyproline or 3-hydroxyproline, and the third enzyme converts some lysyl residues to 5-hydroxylysine (Olsen, 1991). Their reactions require ferrous iron, 2-oxoglutarate, O₂ and ascorbate.

\[
\text{Peptide-bound proline (lysine) + O}_2 + 2\text{-oxoglutarate} \quad \text{Fe}^{2+} + \text{ascorbate} \\
\downarrow \\
\text{Peptide-bound hydroxyproline (hydroxylysine) + CO}_2 + \text{succinate}
\]

The 2-oxoglutarate is stoichiometrically decarboxylated during hydroxylation to form succinate. Ascorbate is a highly specific requirement and appears to be necessary to reduce \( \text{Fe}^{3+} \) (formed during the reaction) to \( \text{Fe}^{2+} \) (Olsen, 1991; Peterkofsky, 1991). In ascorbate deficiency (scurvy), the chains of fibrillar procollagens are underhydroxylated, and do not form stable triple helices at 37 °C. This leads to intracellular degradation of unfolded chains or delayed secretion of non functional protein. Ascorbate also acts at the level of transcription, specifically increasing mRNA levels (Chan et al., 1990; Houglum et al., 1991). All three hydroxylases are sequence specific and require a peptide bound substrate in a random coil conformation.

Prolyl 4-hydroxylase converts prolyl residues in Gly-Xaa-Pro sequences to 4-hydroxyproline. Under physiological conditions the formation of a stable triple helix
require about 100 hydroxyprolines per chain. Therefore nucleated growth of the triple helix is delayed until the chain acquires the necessary content of hydroxyproline. The delay ensures that nucleation and propagation of the triple helix occurs in an orderly manner (Prockop, 1990; Kuivaniemi et al., 1991). The active prolyl 4-hydroxylase is an \( \alpha_2\beta_2 \) tetramer of 240 K, where \( \alpha \) and \( \beta \) are two types of subunit (both glycoproteins) of around 60 K. A proportion (30-99 \%) of the enzyme remains inactive, and the ratio of active to inactive enzyme is proportional to the rate of collagen synthesis. The conversion of inactive to the active form may be important in regulating synthesis. The enzyme also has an associated protein-disulphide-isomerase activity (Olsen, 1991; Pihlajaniemi et al., 1991). Prolyl 3-hydroxylase hydroxylates prolyl residues at the Xaa position in Gly-Xaa-Hyp sequences to 3-hydroxyproline. Very little 3-hydroxyproline is found in collagen and its physiological role is not known.

Lysyl 5-hydroxylase is a dimer composed of two 90 K subunits, the enzyme hydroxylates lysyl residues at the Yaa position in Gly-Xaa-Yaa sequences to 5-hydroxylysine (Olsen, 1991; Jiang & Ananthanarayanan, 1991). Around 5-10 hydroxylysines are produced in each chain, some of which are required for the glycosyl transferase enzymatic addition of O-linked carbohydrate units to the \( \varepsilon \)-hydroxyl groups on the lysine side chain. However within the telopeptide there is an exception to the sequence specificity, which may suggest that a different enzyme is required. The hydroxylysines synthesised within the telopeptides are of particular importance because they are involved in intra- and inter-molecular cross-links which stabilise the assembly of the triple helical monomers in collagen fibrils. Recent data has suggested that the conformational requirement for lysyl hydroxylation in collagen is the presence of a “bent” structure, such as a \( \beta \)-turn with lysine in the second position of the turn, and that the presence of a polyproline-II type structure aids the effective binding of the substrate at the enzymes active site (Jiang & Ananthanarayanan, 1991).

All modifications cease as soon as the protein folds into a triple helix as the post-translational enzymes cannot interact with the triple helical protein. In effect, the degree
of protein modification is regulated by its folding into a triple helical conformation (Kuivaniemi et al., 1991; Torre-Blanco et al., 1992).

1.5.2.2 Glycosylation of Asparaginyl and Hydroxylsyl Residues

O-glycosylation of collagen occurs by the manganese-dependent enzymatic addition of galactose or of glucose and galactose utilising UDP-sugars as substrate. The two enzymes responsible, galactosyl-hydroxylsyltransferase and glucosylgalactosyl-hydroxylsyltransferase, modify peptide bound hydroxylsines, which are in non-helical conformation. The modification lags behind chain synthesis by about 200-300 residues (Prockop & Kivirikko, 1984; Byers, 1990).

The biological function of O-glycosylation is not known, it is clear however that it is not essential for secretion of the molecule. Two possible roles have been proposed, one involved in directing molecular assembly (Rogozinski et al., 1983) and the second in modulating cross-link formation (Robins & Bailey, 1974). Amudeswari et al. (1987) showed that the kinetics of in vitro fibril assembly decreased after glycosylation and increased after deglycosylation. A mechanism was proposed in which the sugar chains have to be redirected axially before fibril formation could proceed. It was also suggested that the glycosylation may have some influence on fibril structure; specifically it has been suggested that an increase in glycosylated hydroxylsine residues will decrease the diameters of in vitro formed fibrils because of steric effects. The extent of glycosylation may also effect collagen/matrix interactions as well as interactions with the cell surface, but these remain to be critically tested (Kagan, 1986; Torre-Blanco et al., 1992).

The C-propeptide contains an asparagine linked oligosaccharide. The biosynthesis of asparaginyl N-linked units occurs in the RER by transfer of a mannose-rich oligosaccharide side chain to an acceptor site containing the sequence Asn-Xaa-Ser/Thr. The transfer has been shown to involve a lipid carrier (Kivirikko & Myllyla, 1982; Olsen, 1991).
1.5.2.3 Disulphide Bonding and Triple Helix Formation

Both propeptides of procollagen I contain intramolecular disulphide bonds, intermolecular disulphides are present only in the C-propeptide. The stabilisation of the C-propeptide by disulphides is required for efficient folding of the triple helix (Koivu & Myllyla, 1987). Protein disulphide isomerase plays a key role in catalysing disulphide exchange in the molecular assembly of collagen. Remarkably, protein disulphide isomerase is identical to the β subunit of prolyl 4-hydroxylase (Freedman et al., 1989). Disulphide bonding accompanies chain registration (the association of pro α-chains in the correct stoichiometry) and precedes helix formation (Hulmes, 1992). In support of this is the finding that chain association and interchain disulphide bonding are processes that can proceed in the absence of prolyl hydroxylation and triple helix formation.

Procollagen assembly differs from the folding of any other protein and begins with the association of the C-propeptide domains (Kuivaniemi et al., 1991). For the helix to extend the full length of the α-chain it is important that the molecules are in register, in the sense that the Gly-Xaa-Yaa tripeptide unit in one chain is hydrogen-bonded to the corresponding unit in the other chains. Primary associations through the C-propeptide provides the correct registration. The folding of the helix then occurs through a zipper-like mechanism of nucleated growth in which a nucleus of triple helix is formed at the C-terminus which is then propagated to the N-terminus (Engel & Prockop, 1991). Because pro α-chains contain a large number of sterically symmetrical gly-pro and pro-hypro bonds, spontaneous isomerisation from the trans to cis configuration is a frequent occurrence. Therefore chain folding occurs at a rate determined by cis/trans isomerisation which probably requires catalysis by a specific isomerase (Engel & Prockop, 1991).

Several mutations that substitute glycine residues for amino acids with bulkier side chains have been defined. These mutations distort the conformation of the helix and delay its folding N-terminal to the site of the mutation. Because the hydroxylases and
glycosyl transferases are only active on random coil collagen substrates, a delay in folding leads to over-modification of the pro α-chains, as observed in a number of heritable disorders (Byers, 1990; Kuivaniemi et al., 1991; Tsuneyoshi et al., 1991).

1.5.3 Intracellular Transport and Secretion of Procollagen

Once the procollagen molecules are completely folded they are transported from the RER through the Golgi system and then to the cell surface where they are secreted (Marchi & Leblond, 1984). Secretion is reduced ten fold by α, α'-dipyridyl, an inhibitor of hydroxylation, therefore helix formation is required for secretion. Prior to secretion, procollagen is concentrated in secretory vesicles where, unlike the D-staggered assemblies that occur in collagen fibrils, procollagen molecules align in register (Hulmes, 1992). Environmental factors strongly influence the form of procollagen assembly since high concentrations of purified procollagen form D-staggered arrays in vitro. Both propeptides appear to be located at the surface of the assembly (Mould et al., 1990).

1.5.4 Proteolytic Processing of Procollagen

Following secretion of procollagen molecules into the extracellular matrix, both the N and C-propeptides must be removed, for the collagen molecules to form stable fibrils with normal morphology. Three distinct enzymes have been described so far that are responsible for processing procollagens I-III. The enzymes are procollagen N-proteinases (removal of N-propeptides) and procollagen C-proteinase (removal of C-propeptide). The incorporation of collagens I and III into fibrils requires a different N-proteinase for each type. The need for two different N-proteinases allows for independent control of processing, and assembly in tissues containing both types. (Peltonen et al., 1985; Hulmes, 1992)

All the processing enzymes described so far are sequence-specific, neutral, calcium-dependent metalloproteinases. Procollagen I/II N-proteinase is a high
molecular weight (500 K) complex. The complex consists of four subunits, ranging in molecular weight between 60 K-160 K, where the two largest subunits probably possess the catalytic activity (Hojima et al., 1989). Unlike C-proteinase, the enzyme has a specific requirement for the native conformation of the cleavage site which apparently consists of a hairpin loop in each of the three pro α-chains (Torre-Blanco et al., 1992). Further to this, it has been shown that the absence of the 18 amino acid residues that surround and include the N-proteinase cleavage site in the pro α2(I) chain drastically slows the rate of cleavage of the pro α1(I) chains (Watson et al., 1992). These observations demonstrate that cleavage of the pro α1(I) chains by procollagen N-proteinase occurs at a maximum rate only when the procollagen molecule contains three pro α-chains and when the cleavage sites have the correct spatial organisation. When N-terminal processing is defective, fibrils with an altered morphology are often observed. Defects may arise from reduced proteinase activity (e.g. dermatospororaxis) or mutations which alter the N-proteinase cleavage site (e.g. Ehlers-Danlos syndrome type VII), rendering the mutant procollagen resistant to cleavage (Watson et al., 1992). Because the collagen triple helix folds in a C-N terminal direction any in-frame deletion anywhere in the helix is transmitted to the N-propeptide (Dombrowski et al., 1989). As well as impaired fibril formation, there may also be a disturbance in the suggested feedback regulation of collagen synthesis by the free N-propeptides which may affect the quantity or quality of the secreted molecules. Procollagen C-proteinase is a monomer of 90 K, enzymic activity has been shown to be enhanced by a 34-55 K connective tissue glycoprotein (Kessler et al., 1990).

The pathway of procollagen processing (i.e. via pN-collagen, procollagen lacking the C-propeptides; or pC-collagen, procollagen lacking the N-propeptides) appears to vary with collagen type (Fessler et al., 1981) and stage in development (Mellor et al., 1991). Processing intermediates can persist in normal tissue (Veis et al., 1973), and immunoelectron microscopy has revealed an association of N-propeptides and C-propeptides with small and large diameter fibrils, respectively (Fleischmajer et al.,
1987). By *in vitro* reconstitution experiments, it was demonstrated that persistence of the N-propeptide distorts fibril morphology from normal cylindrical to fluted and sheet-like structures of thickness 8 nm (Hulmes *et al.*, 1989a; Holmes *et al.*, 1991), some of which resembled abnormal fibrils seen *in vivo*. For example, structures were observed which resembled the “spider-like” fibrils seen in dermatosporotic animals. Holmes *et al.* (1991) demonstrated that the N-propeptides are found at the surface of the growing assembly in a “folded-back” conformation, and may block further growth by preventing the side-by-side association of collagen molecules. It has since been demonstrated that treatment of hieroglyphic fibrils, that are copolymers of collagen and abnormal pN-collagen (pN-collagen$^{\text{ex6}}$, pN-collagen that lacks the 18-amino acid residues encoded by exon 6 of the COL1A2 gene; Watson *et al.*, 1992) with N-proteinase results in partial cleavage of the pN-collagen$^{\text{ex6}}$ in which the abnormal pNα2(I) chains remain intact (Watson *et al.*, 1992). The further observation that the hieroglyphic fibrils were resolved to cylinders supports the view that there is considerable fluidity between collagen molecules within a collagen fibril (Chapman, 1989). That the relative proportions of collagen to pN-collagen$^{\text{ex6}}$ did not change following cleavage of the pNα1(I) chains by N-proteinase and that partially cleaved pN-collagen$^{\text{ex6}}$ molecules were present in large diameter fibrils suggest that some of the abnormal molecules must be present within the body of the cylindrical fibril. It seems that the partially cleaved N-propeptides adopt a more extended conformation that permits their incorporation into fibrils (Kadler, personal communication).

1.5.5 Ordered Aggregation and Collagen Cross-Link Formation

Procollagen I is relatively soluble and is found in the medium of cultured fibroblasts as monomers with solubilities of 1 mg/ml or more (Kadler *et al.*, 1990). The high solubility of procollagen appears to be conferred mainly by the C-propeptide domain, since pN-collagen, produced by cleaving the C-propeptide, has a solubility of approximately 0.15 mg/ml (Mould *et al.*, 1990). The solubility of the protein is
decreased around 2000 fold following processing of the propeptides to generate collagen. Collagen that leaves solution spontaneously polymerises into highly organised fibrils. Fibrillogenesis therefore appears to be largely controlled by the proteolytic cleavage of the propeptides (Prockop, 1990; Kuivaniemi et al., 1991). Fibrillogenesis in vivo is much more complex and is highly regulated by a variety of factors. Fibril assembly is discussed in section 1.8.1.

1.5.5.1 Fibril Structure

Much of today's understanding of collagen fibril structure has come from both electron microscopy (Chapman & Hulmes, 1984) and X-ray diffraction studies (Brodsky et al., 1988). Tendon collagen (predominantly type I), occurs in a highly ordered form in rat tail, and this has proven to be an ideal material for study by X-ray diffraction techniques.

1.5.5.1.1 Axial Structure

Collagen axial structure refers to the molecular structure of the fibril projected onto the fibril axis. Refinements made with respect to staining techniques for electron microscopy together with X-ray diffraction data culminated in the Hodge-Petruska model for the one dimensional arrangement of collagen molecules (Hodge & Petruska, 1963; Fig. 1.3). The collagen fibrils show a D periodicity along their length, this periodicity results from collagen molecules which are staggered with respect to each other by 67 nm (Parry, 1988). However the length of the collagen molecule (300 nm) is not an integral multiple of the D period and it follows that the fibril structure will have alternating regions of dense molecular packing (the overlap regions of length 0.47 D) and less dense molecular packing (the gap regions of length 0.53 D; Fig. 1.3). The 67 nm repeat produces a dramatic diffraction pattern when tendons are placed in a narrow X-ray beam. The intensities of the meridional reflections derive from the axial electron density distribution within the D period. The axial structure is reflected by the
Figure 1.3 One-dimensional molecular packing in collagen fibrils
Diagramatic representation of a negatively stained collagen fibril (a). The pattern of
alternating light and dark bands reflects the axial packing of collagen molecules (300
nm long) shown in (b), where the dark bands correspond to the heavy metal stain
entering the gaps between the molecular ends. The molecules in (b) are staggered by 67
nm with respect to each other. Each collagen molecule (c) may be regarded as
composed of five segments (labelled 1-5) each of length 67 nm except for segment 5
which is 0.47 x 67 nm long. The same numbering of the segments is used in Fig.1.4
(adapted from Miller, 1982).
distribution of amino acids along the length of the collagen molecule. These amino acids form a pattern which repeats every 67 nm (or 234 ± 1 residues). Oppositely charged amino acids tend to occur close enough to each other to form ion pairs and hydrophobic interactions are maximised. Hence, when one collagen molecule is displaced by 67 nm with respect to another, a strong interaction can be expected between them (Hulmes et al., 1973; Miller, 1982; Jones & Miller, 1991). The one dimensional model for the packing of collagen molecules has been confirmed at low resolution (5 nm) from the pattern of bands in positively stained collagen fibrils and at higher resolution (1.6 nm) by a correspondence between the observed X-ray meridional reflection intensities and the predicted intensities calculated from the amino acid sequence (Hulmes et al., 1977).

1.5.5.1.2 Lateral structure

Evidence comes mainly from electron microscopy and medium angle X-ray diffraction for a well defined microfibrillar substructure in native collagen fibrils. In the original microfibril model proposed by Smith (1968), the structural unit of packing was a group of five molecules closely packed with helical symmetry. These discrete structures (microfibrils) are D-periodic with approximate diameter 3.8 nm, and were thought to be packed together in a tetragonal, three-dimensional lattice. Although consistent with most of the EM data and some of the diffraction data, the model is not proven. The structure proposed by Hulmes and Miller (1979) based on a reinterpretation of X-ray data from rat tail tendon is consistent with quasi-hexagonal molecular packing of individual collagen molecules (Fig. 1.4). The diffraction patterns of rat tail tendon show a series of sharp equatorial or near equatorial Bragg reflections which are derived from the lateral arrangement of molecules in the direction perpendicular to the fibril axis. These reflections represent a discrete sampling of an ordered lattice which demonstrates that the collagen I fibril is a true three dimensional crystal with lateral as well as axial order (Brodsky et al., 1988). The parameters of the
Figure 1.4 Quasi-hexagonal lateral arrangement of molecules in the collagen fibril
This represents a transverse section through a fibril, and shows the lateral arrangement of collagen molecules in the structure proposed by Hulmes and Miller (1979). The numbers refer to the five segments (labelled 1-5 as in Fig. 1.3) of the collagen molecule. The crystallographic unit cell ($a = 3.9$ nm, $b = 2.67$ nm, $\gamma = 104.6^\circ$) is shown by broken lines (adapted from Miller, 1982).
triclinic unit cell which describes this structure is interpreted in terms of the quasi-hexagonal arrangement of collagen molecules shown in Figure 1.4. The fanning of the near equatorial reflections in the diffraction pattern is consistent with a straight molecular tilting of around 4° when viewed perpendicular to the fibril axis (Hulmes & Miller, 1979). The absence of reflections at high angles attributable to the molecular segments within the gap region led to the suggestion that these segments were more mobile than those in the overlap region (Fraser & Trus, 1986; Fraser et al., 1987). It has been proposed that the gap region may be required for lysyl oxidase to diffuse into the interstices of fibrils and initiate cross-link formation (Linsenmeyer, 1991). A network of aromatic residues in the telopeptide may also be vital in stabilising the correct molecular orientations in preparation for cross-linking (Jones & Miller, 1991).

Crystalline molecular packing has also been shown for collagen II in the fibrils of lamprey notochord sheath (Eikenberry et al., 1984). However the quasi-hexagonal triclinic unit cell is not consistent with that for collagen II (Eikenberry et al., 1984), as the equatorial x-ray diffraction pattern for notochord sheath appears to indicate that the spacing between planes of molecules are larger than those in rat tail tendon. X-ray diffraction patterns of bone and skin show only a single broad equatorial maximum, which indicates a low degree of order in these tissues. In crystalline fibrils, some diffuse equatorial scattering is also apparent and constitutes a background to the discrete reflections. This diffuse scatter may indicate lateral disorder which may be static (i.e. due to the presence of a number of fixed conformations) or dynamic (i.e. due to the motion of molecules). Experimental results from nuclear magnetic resonance spectroscopy (Torchia, 1982; Sarkar et al., 1983) indicate that fluidity of contact exists between adjacent collagen I molecules, and implies that molecules in fibrils continually reorientate about their long axes.

Although the quasi-hexagonal model is consistent with measurements of fibril density and accounts for most of the diffraction data, it is in conflict with other models since it does not provide for an ordered intermediate substructure (Piez & Trus, 1981).
As a compromise, the compressed microfibril model was proposed (Piez & Trus, 1981). The idea was to compress a 5-stranded microfibril and place it in the quasi-hexagonal unit cell, the molecules of the compressed microfibril having a left handed supercoil of pitch 400-700 residues. Recently a new structure has been proposed which also has a microfibrillar level of organisation, with the molecules in the microfibrils packed in a slightly distorted hexagonal lattice (Kajava, 1991). In this model several closely packed molecules form a bunch which are linked “head to tail” and penetrate each other a distance of 30 nm forming a microfibril. These microfibrils then associate together such that different zones with different densities appear within the fibril which acquires axial regularity with a 67 nm repeat (Kajava, 1991). However the model does not account for the biochemical data on the position of several collagen cross-link sites, which presupposes a 4D stagger configuration between individual molecules (Reiser et al., 1992). Kajava’s model also predicts reflections on the X-ray diffraction map, which show a poor correspondence with the observed X-ray pattern (Wess, personal communication).

Tissues such as skin and aorta which contain collagens I and III have a slightly shorter (65 nm) D-period, this smaller axial periodicity has been shown to be directly related to the tilt of the molecules. The measured mean tilt angle of 9° causes an axial contraction, which results in twisting of the collagen fibril (Folkhard et al., 1987). Raspanti et al. (1989) found that several tissues contained two distinct classes of fibril, one having helical microfibrils at an angle of about 18° to the fibril axis, the other having slightly wavy or zig-zag microfibrils. The helically arranged fibrils were present in tissues rich in collagen III and tended to form small fibrils with a uniform diameter, whereas the zig-zag morphology was characteristic of the large heterogeneous population of collagen I fibrils in tendon. These observations suggested that the overall morphology may identify the different types of collagen fibril (Raspanti et al., 1989). Collagen networks may present different types of twist dependent on the variation of fibrillar orientation in the collagen network. So far three types of twist have been
identified, planar, cylindrical and toroidal. Both planar and cylindrical twists are observed in vitro and in vivo, whereas the toroidal twist has only been observed in vitro (Gaill et al., 1991).

1.5.5.2 Lysyl Oxidase

Lysyl oxidase (Protein-lysine-6-oxidase; EC 1.4.3.13) is a specific copper dependent amine oxidase that functions extracellularly. The enzyme initiates the formation of aldehyde-derived inter- and intra-molecular cross-links in collagen and elastin by the oxidative deamination of certain lysine or hydroxylysine amino acid residues. Cross-links form spontaneously by condensations involving side chain aldehydes and unreacted ε-amino groups. Continuous inter-molecular condensations of this kind convert soluble monomers of collagen or elastin into insoluble fibres in the ECM (for reviews on lysyl oxidase and collagen cross-linking see: Kagan, 1986; Ricard-Blum & Ville, 1989; Last et al., 1990; Kagan & Trackman, 1991; Reiser et al., 1992). The biomechanical stability and turnover of collagen is critically dependent on cross-linking, so lysyl oxidase plays a key role in collagen function and metabolism.

The activity of lysyl oxidase on a collagen substrate in vitro is markedly increased by prior assembly of the molecules into fibrils (Siegel, 1974). This indicates that biosynthesis of the aldehyde cross-link intermediate probably occurs after the onset of fibril formation in vivo. Therefore cross-linking may be related to the rate of fibril assembly as well as the activity of lysyl oxidase. Hence factors which modulate the kinetics of fibril formation may also modulate the extent of subsequent cross-linking.

The binding of lysyl oxidase to preformed collagen fibrils has been characterised (Cronlund et al., 1985). The results indicate that lysyl oxidase binds to the triple helix, with the catalytic site positioned on the non-helical telopeptide. The above observations led to the suggestion that lysyl oxidase binds to the collagen molecules at sites remote from the lysines (or hydroxylysines) to be oxidised, but which become juxtaposed to these residues when molecules are assembled in D-staggered arrays.
Lysyl oxidase has been purified from several sources including bovine aorta and lung (Kagan et al., 1979; Cronlund & Kagan, 1986), human placenta (Kuivaniemi et al., 1984) and porcine skin (Shackleton & Hulmes, 1990a), these preparations yielding $M_i$ values of 30-34 K on SDS-PAGE. The presence of multiple ionic variants have been observed in enzyme preparations from bovine aorta (Kagan et al., 1979; Williams & Kagan, 1985), bovine cartilage (Sullivan & Kagan, 1982), human placenta (Kuivaniemi et al., 1984) and porcine skin (Cronshaw et al., 1993). By SDS-PAGE, these variants have the same molecular weight and exhibit a high degree of structural similarity as evidenced by the nearly identical peptide maps obtained from proteolytic digests (Sullivan & Kagan, 1982; Williams & Kagan, 1985) or CNBr digests (Cronshaw et al., 1993) of each. Moreover substrate specificities (toward collagen, elastin and alkylamines), inhibition profiles, and kinetic parameters of each of the variants are similar, consistent with the same catalytic mechanism for each of these enzyme forms (Kagan et al., 1979; Sullivan & Kagan, 1982). The origin of the enzyme variants is not known, since only one gene for lysyl oxidase has been found in rat genomic DNA (Trackman et al., 1990). Thus multiple forms of lysyl oxidase may stem from post-translational modifications. Studies with purified preparations of lysyl oxidase confirm that copper provides a catalytic or structural role in the enzyme that is essential to the expression of enzyme activity (Iguchi & Sano, 1985; Gacheru et al., 1990). The highly purified enzymes of chick bone (Iguchi & Sano, 1985) and bovine aorta (Gacheru et al., 1990) contained 1 g atom of tightly bound copper per 32 K monomer, whereas the metal free enzymes are catalytically inert. Nutritional studies indicate that copper deficiency results in both a reduction in cross-link formation and an increase in elastin degradation, while restoration of copper reverses these effects (Gacheru et al., 1990; Tinker et al., 1990). Thus the activity of lysyl oxidase appears to be essential to the accumulation as well as the prevention of loss of connective tissue protein. Although the enzyme also requires a functional carbonyl cofactor, its chemical identity has not been firmly established. Early investigations involving spectral
analyses of chick aorta lysyl oxidase (Bird & Levene, 1982) led to the suggestion that pyridoxal phosphate was the organic cofactor for lysyl oxidase. More recently, resonance Raman spectroscopy of an active site peptide of the bovine aortic enzyme implicated pyrroloquinoline quinone (PQQ) as the functional carbonyl (Williamson et al., 1986). Although PQQ had previously been reported to be the cofactor of one other mammalian enzyme, i.e. bovine serum amine oxidase (Lobenstein-Verbeek et al., 1984), a recent report contradicts the presence of PQQ and, instead, provides strong support for the presence of 6-hydroxydopa at the active site of the enzyme (Janes et al., 1990). Although a detailed analysis of the organic cofactor in lysyl oxidase has still to be done, it is of interest that a dietary deficiency of PQQ in rats decrease the accumulation of lysyl oxidase, and produce lathyritic symptoms (van der Meer & Duine, 1986). Feeding of PQQ reverses these effects (van der Meer & Duine, 1986), therefore a quinone possibly 6-hydroxydopa serves as a catalytic centre for the enzyme (Kagan & Trackman, 1991; Romero-Chapman et al., 1991).

Lysyl oxidase appears to follow a “ping-pong” kinetic course of reaction with the order of substrate binding and product release as shown below (possible enzyme intermediates are represented below the line; Kagan & Trackman, 1991).

\[
\begin{align*}
RCH_2NH_2 & \quad \xrightarrow{E_{ox}} \quad (E_{RCH_2NH_2} \xrightleftharpoons{E_{RCHO}} E_{RCHO}) \\
\xrightarrow{E_{NH_2}} RCHO & \quad \xrightarrow{O_2} E_{NH_2} \\
\xrightarrow{(E_{NH_2} \xrightleftharpoons{E_{NH}} E_{NH})} H_2O_2 & \quad \xrightarrow{NH_3} E_{ox}
\end{align*}
\]

This proposed chemical mechanism assumes a PQQ-like o-quinone cofactor in which the amine substrate is initially oxidised to the aldehyde during which the carbonyl cofactor is reduced by the passage of two electrons from the α-carbon of the substrate (Kagan & Trackman, 1991). Upon binding of O₂, the enzyme is reoxidised to form and release H₂O₂. The role of copper has still to be firmly established, however it was
shown that the metal-free apo-enzyme cannot catalyse the first half of the reaction in which the aldehyde is formed (Gacheru et al., 1990).

Romero-Chapman and co-workers isolated 40 and 32 K forms of the enzyme from rat skin, both of which showed apparent lysyl oxidase activity. Antibodies raised against the 40 K form detected the 32 K form in immunoblots, the inference being that the 32 K form is processed from the 40 K form (Romero-Chapman et al., 1991). The above observations complement those of Trackman et al. (1990; 1991) who have characterised lysyl oxidase from a rat aorta cDNA library. Their observations suggest that lysyl oxidase is first synthesised as a 46-48 K precursor with obvious tryptic cleavage sites that could generate products of 32 K and 40 K. In a study by Wakasaki and Ooshima (1990), a 48 K form of lysyl oxidase was observed after cell-free translation.

Although lysyl oxidase doesn’t appear to be glycosylated (Sullivan & Kagan, 1982), an N-glycosylation site was found in the sequence encoding the 32 K protein, which is immediately followed by a thr- and ser-rich sequence. Further glycosylation sites are found in the sequence encoding the precursor, thus raising the possibility that N and O-glycosylation of the pro-lysyl oxidase molecule may occur (Trackman et al., 1990; Trackman et al., 1991; Kagan & Trackman, 1991; Hamalainen et al., 1991). Serafini-Fracassini et al. (1981) isolated a 34.4 K glycoprotein from bovine ligamentum-nuchae. This preparation displayed lysyl oxidase activity towards an elastin substrate, however the protein was unusually resistant to BAPN. The 34.4 K protein was however similar to structural glycoproteins closely associated with nascent elastic fibres, thus raising questions concerning the role of this catalytically active glycoprotein in connective tissue fibre synthesis. Recently the human lysyl oxidase gene has been mapped to the long arm of chromosome 5 (Hamalainen et al., 1991). The human enzyme appears to be synthesised as a 45 K protein containing a putative signal peptide of 21 residues and an N-terminal propeptide, which is cleaved to produce a 30 K enzyme.
Lysyl oxidase has been localised and found to be associated with the microfibrillar network surrounding elastic fibres, and within elastic fibres themselves (Kagan & Trackman, 1991). Other workers have localised the enzyme on and within elastin and collagen fibres of human placenta, skin and aorta (Baccarani-Contri et al., 1990). The apparent association of lysyl oxidase with the microfibrillar network implies that new tropoelastin molecules are cross-linked as they are added to the growing elastic fibre.

The level of enzyme activity is markedly decreased in two X-linked recessively inherited human connective tissue diseases, Ehlers-Danlos syndrome type IX and Menkey's syndrome (Kagan, 1986). On the basis of these findings it was assumed that the lysyl oxidase gene was located on the X chromosome. Nevertheless both disorders appear to be associated with disturbances in copper metabolism (Kagan & Trackman, 1991). Hence it was not known whether the decrease in activity was due to a reduction in intracellular copper or impaired synthesis of lysyl oxidase. Recently the assignment of the lysyl oxidase gene to chromosome 5 indicates that the low enzyme activity associated with both disorders appears to be secondary to a decrease in available copper (Hamalainen et al., 1991). The non-availability of copper leads to a further consequence in the synthesis and or degradation of lysyl oxidase as patients with type IX Ehlers-Danlos syndrome contain virtually undetectable levels of tissue enzyme (Byers et al., 1980).

The activity of lysyl oxidase responds to changes in levels of certain hormones: for example, testosterone has been shown to increase the activity approximately 5 fold in cultured aortic smooth muscle cells (Bronson et al., 1987). A lysyl oxidase enhancer from porcine skin has also been found (Shackleton & Forbes, unpublished observations). The enhancer, which has no lysyl oxidase activity on its own, but which can increase lysyl oxidase activity several fold, has a molecular weight greater than 10 K, is relatively heat stable but is inactivated by freezing.
1.5.5.3 Collagen Cross-links

The nature and position of cross-links has long been the subject of conjecture, the ultimate cross-link produced reflecting the steric relationship of the individual collagen molecule within the fibril structure. Oxidation of lysine (or hydroxylysine) in the interstitial collagens is restricted to one lysine in the N-terminal telopeptide (residue 9N) and one in the C-terminal telopeptide, the lysines in the triple helical region are not normally oxidised. Native isolated cross-links are consistent with cross-linking between the C-terminal telopeptide lysine (residue 16C) of both α1 chains, and triple helical lysine (residue 87) on both α1 and α2 chains of neighbouring molecules. The α2(I) and α2(V) chains lacks the C-terminal aldehyde site (Eyre et al., 1984; Yamauchi et al., 1989). The same homologous sites of cross-linking are conserved for collagens I, II, III and V. Only four locations have been established: two non-helical aldehyde sites, one at each end of the molecule, and two triple helical sites which correspond to hydroxylysine residues (Ricard-Blum & Ville, 1989). In the α1 chain of collagen XI, three of the conserved sites have been identified (Bernard et al., 1988). Conserved regions in the triple helix between residues 941-951 and 101-111, which are adjacent to Lys 9N and Lys 17C respectively, both bear an overall positive charge (Kagan et al., 1984). Since lysyl oxidase is more active on a positively charged substrate, these conserved regions may provide the appropriate electrostatic potential for binding of the enzyme (Kagan 1986). Hence the correct binding and orientation of lysyl oxidase to the susceptible lysine residues may necessitate the requirement for the quarter stagger relationship between monomers in collagen fibrils. To account for the arrangement of collagen cross-links, Davison and Brennan (1983) proposed a model for the organisation of collagen molecules in the fibril. Their model supported the postulated lamellar arrays of in register molecules with consistent polarity, as described in the quasi-hexagonal model of Hulmes and Miller (1979).

Collagen cross-links appear to arise from one of two general biosynthetic pathways, those initiated by lysyl oxidase, and those derived from non-enzymatically
glycosylated lysine and hydroxylsine (Reiser et al., 1992). These early glycation products undergo a series of reactions to form what is referred to as advanced Maillard products. These cross-links appear to be prevalent in diabetic subjects and have been associated with abnormalities in the physiochemical properties of collagen with long term clinical complications (Buckingham & Reiser, 1990; Reiser et al., 1991; Reiser et al., 1992). Relatively little is known about the chemistry of sugar derived cross-links compared to the lysyl oxidase initiated cross-links. However both pathways involve lysine and hydroxylsine, so it is likely some relationship exists between them.

The first step in lysyl-oxidase-mediated cross-linking involves the oxidative deamination of specific lysine and hydroxylsine residues to their corresponding aldehydes called allysine and hydroxyallysine respectively (Fig. 1.5). These aldehydes stoichiometrically condense with other lysyl or hydroxylysyl residues to form Schiff bases (aldimines) or with other aldehydes to form aldol cross-links (Ricard-Blum & Ville, 1989). Some of these reducible cross-links are more stable than one might expect for a Schiff base, as they may undergo an amido-rearrangement to form ketoamines (Last et al., 1990). Intramolecular cross-links are formed when two allysine residues within the same molecule combine to produce a condensation product called allysine aldol. During the last twenty years, a variety of di, tri and tetrafunctional cross-links have been identified in collagen I. These cross-links are produced by reactions involving lysine, hydroxylsine and histidine residues. Cross-links between different collagens (e.g. I and III) have also been isolated (Henkel & Glanville, 1982) and in cartilage pyridinoline residues covalently link molecules of collagen IX to molecules of collagen II (Eyre et al., 1987).

Aldehyde-derived intermolecular cross-links are conveniently divided into two classes. The initial cross-links on both pathways are reducible aldimines or ketoamine rearranged products. Acid-labile histidine based cross-links which are derived from lysine aldehydes predominate in skin, whereas hydroxylsine aldehydes which form the more stable pyridinium cross-links predominate in bone and cartilage (Fig. 1.5).
Figure 1.5 Collagen cross-links
Tissue specificity and maturation changes in cross-linking, showing the structures of the principal cross-links in mature tissue (adapted from Kagan, 1986; Ricard-Blum & Ville, 1989).
Tendon may contain both types, the dominant form depending on the location of the tissue. Variations in mechanical and physical properties among different connective tissues may be due in part to differences in cross linking. In general the hydroxyllysine route predominates in tissues that bear large mechanical loads (Eyre et al., 1984). The amounts of the reducible cross-links have been shown to decrease with age; this led to the hypothesis that the reducible components were intermediates that were converted to a mature, stable, non reducible form, thus accounting for the increased stability of mature tissue (Barnard et al., 1987).

The functional importance of cross-links has been demonstrated both in animal models and in cross-linking experiments in vitro. β-aminopropionitrile (BAPN), is a potent irreversible inhibitor of lysyl oxidase (Tang et al., 1983). The ability of BAPN to inhibit collagen cross-linking results in an increased fragility of all connective tissues, with a concomitant elevation in the solubility of collagen. The kinetics of the inhibition is consistent with that of an active-site-directed inhibitor (Tang et al., 1983). The control of cross-linking is potentially of great clinical significance as there are several disorders including rheumatoid arthritis and osteoarthritis in which the symptoms may be alleviated by mobilising the rapid proliferation of collagen that characterise these conditions (Robins, 1982; Kagan, 1986). Decreasing the number of cross-links not only reduces the tensile strength of collagen fibres, but also renders them more susceptible to degradation by collagenase (Vater et al., 1979). Hence the dose of BAPN must be balanced against potential reductions in tissue tensile integrity and other undesirable effects.

In addition to the lysine-derived cross-links, disulphide bonds are found in some collagens. Collagens III, IV, VI and VII are stabilised by both intra- and inter-molecular disulphide bonds whereas collagens IX and XII contain only intramolecular disulphides (Ricard-Blum & Ville, 1989).

Over the past two decades significant advances have been made in the understanding of collagen cross-link biochemistry. For example, it has been shown
that the cross-linking pattern in the collagens differs widely depending on the tissue and age of the organism, and that cross-link maturation results in improved collagen resistance to catabolic enzymes. However many questions still remain unanswered, such as the biological significance of the various cross-links. The regulation of cross-link biosynthesis is still poorly understood, as is the relationship between lysyl oxidase initiated cross-links and sugar derived cross-links.

1.6 Disorders of Collagen

Collagen biosynthesis and degradation are highly complex processes comprising many steps, disruption at any of these steps become manifest as diseases. Collagen may also be influenced by non-collageneous components and abnormalities of these components can result in a number of other disorders (Tzaphlidou, 1992). Point mutations in fibrillar collagens result in a variety of skeletal and tissue abnormalities such as osteogenesis imperfecta and certain Ehlers-Danlos syndromes (Steinmann et al., 1991; Watson et al., 1992). Disease may also result when aberrant cells infiltrate pre-assembled extracellular matrices to mount an inflammatory response such as that found in rheumatoid arthritis.

Osteogenesis imperfecta (OI) is a heritable disorder characterised by brittleness of bones. Over 80 mutations have been described to date in the genes encoding for procollagen I. Single base changes that substitute glycine for amino acids with bulkier side chains account for most of the mutations and reflect the strict requirement for glycine at every third position along the collagen triple helix (Steinmann et al., 1991). Mutations that cause OI also include insertions and deletions of DNA as well as splicing mutations which lead to exon skipping (Deak et al., 1991; Kuivaniemi et al., 1991). The severity of the disease is varied and appears to correlate with the position of the mutation (Byers, 1990; Khillan et al., 1991). In general mutations near to the C-terminal are lethal, whereas mutations approaching the N-terminus produce milder forms of the disease. This affect may be ascribed to the zipper-like folding of pro α-
chains from the C to N-terminus (Engel & Prockop, 1991). Any mutation which interferes with the folding can destabilise the triple helix C-terminal to the substitution causing the chains to spend longer in non helical conformation. Because the degree of post-translational modification is increased by any delay in folding, over-modified proteins are produced. The extent of the over-modification is therefore inversely related with distance of the substitution from the C-terminus. Post-translational over-modification is seen in a series of mutations which cause OI, although it remains to be established what contribution it makes to the phenotypic changes seen in the disease (Torre-Blanco et al., 1992). Most mutations lower the thermal stability of the collagen molecule such that folding is impaired and secretion prevented. Unfolded molecules accumulate intracellularly and are slowly degraded, so less ECM is produced. Normal pro α-chains may also associate and become disulphide-linked to mutant chains. Thus both normal and mutant chains are degraded intracellularly in a process called procollagen suicide. Alternatively, when mutant procollagen molecules allow folding of the three chains, a conformational change such as a flexible kink can be introduced into the triple helix. When these distorted molecules are secreted, they copolymerise with normal collagen which disrupts their self assembly into fibrils (Khillan et al., 1991).

Ehlers-Danlos syndrome (EDS), a heterogenous group of heritable disorders characterised by hypermobility of joints and abnormalities in skin, is classified into 11 types based on clinical and biochemical findings. By virtue of marked joint hypermobility and frequent joint dislocation, EDS type VII is distinct from other forms of EDS. The biochemical basis of the disorder is a failure to process the N-propeptide of procollagen I. Hence EDS type VII was thought to be the human counterpart of dermatsporaxis (fragile skin), originally described in cattle and sheep. Dermatosporaxis is caused by the absence of N-proteinase activity; however in EDS type VII a mutation causes skipping of exon 6. Exon 6 contains the coding sequence for the N-propeptide cleavage site and therefore cleavage of the procollagen by procollagen N-proteinase is prevented in molecules which contain the mutation. The
persistence of the N-propeptide in collagen drastically alters fibril formation so that fibrils are irregular in cross section (Hulmes et al., 1989a; Kuivaniemi et al., 1991; Watson et al., 1992).

1.7 Matrix Degradation

Two principal methods exist for matrix degradation: extracellular secretion of catabolic enzymes (e.g. neutral metalloproteinases) and the internalisation of matrix molecules followed by lysosomal degradation (for reviews, see Alexander & Werb, 1989; Matrisian, 1990; Alexander & Werb, 1991; Woessner, 1991).

Matrix metalloproteinases (MATRIXINs) comprise of at least seven members which range in size from 28-92 K. These zinc-containing enzymes are responsible for degradation of ECM and basement membrane components during normal embryogenesis, tissue remodelling, and in many disease processes such as arthritis and cancer. Members of the MATRIXIN family share the following characteristics:

(i) They contain a zinc ion at the active centre.
(ii) The proteinases are initially secreted in zymogen form, which are activated upon autocatalytic removal of a 10 K amino propeptide.
(iii) The enzymes cleave one or more constituents of the matrix.
(iv) They are inhibited by tissue inhibitors of metalloproteinases (TIMPs).

The matrix metalloproteinases can be divided into three groups which comprise the collagenases, gelatinases and stromelysin. The collagenases and stromelysins have three functional domains; an amino propeptide of around 80 amino acids containing a free cysteine residue which may be required for the maintenance of latency of the proenzyme form, a catalytic domain of about 180 amino acids which is required for peptide bond cleavage as well as co-ordinating calcium and zinc binding to the enzyme, and a C-terminal domain containing around 200 amino acids which is thought to be involved in matrix binding (Murphy et al., 1992).

Interstitial collagenase cleaves collagens I, II and III. In collagen I, cleavage in the
triple helical domain produces a three quarter and one quarter length fragment called TCa and TCb respectively, these fragments rapidly denature and become suitable substrates for gelatinase and stromelysin. The cleaved peptides may be further fragmented in the matrix or degraded by cysteine proteases (cathepsins) in lysosomes. The observations of Murphy et al. (1992) showed that the specific action of collagenase on triple helical collagen was determined by the presence of the C-terminal domain, which appears to orientate the enzyme’s catalytic domain at the single cleavage site. The discovery of the FACIT collagens changed ideas about degradation of collagen matrices. These collagens may well coat fibril bundles and protect the internal fibrils from attack by interstitial collagenase. Therefore degradation may involve an initial cleavage of the FACITs by stromelysin. The regulation of proteolysis is under tight control at several levels. Transcription of MATRIXIN genes may be stimulated by growth factors and cytokines. This induction, can when necessary, be blocked by biological agents such as transforming growth factor-beta (for review see Roberts et al., 1990). The activity of the metalloproteinases may also be regulated during conversion from the latent to the active enzyme form. Finally, proteinase activity is dependent on the balance between the enzymes and their inhibitors (e.g. TIMPs, α2 macroglobulin).

TIMP (also called TIMP 1) is a 28.5 K glycoprotein which binds reversibly and with high affinity, forming a 1:1 complex with interstitial collagenase (Woessner, 1991). The mechanism of inhibition is unknown, although it was suggested that TIMP may interact with the active site zinc ion to render the enzyme inactive. With the exception of elastin, which is efficiently digested with neutrophil elastase, the MATRIXINs are capable of degrading most of the ECM components. However a macrophage elastase which is inhibitable by TIMP has been found (Banda & Werb, 1981). In the absence of sequence data, it is not yet possible to assign this enzyme to the MATRIXIN family.
1.8 Matrix Assembly

Matrix assembly is a dynamic process which occurs when an organism undergoes morphogenesis, growth and regeneration (for a review see: Birk et al., 1991). Since over 90% of the organic matter in skin, tendon, ligament and bone is collagen, the physical properties of tissues are determined to a large extent by the three dimensional organisation of collagen fibrils. Fibrillar arrays of collagen show a variety of tissue specific patterns that range from the tight, orthogonal network in bone to the bundles of parallel fibrils found in tendon. Even in tissues which show the same heterotypic mixtures of fibrillar collagen molecules, the collagen fibrils still exhibit diversity in their spatial organisation. Hence tissue specific variation cannot be explained simply by the tissue-dependent expression of unique fibrillar collagen types. However, it is likely that molecules or domains that are exposed on the fibrillar surface are involved in complex interactions which determine the spatial organisation of fibrils. It has been suggested that the tissue specific expression of non-fibrillar collagens contribute to histological variability. Matrix assembly is thought to take place initially via non-covalent interactions of the molecular components, which are later stabilised by covalent bonds (Yurchenco & Schittny, 1990). PGs in particular HSPG probably play a vital role in this process due to their ability to bind several matrix components (including collagen, laminin and fibronectin). In addition to the integrins, cell surface proteoglycans may also provide a vital role in connecting ECM components to the cytoskeleton (Carey, 1991). Finally, cell-mediated control over the stoichiometry of the different ECM components as well as the discharge of macromolecules into the extracellular space is important in determining matrix architecture. Therefore the cellular regulation of mixing during packaging or at the site of secretion provides a mechanism where a variety of components can be combined in several ways to produce a huge diversity of connective tissue structures (Birk et al., 1990b).
1.8.1 Fibril Formation

Generally, collagen fibrillogenesis is the formation of fibrillar structures by the ordered assembly of collagen molecules. However, the term cannot be used to describe the assembly of all collagens, because not all collagens form structures recognisable as fibrils. Unlike the molecules of collagens I, II, III, V and XI, for example, the FACIT collagens do not form fibrils, but do assemble in specific ways to form complex networks most suited for their biological role (Fig. 1.1).

The *in vitro* assembly of collagen molecules into fibrils is a classic example of an entropy-driven self-assembly process. At physiological ionic strength and pH, and at a temperature above 30 °C, the axial registration of molecules is established and leads to the formation of D-periodic fibrils (George & Veis, 1991). Thus it is evident that the information to build D-staggered fibrils is contained in the amino acid sequences of the fibril-forming collagens (Vies & Payne, 1988).

Assembly of collagen fibrils *in vivo* is more complex and is probably influenced by other components of the ECM (e.g., proteoglycans), as well as by post-translational modifications of the collagen molecule. It is now well documented that many collagen fibrils are composed of two or more collagen types. Heterotypic interactions between different collagens have been proposed as a possible mechanism for fibril diameter limitation (van der Rest & Garrone, 1991). It has also been proposed that proteoglycans bind to specific regions on collagen fibrils (Scott 1991), and regulate their assembly and final diameter (Brown & Vogel, 1989). The rate of removal of the N-propeptide strongly influences fibrillogenesis, and limited persistence of pN-collagen is associated with small diameter fibrils (Mellor *et al.*, 1991). In addition, cells influence extracellular events through their control over secretion of the different macromolecular components (Ploetz *et al.*, 1991). It is evident from the sharp diameter distributions of collagen fibrils in a variety of connective tissues that a stringent control over lateral growth can be exerted *in vivo* (Vies & Payne, 1988; Chapman, 1989), though the factors which control lateral and axial growth and hence determine fibril...
diameters remain to be elucidated. It is also clear that the orientation and interfibrillar spacing of matrix assemblies are subject to cell-mediated, tissue-dependent control, although the exact factors which regulate and determine the supramolecular architecture of fibrils in different tissues are poorly understood (Ploetz et al., 1991; Sage & Bornstein, 1991).

1.8.1.1 Collagen I Fibrillogenesis in Vitro

Collagen I fibril assembly has been studied for several decades (Wallace, 1990). In the classical system, fibrils are reconstituted in vitro by taking a cold acidic solution of collagen (e.g. 4 °C; pH 2.5 - 3) to warm neutral conditions (e.g. 34 °C; pH 7.4). The fibrils which ultimately form are usually well ordered D-periodic fibrils similar to those observed in vivo, although it is usually more difficult to reproduce fibril diameters (Veis & Payne, 1988; Hulmes, 1992). Over the years, the nature of the collagen preparations used has changed as understanding has advanced. Preparations containing aggregates derived from cross-linking or containing molecules that had been altered by proteolysis were used in early studies of fibrillogenesis. More recently workers prefer to use largely monomeric collagen solutions which are as free from degradation as possible; these efforts have resulted in a significant advance in understanding fibril assembly (Wallace, 1990).

1.8.1.1.1 Fibrillogenic Pathways

Holmes et al. (1986) analysed and defined three general routes by which fibrils reconstitute in vitro: cold start, warm start and simultaneous. In a typical warm start procedure, equal volumes of acidic collagen solution and double strength neutral buffer are preincubated to the desired temperature and then mixed rapidly. In the cold start procedure, the solutions are first mixed and then rapidly heated to the desired temperature. Although both pathways have the same end point of fibrillogenic conditions, the two routes do not yield identical kinetics for fibrillogenesis and the
fibrils formed are different in terms of diameter distribution (Holmes et al., 1986; Veis & Payne, 1988).

The kinetics of assembly as followed by turbidity shows the presence of a lag phase during which there is no apparent increase in optical density followed by a rapid sigmoidal growth phase and a stable gel which slowly matures. The length of the lag phase is inversely related to both the total collagen concentration and the amount of oligomers in the solution (Na et al., 1986a). Data has also shown that the final absorbance can be correlated with the diameter of the fibrils (McPherson et al., 1985). A critical concentration for polymerisation can also be demonstrated (Kadler et al., 1987).

Decreasing the temperature results in rapid fibril disassembly. Upon rewarming the solution growth begins almost instantaneously but with a shortened lag phase (Na, 1989; Na et al., 1989). This shortening of the lag phase upon second heating is called the “thermal memory effect” and its existence suggests that some change must take place which imprints the prior thermal history of the preparation. In a pure collagen I system, homogeneous nucleation theory would predict that the lag phase is related to the probability of forming an aggregate of sufficient size to be capable of supporting fibril growth. When it disassembles the collagen may still contain nucleation centres or some oligomers which can readily form centres later. Because the lag phase may be restored by transferring the solution to acidic pH, this may also suggest that any residual structures are stabilised by acid-labile, Shiff-base cross-links (Na, 1989), although it does not explain why thermal memory is lost as the solution is allowed to stand over several days at 4 °C (Veis & Payne, 1988). A further explanation of the “memory” effect is that a thermally driven conformational transition occurs either in the helix and/or the telopeptides of the collagen molecule. Using dynamic light scattering, Payne et al. (1986) showed that no significant differences existed between “naive” solutions of collagen and ones which already exhibited thermal memory, a result which suggests that a subtle change in the state of the collagen molecules rather than extensive
changes in the degree of aggregation are responsible for the memory effect. It was also demonstrated that no large scale changes in the distribution of monomers or aggregates occur until near the end of the lag phase (Payne et al., 1986). Recent data from FTIRS (Fourier Transform InfraRed Spectroscopy) also appears to support this by showing that there is no apparent change in the hydrodynamic parameters of collagen monomers during the lag phase (George & Veis, 1991).

Fibrils which are formed at 35 °C and above tend to be narrow in diameter and loosely packed. However by using a new system for reconstituting collagen, larger diameter fibrils with tighter packing can be assembled following the enzymatic removal of the C-propeptide from pC-collagen by purified procollagen C-terminal proteinase (Kadler et al., 1990b). The kinetics of assembly are similar to fibrils which are reconstituted following neutralisation and warming of acidic collagen solutions, though the rate of enzyme processing provides an additional tier of control. Collagen fibrils generated in this way made it possible for the first time to follow their growth with time lapse photography. Using dark-field light microscopy the first formed fibrils display shape polarity having one blunt end and one highly tapered tip (Kadler et al., 1990a). The fibrils grow longitudinally by the addition of monomers to the tapered tip and laterally by further additions of molecules to fill in the highly symmetric tips (Kuivaniemi et al., 1991). Later in the process of fibril assembly, pointed projections appear at the blunt ends and the fibril then grows in both directions. Mass measurements by STEM (Scanning Transmission Electron Microscopy) revealed that the mass per unit length along both kinds of tips increased linearly over distances of around 100 D-repeats from the fibril end. Fine tips from different fibrils exhibited near identical growth behavior, whereas course tips displayed less regular growth patterns (Holmes, personal communication). The pattern of cross striations in the pointed tips indicate that all the molecules were orientated such that the N-termini were directed towards the tips, thus indicating a switch in molecular polarity within the fibril (Kadler et al., 1990a). These results are consistent with the growth of fibrils from acid
extracted collagen which display symmetrical tips. The above results are also consistent with the *in vivo* observations of Birk *et al.* (1990b) who demonstrated using three dimensional reconstructions from serial sections of embryonic chick tendon which identified individual fibrils (diam. 40 nm) of length approximately 10 μm, and the fibrils had a rapidly tapering and a gradually tapering end.

**1.8.1.1.2 Parameters Governing Collagen I Self-Assembly**

A number of studies have indicated that the collagen triple helix in solution is a semiflexible structure with several subdomains of enhanced flexibility, the hypothesis being that molecules first have to bend to fit within fibrils (Veis & Payne, 1988; George & Veis, 1991). The short N- and C-terminal telopeptides constitute smaller but very important domains. It has already been proposed that both specific ionic and hydrophobic interactions are involved in the initial reaction between the extrahelical peptides and the helix, and this is believed to be the first step in ordered fibril formation (George & Veis, 1991). The importance of the telopeptide regions during the nucleation and growth of collagen fibrils has been demonstrated by disrupting these regions with proteases such as pepsin. Pepsin-treated collagen apparently cannot participate in lateral growth as efficiently or effectively as does intact collagen (McPherson *et al.*, 1985), and the pepsinised molecules (with partially cleaved telopeptides) assemble to form fibrils with little or no D-periodic banding (Gelman *et al.*, 1979). The N and C-terminal extrahelical regions lie in an axially condensed form and appear to have different functions. In the C-terminal telopeptide, there is a hydrophobic region ("the hydrophobic cluster") which is thought to direct the lateral assembly of early aggregates and hence control final fibril diameter, whereas the N-terminal region appears to direct linear growth (Capaldi & Chapman, 1984). However the fact that fibrils can be formed from molecules which lack the extrahelical peptides indicate that the intrinsic molecular properties necessary for fibril growth must be inherent to a certain extent on interactions between the collagen helices. The presence
of aldehydes in the collagen molecule also influences the kinetics of fibrillogenesis. It appears that, in vivo, aldehydes are generated only after the final assembly of the molecules into fibrils, so that any modelling of the natural fibrillogenic process should be done with lathyritic collagen (Brennan & Davison, 1981).

In general, the assembly of monomeric protein molecules into aggregated structures may be mediated by hydrogen bonding, hydrophobic effects, electrostatic interactions and entropic and dispersive forces (Wallace, 1990). Reconstitution of pure type I collagen can generate fibrils of widely differing morphologies depending on the conditions of fibrillogenesis (McPherson et al., 1985). For collagen, the rate of fibril assembly is markedly dependent on temperature and ionic strength, which implies that hydrophobic and electrostatic forces respectively play a prominent role. In general, increasing the salt concentration or lowering the pH lengthens the lag phase. This effect may be attributed to a shielding of intermolecular electrostatic interactions. However if a distortion of the triple helix is required for molecules to be incorporated into fibrils, perhaps fibrillogenesis is inhibited as much by restricting the conformational transition (Fraser et al., 1987; George & Veis, 1991). Phosphate ions are unique and exhibit pH dependent effects, producing maximum retardation at the initiation of the growth phase between pH 7.1 to 7.5. Above physiological concentrations of phosphate, the fibrils displayed larger diameters (Nemeth-Csoka & Tasnadi, 1981; Veis & Payne, 1988).

Many other simple compounds modulate the kinetics of fibrillogenesis. Glucose (Rathi et al., 1989) and urea (Fessler & Tandberg, 1975) were both found to inhibit fibril assembly in a concentration-dependent manner. The glucose appears to act by weakening intermolecular forces within the fibril, while urea seems to dissociate nuclei in the process of fibril growth (Hayashi & Nagai, 1972). It has been suggested that glucose binds to collagen and interferes with the interaction between the telopeptide regions and the triple-helical receptor sites on adjacent 4D staggered molecules (Rathi et al., 1989). Since it has already been shown that lysyl oxidase activity on a collagen substrate is increased by prior assembly of the molecules into fibrils (Siegel, 1974),
glucose may also reduce subsequent cross-linking through the inhibition of fibril formation. Lien et al. (1984) proposed that the high glucose environment in diabetic subjects may prevent cross-linking of newly formed collagen and thus lead to a selective depletion of these molecules. Their results were supported by animal models which in diabetic rats showed an enhanced catabolism of newly synthesised collagen.

1.8.1.1.3 Kinetics and Thermodynamics of Self-Assembly

Self-assembly of collagen monomers into fibrils is primarily entropy driven with thermodynamic parameters similar to the polymerisation of other proteins (e.g. actin, flagellin; Kadler et al., 1987). The endothermic nature of in vitro fibril assembly implies that hydrophobic interactions are the major interactions governing the assembly process. These interactions are driven by the entropy increase resulting from the loss of surface water from the monomer as the molecule enters the fibril aggregate phase (Kuivaniemi et al., 1991). Increasing the temperature from 29 to 35 °C accelerates the rate of assembly in a predictable manner (Kadler et al., 1990b). However between 37 to 41 °C the rate of fibril formation is markedly limited by micro-unfolding of the monomer (Kadler et al., 1988).

Because fibril reconstitution displays two-phase kinetics (a lag phase followed by an exponential growth phase) a useful way to think of assembly is in terms of nucleated growth (Wood & Keech, 1960; Hulmes, 1992). The rate-limiting step is the formation of a critical nucleus from a small number of monomers. Once formed, growth proceeds by the further addition of molecules. At any given time, depending on the reaction conditions, these two processes may proceed simultaneously. Such a model has been widely used in the assembly of other proteins (e.g. actin, tubulin). However collagen assembly is distinctly different in that the final polymeric structure is formed by the addition of monomers in both lateral and longitudinal directions, whereas in actin and tubulin growth only occurs in length (Kadler et al., 1990a; Hulmes, 1992).
1.8.1.1.4 Molecular Specificity of Self-Assembly

As already pointed out, the telopeptide regions have major, but different effects on the course of self-assembly. In native collagen aldehydes are present in the telopeptide domains. If these aldehydes are reduced prior to the initiation of fibrillogenesis, then thermal memory is retained, but the aggregates formed do not have the long term stability exhibited when the aldehydes are present (Veis & Payne, 1988). Reduction following nucleation was found to stabilise the memory effect, even after dialysis into acetic acid. This data suggests that aggregates of some kind form during nucleation. It has been proposed that nucleation is dependent on the telopeptides assuming specific conformations favourable for the 4D-overlap interactions (Helseth et al., 1979; Fleischmajer et al., 1991b). In the model of Helseth et al. (1979), the N-telopeptide forms a β-turn placing the potential cross-linking lysine at the end of the turn. The interaction registering the N-telopeptide to its receptor region at residue 930 of a neighbouring molecule results from a specific alignment of ionic and hydrophobic groups. A similar interaction between the C-telopeptide and its receptor site at residue 87 of α1(I) has also been suggested (Capaldi & Chapman, 1982). A nucleus formed in this way not only dictates the molecular assymetry of the resultant fibril, but must exhibit a unique topology which is important for fibril growth.

1.8.1.1.5 Investigation of the First Formed Aggregates During Self-Assembly

Many workers have endeavoured to experimentally determine the nature of the nuclei or the first formed aggregates in the fibril forming process. The emphasis of these studies has been to determine the length and molecular weight of these structures, the problem however is the low concentration of nuclei. Gelman et al. (1979) proposed that, after a temperature dependent conformational change, intermediate subassemblies form that are less than 8 nm in diameter, but with lengths greater than 1500 nm. However Silver and Trelstad (1980) concluded from their dynamic light scattering
(DLS) studies that 4D-staggered dimers and trimers represent the first formed aggregates. Although more recent DLS data was unable to establish the existence of any new lag-phase aggregates, this does not mean that they have failed to form, since it has been shown that DLS is unlikely to detect the presence of less than 5% by weight of dimers and trimers (Payne et al., 1986). The presence of very small numbers of aggregates is entirely consistent with nucleation theories.

Electron microscopy data using the cold start technique to initiate fibril reconstitution showed that late in the lag phase long, narrow, filamentous aggregates without any apparent banding were present. These subassemblies then proceed to assemble into long thin D-periodic filaments. The lag phase appears to terminate when these filaments begin to aggregate laterally (Gelman et al., 1979). Holmes et al. (1986) made a direct comparison of the initial structures which form mid way through the lag phase. At this point non banded thin filaments predominated in the cold start, whereas the warm start produced thin D-periodic fibrils. Other workers have employed formaldehyde fixation to arrest the self assembly process and showed that the first formed aggregates are dimeric, with the most prevalent dimer having a 4D-stagger between constituent molecules (Kadler & Chapman, 1986; Ward et al., 1986).

1.8.1.1.6 Models for Self-Assembly Pathways

There is disagreement about whether the aggregates formed during the lag phase are unstable nuclei that grow by monomer addition or subfibrillar components that grow in a multistep process. In a nucleated growth process, the nature of the fibrils formed would depend on the concentration of stable nuclei. In a multistep process, monomers build intermediate aggregates which then coalesce linearly and laterally to form D-periodic fibrils (Trelstad, 1982). Silver (1981) concluded that the lag phase terminated when the molecular weight of the aggregate exceeded 930 K and the results were consistent with the formation of a linear 4D staggered trimer, further growth occurring by linear and lateral addition of trimeric units. By using glycerol to inhibit
fibril formation, Na et al. (1986b) demonstrated that a minimal or critical concentration of collagen for fibril assembly is required. Further to this, collagen was shown to be monomeric at subcritical concentrations. Electron microscopy data showed the formation of unbanded microfibrils with diameters in the range 3-15 nm early in the lag phase, whereas larger diameter banded fibrils coexisted with the microfibrils near the end of the lag phase. These results suggested a cooperative nucleation growth mechanism adopting as an intermediate the 5-stranded microfibril originally conceived by Smith (1968). It appeared that the banded fibrils were formed through the lateral packing of the microfibrils (Na et al., 1986b; Na et al., 1989). However the evidence for microfibrils in intact fibrils is inconclusive (Hulmes, 1992).

1.8.1.1.7 In Vitro Fibril Formation of Collagen I from Different Tissues

As it is known that collagen I fibrils have different ranges of diameters in various tissues, it is of interest to carry out a comparative analysis of in vitro fibrillogenesis for collagen I extracted from different tissues. Collagen I extracted from mammalian corneas showed smaller rate constants for both the lag and growth phase, and formed smaller diameter fibrils compared to scleral collagen I (Birk & Silver, 1984a). Fibrillogenesis of corneal collagen, which is characterised by the highest content of hydroxylysine-linked glycoside, appears to suggest that there may be some correlation between glycosylation and kinetics of fibril formation. It was also postulated that the increased glycosylation of corneal collagen I may be a factor controlling fibril lateral growth and thus determine to a certain extent the precise architecture of the corneal stroma. The study by Valli et al. (1986) is also in agreement with these proposals. To explain the data it was suggested that the addition of sugar moieties to corneal collagen decreases the mobility of the collagen molecule and/or an intermediate in the fibril assembly process (Valli et al., 1986).
1.8.1.1.8 *In Vitro* Fibrillogenesis of Different Collagens

Very few comparisons have been made concerning the *in vitro* assembly of the other fibrillar collagens (II, III, V & XI). All four types form fibril structures with characteristic D-periodicity, though the kinetics of assembly differ. The time course of fibril formation was in the order III > II > I (Birk & Silver, 1984b). The diameters for the fibrils formed *in vitro* also correlated closely with those formed *in vivo*. These studies were carried out on pepsinised collagen, thus partial degradation of the telopeptides would have occurred. Birk and Silver (1984b) concluded that differences in helix-helix interactions were responsible for the differences in rate constants and that these interactions may be involved in determining fibril structure. However pepsin treatment of collagen does not remove the entire telopeptide, hence the molecule retains a small component of the telopeptide which may still influence fibril assembly and morphology (Veis & Payne, 1988).

1.8.1.2 Factors Affecting the Kinetics of Fibril Formation, and Regulation of Fibril Diameter

1.8.1.2.1 Interactions Between Collagen Types

It is now well documented that many collagen fibrils are heterotypic and are composed of two or more different collagen types (van der Rest & Garrone, 1991). The study of Keene *et al.* (1987) demonstrated the existence of fibrils containing both collagens I and III. Indirect evidence showed that collagen III may be involved in the regulation of fibril diameter, since collagen I tends to form large diameter fibrils (100-500 nm) whereas collagen III fibrils rarely exceed 60 nm in diameter, and fibrils composed of both collagens have intermediate sized fibrils (Grant & Ayad, 1988; Fleischmajer *et al.*, 1990). Immunolocalisation studies indicate that the fibrils of several tissues including skin and tendon are copolymers of at least collagens I and III (Keene *et al.*, 1987; Birk *et al.*, 1990a). The observation that the amino-propeptide of
pN-collagen III is excised far more slowly than the equivalent peptide of pN-collagen I may suggest that the rate of proteolytic excision of the collagen III amino-propeptide may partially limit collagen assembly and regulate fibril diameter (Keene et al., 1987). It has also been demonstrated that the N-propeptides of collagen III were present at the surface of collagen I fibrils that had apparently reached full growth (Fleischmajer et al., 1990). Studies also suggest that the presence of collagen III serves as a limiting factor in fiber growth during foetal development and in certain pathological conditions (Uitto et al., 1989). Further to this it was postulated that the N-propeptide may act as a chemical link between fibrils and non collagenous components of the ECM (Fleischmajer et al., 1990; Gaill et al., 1991).

Cartilage contains mixed fibrils, composed of collagens II, IX and XI in an 8:1:1 ratio. These collagens are found to be distributed uniformly along the fibril (Mendler et al., 1989). Collagen IX is distributed in a regular D-periodic arrangement along cartilage fibrils, with the chondroitin sulphate chain of collagen IX suggested to be in intimate contact with the fibril (Vaughan et al., 1988). Collagen XI may represent the fibril core as these molecules are buried within the fibril and immunologically masked. In chick embryo hyaline cartilage, fibril diameters are tightly controlled. They are thin (17 nm) and form an apparently random network entrapping large aggregates of proteoglycans and glycoproteins. Collagen X is also found in cartilage, but is restricted to the hypertrophic zone and thus may play a role in the transition of cartilage to bone. The co-assembly of different collagens during fibrillogenesis may be crucial in the regulation of fibril architecture and the modulation of fibril surface properties. While collagen II has an N-propeptide which is cleaved for its insertion into fibrils, collagen XI retains an N-terminal extension composed of a short triple helix and a globular domain. These domains are thought to contribute to the control of fibril lateral growth and diameter (Mendler et al., 1989; van der Rest et al., 1991).

The corneal stroma is a highly organised connective tissue in which rigid control of fibril diameter and the precise arrangement of collagen fibrils are essential for corneal
transparency (Birk et al., 1988; Mellor et al., 1991). Corneal collagen fibrils are of narrow uniform diameter (~25 nm) with a constant centre to centre spacing often with a regular orthogonal arrangement. The corneal stroma is considerably enriched in collagen V relative to other collagen I containing tissues and comprises approximately 20% collagen V. It has been demonstrated that collagens I and V are co-assembled within the same fibril in adult cornea, and that collagen V appears to represent the fibril core (Birk et al., 1988; Birk et al., 1990a; van der Rest et al., 1990). One consequence of the formation of these fibrils may be in regulating the lateral growth of fibrils, evidence from in vitro reconstitution studies appears to support this (Birk et al., 1990a).

Purified intact collagen V was found to form thin filaments with no apparent periodicity, while collagen I fibrils had a broad distribution of large diameters. Mixtures of collagens I and V produced fibrils with intermediate diameters, with the mean fibril diameter decreasing with increasing amounts of collagen V (Birk et al., 1990a). In the absence of the amino terminal globular domain of collagen V, the diameter regulatory activity was substantially reduced.

There are several ways in which a quantitatively minor collagen could function to regulate fibril diameter. Together with the persistence of the terminal globular region, collagen V has a longer helix. These features, although they may permit co-assembly of collagens I and V, may inhibit the further addition of molecules after a certain critical concentration of collagen V is reached. Another possibility is that collagen V forms many thin filaments that serve as nucleation sites for collagen I assembly. The presence of a large number of nucleation sites for a given quantity of collagen I may result in small fibril diameters. Although collagen V was shown to modulate fibril assembly, the fibrils which formed were substantially wider than the 25 nm fibrils characteristic of the corneal stroma (Birk et al., 1990a). Thus other factors such as tissue-dependent cellular control over the synthesis and mixing of components may regulate the final fibril morphology.
Most of the evidence for the co-assembly of different collagens within the same fibril has come from immunolocalisation studies. However covalent peptides containing intermolecular cross-linked sequences from collagens I and III as well as II and IX have been isolated and these further support the existence of heterotypic fibrils (Mendler et al., 1989; Fleischmajer et al., 1990). Thus while structural similarities allow the polymerisation of different collagens within single fibrils, structural differences among the collagens may incorporate specific steric information necessary for initiation of fibril growth and regulation of fibril diameter.

1.8.1.2.2 Procollagen Processing

A number of studies have indicated that the order and extent of procollagen processing can, at least in part, modulate collagen assembly and regulate fibril diameter. (Hulmes et al., 1989b; Mould et al., 1990). Both pN and pC-collagen have been shown to play a role in collagen fibrillogenesis in skin and bone (Fleischmajer et al., 1991b). Collagen, procollagen and both processing intermediates can undergo concentration-dependent supramolecular assembly. The nature of these assemblies are influenced by the preservation or removal of the propeptide domains. Persistence of the N or C-propeptide is associated with the formation of small or large diameter fibrils respectively (Fleischmajer et al., 1988). The persistence of the N-propeptide is also associated with the thin hieroglypic fibrils in dermatosporatic animals (Keene et al., 1987). On the basis of in vitro studies, it has been argued that the diameter of collagen fibrils may depend on the pathway (via pN or pC-collagen intermediates) utilised for processing procollagen to collagen (Fleischmajer et al., 1988; Mellor et al., 1991). For example, if the C-propeptide is removed first, subsequent treatment with N-proteinase results in the formation of thin fibrils, whereas, if the N-propeptide is removed first followed by treatment with C-proteinase, thick fibrils form (Fleischmajer et al., 1987). Hence by regulating the respective activities of both proteinases, cells may exert precise control over procollagen processing and thus regulate fibril morphology. The
preference for both N and C-propeptide domains to be located at the surface of collagen assemblies, suggests that these domains regulate fibril diameter in vivo by blocking surface sites on the growing fibril and hence prevent the further addition of molecules (Mould et al., 1990).

1.8.1.2.3 Proteoglycans and Glycosaminoglycans

As well as having a well-defined mechanical role of their own, such as the maintenance of tissue hydration and providing resistance to deformation of cartilage, it has been proposed that proteoglycans interact specifically with collagen fibrils and regulate their assembly and final diameter (Veis & Payne, 1988). In general, the higher the content of proteoglycan (PG) the more widely dispersed are the collagen fibrils. For example, the ordered array of PG along the collagen fibril has been suggested to stabilise the regular three dimensional arrangement within the cornea (Scott & Haigh, 1985a), whereas, in bone, the distribution of PG is different and may be important in calcification (Scott & Haigh, 1985b). It was postulated that the repulsion of negative charges on the glycosaminoglycan (GAG) chains orientates the protein core perpendicular to the longitudinal axis of the fibril, creating a negatively charged field around the fibre, and thus holds the fibres in defined orientations (Scott, 1991).

Biochemical and electron histochemical studies have shown that collagen fibrils are associated with keratan sulphate (KS), chondroitin sulphate (CS) and dermatan sulphate (DS) proteoglycans. Specific binding sites have also been localised along the fibril (Vaughan et al., 1988; Scott, 1991). It was concluded that DSPG’s are specifically associated with the fibril surface at the gap region and the other PGs are limited to the interfibrillar space.

The effect of PG and GAG components on fibril formation has been assessed during in vitro collagen reconstitution experiments. Due to differences in the nature of the collagen preparations used and the difficulty in fractionating and assessing the GAG and PG components, much of the early data has been difficult to interpret and is
often contradictory. Nevertheless it has been shown that the type of PG or GAG is important, and that each GAG exerts a different effect on the kinetics and extent of collagen turbidity increase (Obrink, 1973a, 1973b; Lilja & Barrach, 1983; Vogel et al., 1984). Obrink (1973a) found that chonroitin sulphate, dermatan sulphate, heparan sulphate, heparin and proteoglycans of chondroitin sulphate and dermatan sulphate accelerated the fibril assembly process when they were added before the initiation of fibrillogenesis. When added later in the lag phase, the proteoglycans markedly delayed fibril formation, while the GAGs (CS and HS) also inhibited fibril assembly but to a smaller extent. This last observation was ascribed to an inhibition of fibril growth from pre-formed nuclei. In a similar study, Oegema et al. (1975) correlated the binding of PG to collagen with its effects, as well as the point of addition of PG during fibril formation. It was found that when PGS (cartilage proteoglycan subunit) was added to collagen I early during the lag phase, fibril formation was markedly delayed, but addition of the same amount of PGS near the end of the lag phase did not further retard self-assembly. However the final turbidity of the collagen gel, related to fibril diameter, was consistently increased in the presence of PGS, independent of the time of addition. If the PGS was present in the solution before fibrillogenesis was initiated, binding of PGS to collagen I occurred at a maximum of 1 proteoglycan molecule to 25-30 collagen molecules. Taken together, these results suggest that the effect of PGS on collagen fibril assembly in vitro is related to a limited number of binding sites on early collagen filaments, as they are being organised into mature fibrils. Snowden and Swann (1984) found that the glycosaminoglycans (dermatan sulphate and hyaluronate) accelerate the process of fibril assembly, whereas chondroitin sulphate had little effect on the kinetics of self-assembly. Snowden and Swann (1984) also found that DSPG delayed fibril formation, and slightly decreased the thermal stability of collagen fibrils formed in the presence of this proteoglycan. The GAG alone had no significant effect on the thermal stability. Vogel and Trotter (1987) showed that the small DSPG from bovine tendon binds both collagens I and II, greatly prolongs the lag phase and results in collagen
fibrils that are significantly thinner in width. This interaction depends on the core protein, since the GAG chains alone have no effect on the assembly, whereas the intact core protein had an identical effect to the small proteoglycan. Large cartilage proteoglycan had no effect on collagen assembly. The small proteoglycan decorin (PG-II), which is widely distributed in a variety of connective tissues (Jarvelainen et al., 1991), binds to the surface of collagen fibrils and may regulate fibril diameter. In a recent study it was reported that DSPGs from eroded osteoarthritic cartilage produced a longer lag phase for type II collagen self assembly than DSPGs isolated from normal cartilage (Karvonen et al., 1992). On the basis of these in vitro studies, it was suggested that changes in the proteoglycans of osteoarthritic cartilage may influence collagen fibrillogenesis in vivo. Defective proteoglycans may lead to abnormal collagen fibril formation and result in a weakened cartilage architecture, thus contributing to an accelerated process of cartilage degeneration (Karvonen et al., 1992). Link protein, a glycoprotein involved in the assembly of PG aggregates, also modulates the kinetics of assembly and modifies the resultant fibres (Chandrasekhar et al., 1984; Smith et al., 1987). Neither link protein or the small DSPG alone had an effect on the self assembly kinetics or the size of the fibrils, but in combination there was a decrease in the rate of fibril formation and the production of wider fibrils.

1.8.1.2.4 Sulphated Proteins

Fibromodulin, a 59 K collagen binding protein and a ubiquitous component of the ECM, appears to have a role in the regulation of collagen fibril assembly (Hedbom & Heinegard, 1989; Antonsson et al., 1991). The amino acid sequence deduced from its cDNA predicts a 42 K protein, consisting largely of homologous 23 amino acid repeats with predominantly leucines in conserved positions. Similar repeats have been identified in the small interstitial proteoglycans decorin and PGS1. In decorin the repeats could be responsible for the binding to collagen or to the cell surface. The leucine repeat in fibromodulin also suggests a role in collagen binding (Oldberg et al.,
Tyrosine sulphate residues were identified in fibromodulin, these sulphated residues being located at the N-terminus (Antonsson et al., 1991). Fibromodulin from tendon, cartilage and sclera contains N-glycosidically-linked oligosaccharides, some of which are extended to keratan sulphate chains. Thus the discrepancy between the molecular weight of 59 K previously determined by SDS-PAGE and the predicted molecular mass of 42 K is probably due to anomalies in the electrophoretic behavior of the protein as a result of carbohydrate substitution. Both fibromodulin and decorin bind to collagens I and II and inhibit collagen fibrillogenesis in vitro, and the fibrils which form in the presence of decorin are thinner. The fibromodulin binding site on the collagen molecule has not been localised but appears to be different to the decorin binding site, since both molecules have an additive effect on collagen fibrillogenesis (Oldberg et al., 1989).

Fibronectin, a sulphated protein, also modulates the kinetics of collagen self assembly but the effect is minimal. This has been attributed to a passive dilution of the collagen-collagen interactions, the equilibrium constant for fibronectin binding is low compared with the self association constant for collagen molecules (Speranza et al., 1987).

1.8.1.3 Fibrillogenesis in Vivo

Molecular interactions driven by physicochemical forces are important during the in vitro polymerisation of collagen I molecules into fibrils. However it has not yet been possible to assemble in vitro a functional group of fibril bundles (fibrils) organised into tissue specific macro aggregates such as regular layers in bone and cornea, irregular layers in skin, and cables in tendons and ligaments (Ploetz et al., 1991). It remains to be firmly established whether collagen fibrils form at the cell surface or deeper into the ECM. It has been suggested that 0D or segment-long-spacing (SLS) packets of collagen molecules may form D-periodic fibrils by stacking of the SLS assemblies in 1D shifts (Hulmes et al., 1983). Radiographic studies on the fibroblasts of the rat foot
pad revealed that after approximately 50 minutes of labelling, label appeared "over" collagen fibrils, indicating that collagen was being added to existing fibrils rather than forming new ones (Marchi & Leblond, 1983). More recent studies employing three dimensional reconstructions from electron micrographs of tissue serial sections suggests that there is a discontinuous assembly of fibrils from smaller fibril segments (Birk et al., 1990b; Ploetz et al., 1991). These fibril segments of around 10 μm in length are assembled via linear and lateral fusions to form a functionally continuous fibril. It has also been postulated that the fusion process is regulated by a surface coat of DSPG. Such discontinuous assembly may also allow for angular rearrangement, intercalatory growth and even repair (Birk et al., 1990b). Collagen fibril formation in situ appears to take place in specific cell-defined compartments. During morphogenesis in both cornea and tendon, the fibroblast partitions the extracellular space into compartments of increasing size containing collagen fibrils, fibril bundles and major aggregates. This compartmentalisation allows for cellular control over post-depositional events which may be important in determining the spatial orientation of regional matrices (Ploetz et al., 1991).

Although it is conceivable that the process of fibril assembly is regulated by the amount of available procollagen, a combination of factors may be involved in regulating fibril structure. It was already observed that there was an 8 nm increment in the diameter distribution in a variety of connective tissues (Parry & Craig, 1979). A model which accounted very plausibly for the 8 nm increments was proposed by Hulmes (1983) and depended on the presence of quasi-hexagonal packing of the molecules. Hulmes suggested that a surface layer of pN-collagen molecules may sterically restrict any further increase in fibril diameter, temporary activation of N-proteinase may then remove the propeptides and allow fibril growth by accretion of further pN-collagen molecules. Either these pN-molecules are freshly secreted into the extracellular compartment or N-proteinase preferentially acts on an aggregated substrate, in which case pN-collagen monomers in the interfibrillar space may not be
significantly processed during the short burst of enzymic activity. Following a further burst of N-proteinase activity, growth continues as a further layer of pN-molecules are laid down in axial register on a quasi-hexagonal lattice with every 4 nm (the breadth of the unit cell) increase in radius, this process continues until a limiting diameter is reached. This model is supported by the observation that the N-propeptides tend to be associated with thin diameter fibrils and hence are thought to be involved in limiting lateral growth (Chapman, 1989). However as pointed out by Chapman (1989) the diameters quoted by Parry & Craig referred to dehydrated tissue, hence the fibril increment in living tissue will be proportionally larger. X-ray diffraction data obtained from hydrated specimens indicate that fibril diameters are multiples of around 11 nm. Therefore an acceptable model has to account for 5-6 nm increments in radius, although no spacing with such high values occur in the quasi-hexagonal lattice.

Chapman (1989) suggested that a growth inhibitor, possibly a proteoglycan, forms a complete circumferential layer around the fibril surface. Cell mediated removal of the inhibitor then allows growth to proceed to a second limiting layer and so on to subsequent limiting layers. Although the mechanism depends on axial order it does not require any specific lateral packing of the molecules.

1.9 The Extracellular Matrix of Skin

Collagen is the major component of skin and represents over 70% of the tissue dry weight. Six different collagens (I, III, IV, V, VI & VII) have been detected in human skin, and these and several other collagens have been shown to be synthesised by cultured human skin fibroblasts in vitro (Uitto et al., 1989). Collagens I and III represent the major interstitial fibril-forming collagens, and, although collagens IV, V, VI and VII are quantitatively minor collagens in skin, they may represent major components in specific regions within the tissue, and they probably have major roles in maintaining tissue structure and integrity. Collagens form a structural framework underlying the epidermis, with which other connective tissue macromolecules interact.
(e.g. elastin, fibrillin, proteoglycans, laminin, nidogen (entactin), fibronectin). Such interactions are necessary for the provision of tissue strength and flexibility (Uitto et al., 1989).

In adult human skin, the ratio of collagen I : III is approximately 6 : 1. In contrast, foetal skin has a higher proportion of collagen III and accounts for over half of the total collagen, suggesting that collagen biosynthesis is developmentally regulated (Grant & Ayad, 1988). Collagen IV is a major component of basement membranes in the dermal/epidermal junction, and differs from collagens I and III by virtue of several non-collageneous segments which interrupt the central triple helix (Fig. 1.1). This feature provides the molecule with enhanced flexibility and permits the assembly of a meshwork-like structure to which other components may interact (Fox et al., 1991). The function of collagen V is poorly understood, although it has been reported that the continued synthesis of this quantitatively minor collagen is a prerequisite for the migration of epidermal cells (Stenn et al., 1979). The beaded filamentous structure characteristic of collagen VI (Fig. 1.1) is highly disulphide bonded, and the presence of large numbers of RGD sequences within the collagen helix suggests that collagen VI plays a key role in cell-matrix interactions (Ruoslahti, 1991a; van der Rest & Garrone, 1991). Collagen VII is a predominant component of anchoring fibrils (Fig. 1.1), structures that extend from the dermal/epidermal junction to the underlying dermis. These fibrils often entrap interstitial collagen fibrils and appear to anchor the basement membrane to the dermis (Uitto et al., 1989; Burgeson et al., 1990).

1.10 Introduction to the Present Study

The number of recognised ECM components has increased rapidly in recent years (Hay, 1991). However, as pointed out by Neame et al. (1989), there are many small molecular weight components of less than 50 K that have not been characterised in detail. In many preparations of lysyl oxidase (Sullivan & Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986; Shackleton & Hulmes 1990a), the enzyme (30-34 K)
is contaminated with one or more low molecular mass proteins (22-24 K). These contaminants have been suggested as being either degradation products of lysyl oxidase (Sullivan & Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986) or unrelated proteins (Kuivaniemi et al., 1984). When lysyl oxidase (34 K) from porcine skin was purified by selective interaction with Sephacryl S-200, a 24 K protein was found to co-purify (Shackleton & Hulmes 1990a). The main aims of this study were to characterise both lysyl oxidase and the 24 K protein (Chapter 3), and to examine the effects of these proteins on collagen fibril formation (Chapter 4).

It is shown that the 24 K protein has no enzymic activity on an elastin substrate, however like lysyl oxidase, several variants of 24 K protein can be separated by Mono Q FPLC. The protein is particularly acidic (pI 4-4.5) and relatively rich in tyrosine, so the name TRAMP (Tyrosine Rich Acidic Matrix Protein; Cronshaw et al., 1993) is used to identify it. No amino acid sequence homology was found between TRAMP and the rat lysyl oxidase sequence recently deduced from cDNA (Trackman et al., 1990; Trackman et al., 1991), but TRAMP appears to be the same as a 22 K extracellular matrix protein from bovine skin which co-purifies with dermatan sulphate proteoglycans (Neame et al., 1989). By amino acid sequence analysis, both TRAMP and the 22 K protein were found to be identical in all but 4 residues (Cronshaw et al., 1993). TRAMP appears to contain sulphated tyrosine residues, and seems to promote cell attachment (Lewandowska et al., 1991). The present study demonstrates that TRAMP binds to collagen I and modulates the process of fibril assembly (Chapter 4).

Although lysyl oxidase does not appear to be glycosylated (Sullivan & Kagan, 1982), an N-glycosylation site was found in the sequence encoding the 32 K protein (Trackman et al., 1990; Trackman et al., 1991; Kagan & Trackman, 1991). By SDS-PAGE porcine lysyl oxidase often appears as a 34-36 K doublet. Preliminary data indicates that there may be two structural forms of the enzyme, with N-linked carbohydrate bound to the 36 K form of the enzyme.
Chapter 2

Materials and Methods
2.1 Purification of Skin Collagen

2.1.1 Reagents

Male Sprague-Dawley rats were supplied by Banton & Kingman, Universal Ltd, Field Station, Grimston, Hull. PMSF, NEM and BAPN (fumarate salt) were obtained from Sigma (Poole, Dorset). EDTA was supplied by BDH/Merck Ltd (Poole, Dorset). DEAE-Sephacel was obtained from Pharmacia/LKB (Milton Keynes, Bucks). All other reagents (analytical grade), unless stated otherwise, were supplied by BDH.

2.1.2 Purification Procedure

The method used was a modification of that of Payne et al. (1986). Semi-sterile conditions were used throughout, i.e. dissection instruments were autoclaved prior to use, and phosphate buffers were filtered through a Millipore 0.22 µm disc filter. All procedures were carried out at 4 °C to minimise bacterial growth, enhance collagen solubility and ensure retention of the native collagen conformation.

Twelve 21-day-old male Sprague-Dawley rats were fed a diet of rat chow and water. The water contained 0.17 % (w/v) BAPN, which renders the animals lathyritic through the inhibition of collagen cross-linking, thus increasing the extractability of native monomeric collagen. The activity of the lathyrogen with respect to cross-linking may be ascribed to an irreversible inhibition of lysyl oxidase (Siegel et al., 1970). Following a three week exposure to BAPN, the rats were sacrificed and their skins removed, dehaired, and pieces of adhering muscle and fat removed. The skins were diced, then homogenised in a Waring blender for three times ten seconds at high speed. The skins were then extracted for 1 hour under cold neutral salt conditions (3 ml/g tissue) using a buffer whose composition was 0.15 M NaCl, 5.4 mM Na₂HPO₄, 1.1 mM KH₂PO₄, pH 7.4, containing enzyme inhibitors, 25 mM EDTA, 1 mM PMSF, 10 mM NEM and 1 µg/ml pepstatin to inhibit each of the four recognised classes of proteinases (Barrett, 1980). (In general fresh proteinase inhibitors were added throughout the purification from concentrated stock solutions.) The buffer also
contained 50 μg/ml BAPN and 0.01 % NaN₃. Neutral salt solutions preferentially extract collagen molecules that are not bound within the fibres through covalent intermolecular cross-links.

Following centrifugation at 10,000 g in a Beckman J2-20 centrifuge for 30 minutes, the supernatant was collected and filtered through several layers of cheesecloth to remove fatty material, and diluted if necessary before the first salt precipitation. Salt precipitation was induced by the slow addition of NaCl to the solution to give a final salt concentration of 2 M (to precipitate collagen III). The solution was left to stand for 1 hour after which it was centrifuged (as above) and then NaCl was added to the supernatant to give a final salt concentration of 3.5 M. The solution was again left to stand for 1 hour, after which the resulting suspension was centrifuged. The white precipitate was collected and gently homogenised in a small volume of phosphate buffer (62 mM Na₂HPO₄, 14.5 mM KH₂PO₄, pH 7.4). The homogenate was then allowed to redissolve in a larger volume of the same buffer. After centrifugation at 100,000 g in a Beckman L60 centrifuge for 4 hours to remove the insoluble material, a second salt precipitation was carried out at 3.5 M NaCl and the precipitate redissolved in 0.2 M NaCl, 0.05 M Tris-HCl, pH 7.5 (DEAE start buffer). The solution was dialysed against two changes of this buffer, and then clarified by centrifugation at 10,000 g for 30 minutes prior to chromatography on DEAE-Sephacel.

A column of DEAE-Sephacel (2.6 x 10 cm) was prepared and packed at a flow rate of 50 ml/hour and equilibrated with DEAE start buffer. The partially purified collagen solution was then applied to the column at a flow rate of 20 ml/hour. After application of the sample, elution with start buffer was continued until no further UV-absorbing material was eluted from the column, as monitored by a two channel 276 nm UV detector (uvicord) and chart recorder. Under these conditions, the relatively basic collagen molecules are not retained by DEAE-Sephacel. Acidic contaminants were retained, which were subsequently eluted with 2 M NaCl, 0.05 M Tris-HCl, pH 7.5 (Miller, 1971). The column was then stored in this buffer which contained 0.002 % hibitane as preservative.
The collagen solution was then dialysed exhaustively against 0.02 M Na₂HPO₄; the white precipitate that formed during dialysis was collected by centrifugation at 10,000 g (as above) and then redissolved in 0.5 M acetic acid. This acid solution was then dialysed against the same solvent. After dialysis, the appropriate volume of prechilled 20 % (w/v) NaCl solution was slowly added to the stirred acid solution to give a final salt concentration of 3.5 % (w/v). The solution was left to stand for 15 minutes. Salt precipitation from an acid solution of collagen was included to preferentially remove large aggregates of collagen, if present (Chandrakasan et al., 1976). The precipitated solution was centrifuged and the supernatant dialysed against 0.5 M acetic acid, followed by dialysis against several changes of 5 mM acetic acid. Finally the solution was centrifuged at 100,000 g for 4 hours to produce a clear solution of collagen for immediate use or storage at -70 ºC (for up to 3 months) and re-centrifugation (100,000 g, 4 h) immediately before use. By hydroxyproline analysis (section 2.3.3), the yield of type I collagen from the skins of twelve rats was typically 60-90 mg.

Amino acid analysis, SDS-PAGE and electron microscopy of reconstituted fibrils, were used to determine the quality and suitability of the preparation for fibril formation studies.

2.2 Purification of TRAMP and Lysyl Oxidase

2.2.1 Reagents

Stillborn piglets were obtained from the Animal Breeding Research Organisation, Dryden-Mountmarle Field Laboratory, Roslin, Midlothian. Chromatography media (DEAE-Sephacel, CM-Sepharose CL-6B, Sephacryl S-200 and Sephadex G25 medium grade) were from Pharmacia/LKB. AG 501-X8 mixed bed resin was obtained from BioRad. All other chemicals (analytical grade) unless stated otherwise were from BDH.
2.2.2 Purification Procedure

Prior to the finding that the bulk of tissue lysyl oxidase was rendered soluble by extraction buffers containing 4 to 6 M urea (Narayanan et al., 1974), purification of lysyl oxidase had been a difficult task often yielding preparations that were significantly contaminated by other proteins (Kagan, 1986). Urea-extractable enzyme has since been highly purified from bovine aorta and lung (Kagan et al., 1979; Cronlund & Kagan, 1986), human placenta (Kuivaniemi et al., 1984), and rat lung (Almassian et al., 1990). In human tissues, Kuivaniemi (1985) demonstrated the richest source of lysyl oxidase was skin. The enzyme is stable in urea-containing buffers, while chromatographic recoveries of the enzyme are also improved (Narayanan et al., 1974). The enzyme is, however, inactive in urea, though activity is restored after dialysis into physiological buffers.

The method initially used was a modification of a new procedure for the purification of lysyl oxidase, employing ion-exchange chromatography and selective interaction with Sephacryl S-200 (Shackleton & Hulmes, 1990a). The extraction and purification steps were carried out under semi-sterile conditions, between 0 and 4 °C. In a typical purification, the skins from 10 stillborn piglets were removed within 24 hours of death. The skins were then either immediately utilised or stored at -30 °C for up to 1 week until required. Diced skin (approximately 900 g wet wt.) was fed through a pre-chilled fine mincer before homogenisation in PBS (0.1 M sodium phosphate, 0.15 M NaCl, 1 mM PMSF, pH 7.5) at 3 ml/g, in a Waring blender for 3 x 10 seconds at high speed. After centrifugation at 10,000 g for 20 minutes, the supernatant was discarded. The extraction was repeated once and the pellet was washed three times in PB (20 mM sodium phosphate, 1 mM PMSF, pH 7.5) at 3 ml/g with centrifugation as above. The pellet was then extracted overnight with 6 M PBU (PB containing 6 M urea, pH 7.5) at 1 ml/g of original material, and this step repeated once. After centrifugation, the extracts were pooled and passed through several layers of cheesecloth, followed by a second filtration through Whatman no. 1 filter paper before the first chromatographic step. (To
remove cyanate, all urea-containing buffers were prepared from fresh stock solutions of 8 M urea that were de-ionised by passage through a column containing AG 501-X8 mixed bed resin. The buffers were then kept at 4 °C to prevent reformation of cyanate."

The extract was loaded onto a 2.6 x 40 cm column of CM-Sepharose CL-6B, previously equilibrated with 6 M PBU, at a flow rate of 50 ml/hour. The flow through solution was then immediately applied to a 2.6 x 40 cm column of DEAE-Sephalcel, previously equilibrated in 6 M PBU, at a flow rate of 50 ml/hour. The DEAE-Sephalcel column was then washed with 6 M PBU to elute unbound proteins, until a steady reading at 280 nm was obtained. Bound proteins were eluted at 40 ml/hour with 6 M PBU containing 0.5 M NaCl, and collected as 10 ml fractions. (Lysyl oxidase is not eluted from DEAE using neutral-salt buffers in the absence of urea. While enzyme binding to DEAE doubtlessly involves ionic interactions with the cationic matrix, the requirement for urea implies that other nonionic, possibly hydrophobic bonds are also involved.) Protein bound fractions were pooled and dialysed exhaustively against 10 mM PB, pH 7.5.

The urea-free protein from the DEAE-Sephalcel column was clarified by centrifugation at 10,000 g for 30 minutes, and then loaded on a 2.6 x 60 cm column of Sephacryl S-200, previously equilibrated with 10 mM PB, at a flow rate of 80 ml/hour. The column was extensively washed with 10 mM PB, before elution at 20 ml/hour with 6 M PBU. The Shackleton and Hulmes (1990a) protocol did not produce mainly lysyl oxidase but instead produced both TRAMP and lysyl oxidase, with TRAMP being the major component, and it could be partially separated from lysyl oxidase by Mono Q FPLC (see results section 3.1.1 for improvements to the purification protocol). The purified proteins were then used immediately or stored at -30 °C. The ultrafiltration assay (section 2.4.3) for lysyl oxidase activity and SDS-PAGE analysis (section 2.3.4) was carried out on the preparation to assess its suitability for further experiments.
2.3 Characterisation of Collagen

2.3.1 Amino Acid Analysis

The purity of collagen preparations were determined by comparing the amino acid composition with the known composition of type I collagen. Glycine is the most abundant amino acid in collagen (approximately 330 residues/1000). Therefore the glycine content of a collagen preparation provides a reliable figure for assessing sample purity.

For fibril formation studies, it is important that both the N and C-terminal telopeptides remain intact following purification, since it has been shown in vitro that these extrahelical extensions are necessary for orderly growth and packing of native, uniform-diameter, polarised D-stagger fibrils (Veis & Payne, 1988). The telopeptides of collagen I contain all 11 tyrosine residues found in the collagen molecule. Therefore the presence of the telopeptides can be indicated by measuring the tyrosine content.

2.3.1.1 Procedure

Collagen was hydrolysed in 6 N hydrochloric acid at 110 °C for 24 h, in sealed evacuated tubes. After hydrolysis, acid was removed in a vacuum desicator containing NaOH pellets, then the samples were re-dissolved in 0.025 % K$_2$EDTA prior to analysis. Amino acid analysis was carried out (by Mr A D Cronshaw) on an Applied Biosystems 420A Derivatiser with an on-line model 120A phenylthiocarbamyl (PTC) analyser. The PTC-amino acids were separated at 38 °C on an Applied Biosystems PTC C-18 column (5 μm particle size; 2.1 x 220 mm) at a flow rate of 0.3 ml/min. The column was previously equilibrated with 50 mm sodium acetate (pH 5.4), and elution was achieved by a gradient to 70 % (v/v) acetonitrile in 32 mM sodium acetate (pH 6.1) over 20 min. The eluate was monitored by absorbance at 254 nm.

2.3.2 Cyanogen Bromide Chemical Cleavage

Hydrolysis of the polypeptide backbone is a prerequisite for any peptide
mapping strategy and will determine the type and amount of information that can be obtained from the map. For example limited proteolysis of a protein using a protease of broad specificity may provide valuable information on the accessibility of a small number of the total possible sites for cleavage, and hence on the conformation of the protein. Sites may be more accessible because they are in an exposed stretch of polypeptide linking two structured regions or domains. Hydrolysis methods for peptide maps must produce peptides ranging in size from a few residues to larger structured fragments of several hundred residues.

2.3.2.1 Procedure

Pure fresh cyanogen bromide (CNBr) was used to cleave collagen specifically on the C-terminal side of methionine residues (Gross & Witkop, 1962). In order for collagen to undergo complete digestion with CNBr, it was necessary to first reduce any methionine sulfoxide present to methionine (Hill & Harper, 1984). Unless this was done, a large percentage of material was incompletely cleaved, particularly in the case of collagen III, so that inconsistent peptide maps were obtained. The collagen was reduced by the method of Adelstein and Kuehl (1970). Lyophilised collagen (1 mg) was reduced using a 15-fold molar excess of 2-mercaptoethanol in 8 M urea, 0.1 M NH₄HCO₃, pH 8.0. The thiol was then removed by dialysis against 0.1 M acetic acid (the low pH also minimises reoxidation of the protein; Jaenicke & Rudolph, 1989) and the collagen freeze dried. The reduced collagen (0.5 mg) was then dissolved in 100 μl of 70 % (v/v) aqueous formic acid. After transferring the sample to a fume cupboard, a 50-fold molar excess over methionine residues of CNBr in 20 μl of 70 % formic acid was added with gentle shaking. The mixture was bubbled through with oxygen-free nitrogen (to prevent reoxidation) and sealed tightly in a 10 ml polythene centrifuge tube. The sample was protected from light and gently agitated for 24 h at room temperature. Following the digestion period, a 15-fold excess of water was added to slow down the reaction and improve subsequent peptide recovery. The material was then dried in a Savant Speed Vac vacuum centrifuge, which was connected to a chemical trap.
containing 2 M NaOH to remove any HCN present. For complete removal of the acid, a further addition of water followed by lyophilisation was necessary. The dried samples were stored at -20 °C until required for SDS-PAGE.

2.3.3 Hydroxyproline Assay

4-Hydroxyproline and 3-hydroxyproline are found almost exclusively in collagens and a few other proteins with collagen-like amino acid sequences (Adams & Frank, 1980). So the presence of hydroxyproline provides a characteristic biochemical marker. The measurement of hydroxyproline has been used to determine not only the quantity of collagen in a given tissue, but also the rate at which the collagen is synthesised or degraded in such tissues.

The colorimetric assay for hydroxyproline was a modification of that of Woessner (1961). The reaction is based on the oxidation of hydroxyproline to pyrrole which is then allowed to react with p-dimethyl-aminobenzaldehyde to form a complex that is detected and quantified as a chromophore having an absorbance at 557 nm. The reagents are as follows:

**Buffer.**
50 g of citric acid monohydrate, 12 ml of glacial acetic acid, 120 g sodium acetate trihydrate, and 34 g sodium hydroxide are made to a final volume of 1 l in distilled water, and adjusted to pH 6.0.

**Chloramine T.**
A 0.05 M solution was prepared on the day of use by dissolving 1.41 g of chloramine T in 20 ml water. 30 ml of 2-methoxy ethanol and 50 ml buffer were then added. The solution was kept in a stoppered flask.

**Ehrlich’s Reagent.**
A 20 % solution was prepared shortly before use by adding 2-methoxyethanol to 20 g
of p-dimethyl-aminobenzaldehyde to give a final volume of 100 ml. The solution was warmed to facilitate solubilisation.

2.3.3.1 Procedure

Freeze-dried collagen samples were hydrolysed in 6 M hydrochloric acid in 10 ml pyrex tubes, sealed with teflon-lined screw caps, for 16 h at 116 °C. After hydrolysis, the hydrochloric acid was removed by evaporation to dryness in a Savant Speed Vac vacuum centrifuge. Each sample was then dissolved in 2 ml distilled water. A series of dilutions were usually necessary to obtain a sample containing 1-5 µg of hydroxyproline in 2 ml. Hydroxyproline standards containing 0-5 µg each in 2 ml were prepared from a stock solution containing 5 mg/ml.

To each sample in order, 1 ml of chloramine T was added to initiate hydroxyproline oxidation. The tube contents were then vortexed and allowed to stand for 20 minutes. The chloramine T was then destroyed by adding 1 ml of a 3.15 M solution of perchloric acid to each tube in the same order as before. The contents were mixed and allowed to stand for 5 minutes. Finally, 1 ml of Ehrlich's reagent was added. After vortexing, the tubes were placed in a water bath at 60 °C for 20 minutes, and then cooled with tap water. The developed colour is stable for at least 1 hour. The absorbance of the solution was determined using an LKB Ultraspec spectrophotometer (1 cm light path). The hydroxyproline values for collagen samples were then determined directly from a standard curve (Fig. 2.1). A value of 10 was used as the constant of proportionality to relate hydroxyproline concentration to collagen concentration (Bornstein & Sage, 1980).

2.3.4 SDS-PAGE

In gel electrophoresis, separation of molecules results from both differences in charge and in size. However only the molecular size is relevant when protein samples containing 2% SDS and 3% β-mercaptoethanol are denatured by heating at 100 °C for 3 min. Disulphide linkages are broken by the mercaptoethanol, the anionic detergent
Figure 2.1 Hydroxyproline standard curve
Colour response curve showing the absorbance at 557 nm generated from hydroxyproline standards (0-6 μg) when treated in the assay of Woessner (1961). Error bars are standard deviations (n=5).
unfolds the native protein, binds to it, and confers a charge proportional to the length of the polypeptide chain. The charge density and the electrophoretic mobility are nearly constant for most proteins, the exception being glycoproteins. Separation due to size depends on the pore size of the gel matrix. The pore size of polyacrylamide gels decreases as the concentration of gel monomer increases. However it is also affected by the extent of cross linking. For a separation range from approximately 200 K to 60 K or 100 K to 15 K, 6 % acrylamide/0.16 % bis-acrylamide or 12 % acrylamide/0.32 % bis-acrylamide in the separating gels were chosen respectively (see Appendix I). When the size range of the sample components was too wide to be sieved by a gel of a single pore size, a gradient separating gel was used.

2.3.4.1 Procedure

The most widely used discontinuous system for electrophoresis of proteins is that described by Laemmli (1970) and was the system used throughout this study. Gels (16 x 14 x 0.15 cm) were cast between 2 glass plates. Gradient gels were cast with the aid of a gradient mixer and peristaltic pump at a flow of 200 ml/h. The solutions were precooled to slow down polymerisation during casting. Prior to electrophoresis, protein sample solutions had one-quarter the volume of 5 x sample buffer added, producing a final buffer composition of: 25 mM Tris-HCl, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol blue, pH 6.8. When protein samples were run under reducing conditions, 3 % (v/v) β-mercaptoethanol was also present. Samples were boiled for three minutes before being applied to the stacking gel. Gels were run on a vertical LKB electrophoresis unit at a constant current of 40 mA per gel (this corresponded to a maximum power of 25 W in each gel). The unit was equipped with water cooling, and the lower buffer chamber was stirred continuously. The duration of the run using bromophenol blue as a tracking dye was generally 3-4 h. When high resolution was not required, for example the screening of a purification procedure, mini gels (7.5 x 8.5 cm) were run on an LKB midget electrophoresis unit with water cooling. Up to 5 (0.75 mm) gels could be set up using the LKB multicast unit, and
because the gels were thinner and smaller the run time was much shorter, as were staining and destaining times. Gels were fixed and stained using several methods which are described in section 2.4.8. Photographs of all gels were taken on polaroid 665 film (positive and negative). When necessary gels were scanned in the wet state as described in section 2.3.5.

A plot of log molecular weight for calibration proteins (Pharmacia) as a function of electrophoretic mobility was used as a standard curve for the determination of sample protein molecular mass.

2.3.4.2 Transfer of Proteins from Gel to Membrane

The binding of electrophoretically separated proteins from polyacrylamide gel to a surface matrix (usually nitrocellulose) permits detection of specific proteins by antibodies. In addition methods have been described for the localisation of glycoproteins on nitrocellulose membrane using lectins as probes (O'Shannessy et al., 1987; Haselbeck et al., 1990).

The most widely used technique for transferring proteins onto a membrane support employs electrophoretic elution, sometimes referred to as electro-blotting. This procedure provides a rapid and more complete transfer of protein compared to the simple blotting methods originally described (Andrews, 1986). It is necessary however to select a pH such that all species will be charged and will migrate towards the same electrode. Methanol should also be present in the blotting buffer as it is necessary for efficient binding of the protein to the nitrocellulose. Once transferred, the proteins appear to be held non covalently to the membrane probably through hydrophobic contacts (Andrews, 1986).

Immediately following SDS-PAGE, separated proteins were electro-blotted onto a nitrocellulose sheet (pore size 0.45 μm; Schleicher & Schuell). Electrophoretic elution was achieved by complete immersion of a gel-membrane sandwich into precooled blotting buffer (20 mM sodium phosphate, 20 % (v/v) methanol, pH 9.4). The gel-membrane sandwich was assembled as follows:
A nitrocellulose sheet (14 x 9 cm) was soaked in blotting buffer and applied directly to the gel (It is important that no air bubbles are trapped between the membrane and the gel, as these greatly reduce transfer efficiency. Also the position of the membrane should not be altered once it is in place, as substantial amounts of protein may have already transferred.) This in turn is supported by two sheets of wet Whatman 3 mm filter paper, one on each side of the gel-membrane. A porous pad (Scotch Brite) presoaked in blotting buffer was then placed on each side of the sandwich, next to the filter paper. Finally the assembly was mounted on a stiff plastic grid (which holds the gel and membrane in close contact) and placed into a transblot cell (Biorad) filled with blotting buffer, ensuring that the nitrocellulose is nearest to the positive anode.

The proteins are transferred from a negative to a positive direction by applying a constant current of 250 mA over 18 hours. To monitor the efficiency of transfer, the blotted gel was stained with Coomassie Blue (section 2.4.8.1) and the blot stained for 1 minute with 0.4 % (w/v) Ponceau S (Sigma) in 3 % (w/v) TCA. Ponceau S binds rapidly to protein and because staining is reversible it is compatible with most antigen visualisation techniques. Destaining of the protein bands is achieved by washing the blot in tris buffered saline (TBS; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5).

2.3.5 Densitometry

To quantify the results of SDS-PAGE separations, stained gels were scanned using a Joyce-Loebl Chromoscan 3 coupled to a DCS microcomputer for digital analysis. The Chromoscan was operated in the absorbance mode with an aperture of 0.3 x 5 mm. The light source was a 100 W tungsten halogen lamp, and scans were carried out with a red (626 nm) filter for blue stained gels and a green (530 nm) filter for PAS stained gels. Before calculating the integrated peak areas, a background subtraction was made.
2.4 Characterisation of TRAMP and Lysyl Oxidase

2.4.1 Bicinchoninic Acid Protein Assay

Protein concentrations were measured using bicinchoninic acid (BCA) first described by Smith et al. (1985). This method combines the reaction of protein with Cu$^{2+}$ in an alkaline medium (yielding Cu$^{1+}$) namely BCA. The purple reaction product formed by the interaction of 2 molecules of BCA with one cuprous ion (Cu$^{1+}$) is water soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein in aqueous solution. The main advantages of the assay include compatibility with ionic and non-ionic detergents, a stable working reagent and less protein-to-protein variation compared to other methods.

2.4.1.1 Procedure

Protein concentrations were measured using the BCA protein assay (Pierce). Working reagent was prepared by adding 1 part solution B (4 % copper sulphate) to 50 parts of solution A which contains the BCA detection reagent. Each standard (BSA) or unknown protein (0.1 ml) was placed in a test tube. For blanks, 0.1 ml of the protein diluent was used. Working reagent (2 ml) was then added to each sample, and mixed well. After incubation at 60 °C for 30 minutes, the tubes were cooled and absorbance at 562 nm measured. Protein concentrations for each unknown were determined using a standard curve of varying BSA concentrations (Fig. 2.2).

Using the above method collagen concentrations could be measured, which was not possible using the assay by Bradford (1976). Presumably the increased temperature used during the assay denatures the collagen allowing an enhanced reaction of the peptide bonds with divalent copper.

A standard curve of varying collagen concentrations was used to determine the amount of collagen in purified preparations (Fig. 2.2). The results were comparable to the hydroxyproline assay for collagen, making the BCA assay the most convenient method for measuring collagen in purified preparations.
Figure 2.2 BCA assay response for collagen and bovine serum albumin
The standard curves shown for the BCA assay were obtained using the 60 °C/30 min protocol (Pierce, manufacturers instruction). Error bars are standard deviations (n=3).
2.4.2 Mono Q FPLC

To test whether lysyl oxidase and TRAMP contained different ionic variants, a procedure similar to that of Burbelo et al. (1986) was used, employing anion exchange FPLC.

Using a Gilson HPLC system at room temperature, lysyl oxidase and TRAMP preparations were separated on a Pharmacia Mono Q HR 5/5 column. Prior to sample application the column was sequentially washed with 5 ml of 6 M TBU, (20 mM Tris-HCl, containing 6 M urea, pH 7.5) followed by 10 ml of 6 M TBU containing 1 M NaCl. Finally, the column was equilibrated in 6 M TBU. Samples were loaded in 6 M TBU and, after washing the column with 6 M TBU, bound proteins were eluted at 1 ml/min with a 20 ml linear gradient 0 to 1 M NaCl in 6 M TBU. Protein was monitored by absorbance at 280 nm and peak fractions were collected and stored at -30 °C. All solutions were prepared from HPLC or analytical grade reagents, and buffers containing urea were prepared from freshly deionised stock solutions of 8 M urea. All buffers and samples were filtered and degassed using 0.22 μm filters.

2.4.3 Ultrafiltration Assay for Lysyl Oxidase

2.4.3.1 Preparation of Elastin Substrate

2.4.3.1.1 Materials

Fertile hen eggs were supplied by Ross Breeders, Newbridge. [4, 5-3H] Lysine (1.18 TBq/mol) was obtained from Amersham. BAPN (fumarate salt) was from Sigma; MEM Select-Amine kit was from Gibco. Unless stated otherwise all other chemicals (analytical grade) were from BDH.

2.4.3.1.2 Procedure

Lathyritic [4,5-3H] lysine-labeled elastin was prepared, using essentially the procedure described by Kagan et al. (1979). Dissection instruments and Erlenmayer
flasks (Nalgene) were autoclaved prior to use. The aortas from 120 seventeen-day chick embryos were dissected out and pre-incubated for 1 h at 37 °C in 125 ml Nalgene flasks (30 aortas per flask) containing 50 mls of sterile MEM without lysine, but supplemented with 50 mg/l valine, 50 mg/l alanine, 50 mg/l glycine, 50 mg/l proline, 50 mg/l ascorbic acid, 50 mg/l BAPN, 10^5 units/l penicillin, 100 mg/l streptomycin and adjusted to pH 7.4. By using culture media supplemented with the most abundant amino acids in elastin, alanine, glycine, proline and valine (Sandberg et al., 1981), the yield was approximately twice that obtained without the supplements (Shackleton & Hulmes, 1990b). The aortas were then incubated on an Labtherm orbital shaker for 20 h in 200 ml of the above medium (50 ml per flask) containing 0.56 MBq/ml [4,5-3H] lysine at 37 °C in an atmosphere of 5 % CO₂/95 % air.

The following procedures were at 0-4 °C. The aortas were washed and homogenised in 50 ml of 0.15 M NaCl and then centrifuged at 10,000 g for 5 minutes. The saline-insoluble aortic pellet is 88 % elastin and 12 % collagen; if necessary the collagen may be removed by treatment with purified bacterial collagenase (Narayanen et al., 1974; Stassen, 1976). The pellet was retained and the saline extraction repeated. Background rates of tritium release may be substantial, due to endogeneous lysyl oxidase tightly bound to the elastin pellet (Kagan et al., 1974). Therefore to inactivate endogenous enzyme, the pellet was further homogenised in 1 N hydrochloric acid and centrifuged as above, this step was repeated. The pellet was then homogenised in assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0) and centrifuged as above, this step was repeated. Finally the pellet was resuspended in assay buffer to give a concentration of 3 x 10^5 dpm in 100 μl. A typical yield from 10 dozen embryos was 2.5 x 10^8 dpm which was sufficient for 800 assays.

2.4.3.2 Ultrafiltration Assay

In vitro assays have been developed to measure the activity of lysyl oxidase against collagen or elastin substrates (Pinnell & Martin, 1968; Siegel et al., 1970; Siegel, 1974) as well as against nonpeptidyl amines (Trackman & Kagan, 1979;
Trackman et al., 1981) and other protein substrates (Kagan et al., 1984). The assay used during this study relies on the addition of lysyl oxidase to the [4,5-3H] lysine-containing form of elastin, this leads to the catalytic release of a tritium ion which can exchange with water. The 3H2O formed is then separated by ultrafiltration (Shackleton & Hulmes, 1990b). Although the method is discontinuous, other methods using synthetic peptide substrates, following oxygen uptake with an oxygen electrode (van der Meer & Duine, 1986), or measuring H2O2 release by a fluorometric-peroxidase coupled reaction (Trackman et al., 1981) are useful for studies on enzyme specificity and mechanism, but are inappropriate for routine assays.

2.4.3.2.1 Procedure

The ultrafiltration assay (Shackleton & Hulmes, 1990b) was initiated in 1.5 ml Eppendorf tubes by the addition of 100 µl of enzyme to 100 µl [4,5-3H] elastin (300,000 dpm) suspended in assay buffer (0.1 M sodium borate, 0.15 M NaCl, pH 8.0). After vortexing, the mixture was incubated at 37 °C for 16 h. Following incubation, the reaction was stopped by placing the tubes on ice, and adding 50 % (w/v) trichloroacetic acid to a final concentration of 5 % (w/v). After centrifugation at 4 °C for 5 minutes at 15,600 g to pellet the bulk of the elastin substrate, 400 µl aliquots of the supernatant were then ultrafiltered at 4 °C for 1 h at 5000 g using Millipore Ultrafree-MC units (10 K cut-off) in a Beckman JA 18.1 rotor. During centrifugation, low molecular weight material passes through the ultrafiltration membrane and collects in the lower chamber of the unit. Tritium release is measured by mixing 0.3 ml of the ultrafiltrate with 2.7 ml of BDH Cocktail T and then counted in a liquid scintillation counter for 10 minutes. Assays were usually conducted in triplicate, control assays contained 0.2 mM BAPN, a specific inhibitor for lysyl oxidase (Tang et al., 1983). Where necessary, results were corrected for partial inhibition by urea (Shackelton & Hulmes, 1990b).
2.4.4 Isoelectric Focussing

Isoelectric focussing separates proteins on the basis of surface charge alone, as a function of pH. The separation is done in a non-sieving medium, in the presence of carrier ampholytes, which establish a pH gradient increasing from the anode to the cathode. Since a protein contains both positive (amine) and negative (carboxyl) charge bearing groups, the net charge of the protein will vary as a function of pH.

A pH gradient is established concomitantly with protein separation. As the protein migrates into an acidic region of the gel it will gain positive charge. At some point the overall positive charge will cause the protein to migrate away from the anode (+) to a more basic region of the gel. As the protein enters a more basic environment it will lose positive charge and gain negative charge, and consequently will migrate away from the cathode (-). Eventually the protein reaches a position in the pH gradient where its net charge is zero (defined as its isoelectric point or pl). At that point the electrophoretic mobility is zero, migration will cease and a concentration equilibrium of the focussed protein is established.

The pH gradient is formed by the presence of ampholytes in the separation medium. Carrier ampholytes are complex mixtures of amphoteric buffers that form a smooth pH gradient in an applied electric field. During electrofocussing these buffers stack according to their individual pI's across the gel, producing a step gradient. In order for the gradient to appear smooth, a large number of these buffering components must be present.

2.4.4.1 Procedure

Analytical isoelectric focussing was carried out essentially according to manufacturers instruction, using a Biorad Mini IEF Cell Model III. Prior to loading, samples were dialysed into 4 M urea and adjusted to a protein concentration of approximately 1 mg/ml. Protein samples (2 µl) were focussed on 5 % polyacrylamide gels (125 x 65 x 0.4 mm) containing 2 % ampholines (Pharmacia / LKB; ampholine mixture of pH 3.5 to 5 (5 parts) and pH 3.5 to 10 (1 part)). A mixture of ampholines
ensures rapid generation of the pH gradient. Gels also contained 4 M urea, which was found necessary to prevent sample precipitation. Focussing was carried out under constant voltage conditions in a stepped fashion; 100 V for 15 min, 200 V for 15 min and finally 450 V for 1 h. Step increases in voltage were necessary to prevent overheating and subsequent dehydration of the gel. When focussing was complete, proteins were stained for 1-2 h in 0.04 % (w/v) Coomassie Brilliant Blue R250, 0.5 % (w/v) CuSO₄, 0.05 % (w/v) Crocein Scarlet 7B, 27 % (v/v) ethanol, 10 % (v/v) acetic acid. Crocein Scarlet, is a highly soluble dye which binds to protein, and is included to assure rapid fixation of the bands. The gels were destained in 12 % (v/v) ethanol, 7 % (v/v) acetic acid, 0.5 % (w/v) CuSO₄ (three changes over 45 minutes) followed by destaining in 25 % (v/v) ethanol, 7 % (v/v) acetic acid until the background cleared. The cupric sulphate in the staining and destaining solutions effectively eliminates any background staining due to the presence of ampholytes. Finally gels were air dried in a dust-free area.

2.4.5 Deglycosylation

The deglycosylating enzymes used in this study were shown to be free of proteolytic activity by unmodified SDS-PAGE migration of electrophoresis calibration proteins (Pharmacia) treated with these enzymes.

2.4.5.1 Neuraminidase

Neuraminidase (Sialidase) hydrolyses terminal bonds which join sialic acid (5-N-acetylneuraminic acid, NANA) to oligosaccharides, glycoproteins or glycolipids (Montreuil et al., 1986).

For neuraminidase treatment, 50 μg of TRAMP in 50 mM sodium acetate, pH 5.5, 4 mM CaCl₂, 100 μg/ml BSA, 0.2 mM PMSF, 0.2 μM TPCK, was incubated in a total volume of 40 μl, with 0.8 mU of neuraminidase (Boehringer, from Vibrio cholerae) for 18 h at 37 °C, followed by a further 6 h at 37 °C with the same amount of freshly added enzyme. BSA in the digestion buffer was included to improve the
stability of the neuraminidase. Fetuin (Sigma) was used as a positive control. The reaction was stopped by the addition of 10 μl of 5 x SDS-PAGE reducing sample buffer and heating for 3 min at 100 °C, followed directly by electrophoresis or storage at -20 °C.

2.4.5.2 Endoglycosidase F/N-Glycosidase F

This mixed (1:1) preparation from Sigma provides a wide range in specificity. Endoglycosidase F (Endo F) cleaves glycosidic bonds in the diacetylchitobiosyl core region of both polymannose and complex type N-linked oligosaccharides, leaving only a single N-acetylglucosamine (GlcNAc) residue attached to asparagine on the polypeptide (Elder & Alexander, 1982). Endo F does not affect O-linked carbohydrates of glycoproteins. The efficiency of digestion depends on unfolding and partial denaturation of the glycoprotein substrate (Plummer et al., 1984).

N-Glycosidase F cleaves an intact oligosaccharide from a native or denatured glycoprotein. The cleavage occurs between the innermost residue of N-N′-diacetylchitobiose and the asparagine to which the oligosaccharide is linked. The enzyme readily cleaves most high mannose oligosaccharides, hybrid oligosaccharides and biantennary, triantennary and tetraantennary complex oligosaccharides (Tarentino et al., 1985; Chu, 1986).

For Endoglycosidase F/N-Glycosidase F treatment, 50 μg of TRAMP was first denatured by heating at 100 °C for 3 min in the presence of 10 mg/ml SDS. The enzyme preparation is inhibited by SDS in the absence of non-ionic detergents, therefore the protein in 10 mg/ml SDS was diluted 10 times with 90 μl of digestion buffer (0.25 M sodium acetate, 20 mM EDTA, 10 mM 2-mercaptoethanol, 6 mg/ml Nonidet P-40, 5 mg/ml CHAPS, 0.2 mM PMSF, 0.2 μM TPCK, pH 6.25). 7 μl of Endoglycosidase F/N-Glycosidase F (0.36 Units) was then added to the mixture, which was incubated at 37 °C for 18 h. Incubations were also carried out at pH 4.5 and pH 7.5 to favour the activities of Endo F and N-Glycosidase F respectively. Fetuin (Sigma) was used as a positive control. Reactions were stopped by the addition of 25 μl of 5 x SDS-PAGE
Reducing sample buffer and heating for 3 min at 100 °C, followed immediately by electrophoresis or storage at -20 °C.

2.4.5.3 O-Glycosidase

O-Glycosidase (Boehringer, BSA free, from *Diplococcus pneumoniae*) releases the disaccharide Gal β(1-3) GalNac from O-glycans, which is bound to serine or threonine as a core unit.

For treatment with O-glycosidase, 200 µg of TRAMP (in 100 µl of 20 mM sodium cacodylate buffer, pH 6.0) was incubated with 1 mU of enzyme for 16 h at 37 °C, and the reaction stopped by the addition of 25 µl 5 x SDS-PAGE reducing sample buffer and heating for 3 min at 100 °C, followed directly by electrophoresis or storage at -20 °C. Fetuin was used as a positive control, and as the presence of sialic acid interferes with O-glycosidase activity, incubations with O-glycosidase were done both with and without prior neuraminidase treatment.

2.4.6 Further Carbohydrate Analysis

2.4.6.1 Concanavalin A Affinity Chromatography

Concanavalin A (Con A) serves as an affinity adsorbent for glycoproteins containing α-D-glucopyranosyl or α-D-mannopyranosyl residues (Montreuil *et al.*, 1986).

Affinity chromatography, with immobilised Con A, was performed at 4 °C as follows: samples of TRAMP and lysyl oxidase from the Sephacryl S-200 column were applied to a 3 x 1.6 cm column of Con A-Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl, 0.5 M NaCl, 1 M urea, 1 mM CaCl₂, 1 mM MnCl₂, pH 7.4 (at room temperature) at 20 ml/h. Elution was achieved using 1 M α-D-methyl mannoside dissolved in equilibration buffer, at 10 ml/h.
Lectins are a group of proteins which possess the ability to react reversibly and specifically with different carbohydrate structures. Thus the detection of glycoproteins on electroblots is frequently achieved using lectins as probes. Several methods of visualising bound lectins have been described which include radioactively labelled, fluorescently labelled or enzyme labelled lectins. (O'Shannessy et al., 1987; Haselbeck et al., 1990). The lectins used in this study (wheat germ agglutinin (WGA); Con A) were provided by Dr D K Apps. The WGA (supplied as a conjugate with horseradish peroxidase; HRP) binds to N-acetyl glucosamine containing carbohydrate structures (Montreuil et al., 1986). Con A has an affinity for HRP (Clegg, 1982), thus glycoproteins were detected with this lectin by a sequential incubation with Con A followed by HRP (Sigma; type VI).

Following SDS-PAGE and transfer of the separated proteins to nitrocellulose (section 2.3.4), the blot was stained briefly (1 min) with Ponceau S (section 2.3.4.2). If necessary individual lanes of protein were cut from the membrane and reacted protein-side up in an incubation tray (Biorad). The incubation tray consists of several wells to allow the screening of many different lectin/glycoprotein reactions under separate conditions.

For glycoprotein detection a modification of the procedures described by several workers were used (Harlow & Lane, 1988; Haselbeck et al., 1990). Excess protein binding sites on the transfers were blocked by incubation in Tris buffered saline (TBS; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5) containing 0.5 % (v/v) Tween-20 (Sigma) for 1 h at room temperature with agitation. (Solutions used for blocking need to be devoid of glycoproteins.) The transfers were then incubated for 1 h at room temperature with a lectin (WGA-HRP or Con A, 10 μg/ml in TBS containing 0.1 % (w/v) BSA, 1 mM MnCl₂, 1 mM CaCl₂, pH 7.5). The membrane was then washed (5 x 5 min) as follows: twice with TBS, twice with TBST (TBS containing 0.1 % (v/v) Tween-20) followed by a final wash in TBS. Blots (labelled with Con A only) were further incubated with HRP (50 μg/ml) for 60 minutes in the same buffer used to dilute the
lectins. Excess enzyme was removed by washing (5 x 5 min) in buffer as before. Bound horseradish peroxidase (HRP) was then located by incubating the blot with freshly prepared chloronaphthol substrate, (40 ml 4-chloro-1-naphthol (3 mg/ml in methanol)) added to 200 ml TBS and filtered immediately before use, followed by 80 µl of 30 % (v/v) H₂O₂ which is converted to a highly insoluble blue-black coloured product. After suitable colour development (usually 10-15 min), the blots were washed in distilled water and dried in the dark.

This method is very sensitive and capable of detecting as little as 60 ng of a purified glycoprotein after electrophoresis. To achieve this level of detection only HRP with a high specific activity should be used. It is also important that buffers do not contain azide as this inhibits HRP activity.

Con A was also labelled with I¹²⁵ (by Dr D K Apps) which was then incubated with protein blots. The conditions during incubation were identical to that already described for the WGA-HRP conjugate. However no chloronaphthol development was required, and Con A (30,000 dpm/ml) was used. For development the blots were covered in cling film and exposed to pre-flashed X-ray film (Kodak XAR 5 X-OMAT; Laskey & Mills, 1975). Films were developed in Devalex solution and fixed with Fixaplus (both obtained from Champion photochemistry) using a Fugi X-ray film processor RGII. Procollagen C-propeptide was used as a positive control and Pharmacia molecular weight electrophoresis standards were a negative control. The specificity of the method was demonstrated by the abolition of Con A binding in the presence of α-D-methyl mannioside.

2.4.7 Desulphation

Tysosine sulphation of TRAMP following SDS-PAGE was suggested by Alcian Blue staining (section 2.4.8.4). To investigate whether TRAMP contained any tyrosine sulphate, purified TRAMP variants were treated with 1 M HCl or sulphatase prior to SDS-PAGE.

The tyrosine sulphate bond is particularly labile to acid hydrolysis (Huttner,
1984). Therefore TRAMP was dialysed against distilled water and diluted with 2 M HCl to a final 1 M HCl, heated for 5 min at 100 °C, freeze dried, and finally dissolved in SDS-PAGE reducing sample buffer, with heating at 100 °C for 3 min, followed immediately by electrophoresis. TRAMP boiled in distilled water for 5 min was used as a control.

To further investigate tyrosine sulphonation, TRAMP was incubated with sulphotases (either type V from limpet or type VII from abalone, both obtained from Sigma).

For sulphotase treatment, 30 µg of TRAMP was dialysed into 0.1 M sodium acetate (pH 5.0), 0.1 unit of sulphotase was added in a total volume of 0.2 ml, and the reaction mixture incubated for 2 h at 37 °C. Aliquots (20 µl) were removed at set times and added to 5 µl of 5 x SDS-PAGE reducing sample buffer, followed by heating at 100 °C for 3 min. The small amount of β-glucuronidase activity that was present as a contaminant in the sulphotases was inactivated by temporarily adjusting the pH to 2.2 (Dodgson & Spencer, 1953). Separate experiments with purified β-glucuronidase (E. coli; Boehringer) at two times the level present in the sulphotase preparations, had no effect on the electrophoretic migration of TRAMP variants or their staining with Coomassie Blue, Alcian Blue or PAS.

2.4.8 Gel Staining Methods

2.4.8.1 Proteins

A typical staining method for proteins after SDS-PAGE involved placing the gel into an excess of staining solution, (0.25 % (w/v) Coomassie Brilliant Blue-R250, 50 % (v/v) methanol, 10 % (v/v) acetic acid). This solution also served to fix the proteins in the gel and therefore avoid loss due to diffusion. Gels were stained for a minimum of 2 h and then transferred to destaining solution (10 % (v/v) methanol, 10 % (v/v) acetic acid) for at least 24 h with several changes of the solution. The staining and destaining times were reduced when carried out at 45 °C. The dye solution was reused several
times, and the lower limit of detection corresponded to about 1 \( \mu \text{g} \) protein per band. Coomassie Brilliant Blue-R250 is about 3 times more sensitive than Amido black 10B and forms electrostatic bonds with \( \text{NH}_3^+ \) groups and non-covalent bonds with non-polar regions in the proteins. The staining is uniform, stable and suitable for densitometry although the binding begins to deviate from Beers law at higher protein levels and it is therefore not suitable for quantitative measurements in gels having widely differing amounts of proteins in the various stained bands.

For some purposes, very high sensitivity is required for adequate localisation of separated proteins. This may be achieved by silver staining which is claimed to be 100 times more sensitive than Coomassie Blue-R250 (Andrews, 1986). The method used for silver staining was based on that of Morrissey (1981). Gels were fixed in 25 % (v/v) methanol, 10 % (v/v) acetic acid for 2 h, followed by washing the gel in distilled water over 1 h. Proteins in the gel were then reduced with 5 \( \mu \text{g/ml} \) dithiothreitol (DTT) for 2 h. This step does not seem to be necessary but does improve the sensitivity a little (Sammons et al., 1981). The DTT solution was then poured off. Without rinsing, the gel was incubated in 0.1 % (w/v) silver nitrate, and after 2 h the gel was rapidly rinsed twice with distilled water. To visualise the protein bands, the gel was developed with 100 \( \mu \text{l} \) of 40 % formaldehyde in 200 ml of 3 % (w/v) sodium carbonate, until the desired level of staining was reached. Finally 10 ml of 2.3 M citric acid was added to stop the reaction, after 30 min the gel was placed in 2 % (v/v) glycerol, prior to photography or drying down. Throughout the procedure gloves were worn, and incubations were done with gentle agitation to produce an even stain. Overstained gels could be destained with photographic reducers, such as kodak rapid fixer, the destaining may then be stopped with 5 % sodium thiosulphate, followed by thorough washing in distilled water.

The precise mechanism of silver staining is not known but it appears that silver ions initially bind to protein zones and then act as nucleation sites for the deposition of further amounts of silver. Silver staining can be applied perfectly satisfactorily to gels that have already been stained with Coomassie Blue (Hallinan, 1983). This can be
useful to detect minor components after the principal ones have been detected with dye staining. Greater reliability and sensitivity of silver staining after preliminary staining with Coomassie Blue, has been observed by several groups. It appears that the provision of extra silver binding sites by sulphonic acid groupings on the dye molecules bound to proteins in the gel are responsible for the improved staining (Nielsen & Brown, 1984).

2.4.8.2 Glycoproteins

Glycoproteins can be stained by the methods described above for proteins, but the presence of carbohydrate means that other procedures can be used, both for their localisation and in order to distinguish which components of a mixture are glycoproteins. The most widely used specific glycoprotein staining method, and the one which was used in this study, is the very sensitive (lower limit of detection about 2-3 µg of protein bound carbohydrate) periodic acid-Schiff (PAS) procedure of Zacharius et al. (1969).

The fuchsin-sulphite (Schiff’s reagent) staining solution was prepared as follows: 2 g basic fuchsin (pararosaniline) were dissolved in 400 ml of distilled water, with warming, and then cooled and filtered. 10 ml of 2 N HCl and 4 g of potassium metabisulphite was then added. The solution was placed in a stoppered amber bottle and kept cool overnight. Activated charcoal (1 g) was stirred in, filtered, and sufficient 2 N HCl added (approximately 10 ml) until a drop dried on a glass slide did not turn red. The solution was stored in a stoppered bottle, protected from light. The stain could be used several times, and was discarded if it turned pink.

The procedure for localising glycoproteins in gels followed a 30 min fixation step in 12.5 % TCA, followed by a brief rinse in distilled water. After oxidation with 1 % periodic acid in 3 % aqueous acetic acid for 50 min, the gel was washed thoroughly in distilled water (at least 6 changes over 2 h). Gels were then transferred into fuchsin-sulphite and stained for 50 min in the dark. After washing with 3 changes (10 min each) of 0.5 % sodium metabisulphite, gels were finally washed in frequent changes of
distilled water to remove excess stain.

After localisation of glycoproteins, subsequent staining with Coomassie Blue revealed other non-glycosylated proteins.

2.4.8.3 Phosphoproteins

Interaction of the cationic carbocyanine dye Stains-All with proteins, nucleic acids and substituted polysaccharides causes a change in the absorption spectrum of the dye. Green et al. (1973) reported that many proteins stained red ($\lambda_{\text{max}}$ 500-520 nm), some proteins did not stain, while phosphoproteins stained blue ($\lambda_{\text{max}}$ 640-660 nm), therefore this could be used for differentiating between them. The sensitivity varies according to the degree of protein phosphorylation but is generally in the range 1-5 $\mu$g. The method used was as described by Green et al. (1973).

Working stain was prepared just before use, from a 0.1 % stock solution of Stains-All (Sigma) in formamide, as follows: 10 ml stock stain, 10 ml formamide, 50 ml isopropanol, 1 ml 3 M Tris-HCl, pH 8.8 and distilled water were combined in a total volume of 200 ml.

Following SDS-PAGE, gels were fixed in 25 % isopropanol at 45 °C for at least 30 min to remove SDS which interferes with subsequent staining. After a brief rinse in distilled water, the gels were placed in the dark and stained overnight. The gel was then destained in the dark with several changes of distilled water, until a good contrast between the bands and background was observed. Phosvitin (Sigma), a phosphoprotein, was used as a positive control.

Similar studies have shown that calcium-binding proteins and sialoglyco-proteins also stain blue with Stains-All (Campbell et al., 1983). Therefore controls should be run using neuraminidase or phosphatase treated samples which remove the sialic acid residues or phosphoryl groups, respectively, and these proteins will then stain red.
2.4.8.4 Proteoglycans

Alcian Blue (8GX) is a cationic dye which stains polyionic groups such as negatively charged glycosaminoglycan (GAG) chains of proteoglycans. In practice however, Alcian Blue gives high levels of background staining, and occasional staining of protein bands which do not contain attached GAGs. By adding MgCl₂ to the stain, this non-specific staining is abolished, since each polyanion has its own “critical electrolyte concentration” (CEC) above which it ceases to take up stain (Scott, 1985).

The method for Alcian Blue staining following SDS-PAGE was as described by Wall and Gyi (1988). To remove SDS, gels were fixed in 50 % (v/v) methanol, 7 % (v/v) acetic acid for 1 h, followed by washing in distilled water for 1 h. These 2 steps were repeated before staining the gels overnight in 0.2 % (w/v) Alcian Blue 8GX, 3 % (v/v) acetic acid, 0.05 M MgCl₂. Gels were then destained in several changes of 3 % (v/v) acetic acid, 0.05 M MgCl₂ until the background was clear. Gels could then be photographed and scanned using a densitometer for quantitative analysis.

2.4.9 Production of Antibodies

Polyclonal antibodies to porcine TRAMP were obtained from female New Zealand white rabbits (City Farms, West Calder) by the methods described by Harlow and Lane (1988). Initial immunisation required an intradermal injection (at 6 sites in the back of a rabbit) of 200 μg Mono Q purified TRAMP diluted in 0.15 M NaCl and emulsified in complete Freund’s adjuvant (Sigma). (The TRAMP was free of lysyl oxidase and other contaminating proteins as judged by the ultrafiltration assay and SDS-PAGE. The adjuvant is included to stimulate the immune response and to protect the TRAMP antigen from rapid catabolism by the host animal). This was followed by a booster injection of 100 μg TRAMP in Freund’s incomplete adjuvant (Sigma) after three weeks. Further boosts of 50 μg TRAMP in Freund’s incomplete adjuvant were given at six week intervals.

Before the immunisations were started and also at ten days after each injection, approximately 10 ml of blood was collected from each rabbit. The blood was obtained
by heart puncture under anesthesia after the last injection. The blood was allowed to clot for 30 minutes at 37 °C. Following overnight storage at 4 °C, to allow the clot to contract, the serum was collected after centrifugation at 10,000 g for 10 minutes. The sera was then stored at -20 °C until required. Technical support was provided by staff in the departments animal house.

2.4.9.1 Enhanced Chemiluminescence (ECL) Detection of TRAMP on Immunoblots

TRAMP antigen immobilised on nitrocellulose was detected by the ECL system (Amersham), which is a light emitting non-radioactive technique. The main advantage of ECL over other methods is its sensitivity, with detection limits of less than 1 pg of antigen. Thus low-affinity antibodies or small amounts of antibody can be used.

Purified TRAMP and crude tissue extract containing TRAMP were subjected to SDS-PAGE followed by transfer of the separated proteins onto nitrocellulose (section 2.3.4). Antigen detection was carried out according to manufactures instruction but with some modifications. Antibody dilutions were first optimised to give the highest signal with minimum background. Generally, membranes were blocked with 5 % (w/v) dried milk in TBST for 1 h at room temperature with agitation, followed by washing three times in TBST (1 x 15 min; 2 x 5 min). The antisera containing the primary antibody directed against TRAMP, was diluted 1:5000 in TBST and incubated with the blot for 1 hour at room temperature, followed by washing (as before). A 1:5000 dilution of the second antibody in TBST (HRP-anti rabbit IgG; supplied by the Scottish antibody production unit, Law hospital, Carluke, Lanarkshire) was then incubated with the transfer for 20 minutes. Following a further wash (as before), antigen was detected on the membrane surface by mixing the two ECL reagents (1:1) and incubating for precisely 1 minute. Excess reagent was drained off and the blots covered with cling film, care was taken to smooth out any air pockets. Blots were immediately exposed to pre-flashed X-ray film for 30 seconds-10 minutes, and developed (section 2.4.6.2). Sera taken from rabbits before the TRAMP immunisations began was used to correct
for non-specific binding. To ensure there was no spurious binding of the second antibody directly to the blotted proteins, the procedure above was repeated but with no primary antibody.

2.5 *In Vitro* Fibril Formation

Purified lathyritic rat skin collagen, free of aggregates and with intact non-helical telopeptides, was used to study the effect of TRAMP and lysyl oxidase on collagen fibril formation.

Fibrils were reconstituted by the "warm start" technique (Holmes *et al.*, 1986), in which collagen solutions in 5 mM acetic acid (pH 2.5), and double strength fibril formation buffer (60 mM disodium phosphate, 60 mM TES, 270 mM NaCl, pH 7.4; Williams *et al.*, 1978) were preincubated separately at 34 °C before mixing 1:1 to initiate fibril formation. Purified variants of TRAMP were dialysed into double strength fibril formation buffer and diluted in the same buffer prior to mixing with collagen. Turbidity at 313 nm (using a Philips PU 8720 UV/VIS scanning spectrophotometer) monitored the kinetics of fibril formation, and the products were examined by electron microscopy, or SDS-PAGE.

2.5.1 Electron Microscopy

2.5.1.1 Negative Staining of Collagen Fibrils

Negative staining is used as a method for contrast enhancement in electron microscopy of biological specimens. It is a simple and rapid method for studying the morphology and structure of particulate specimens, such as viruses, cell components, and macromolecules. The sample shows well preserved order, since the stain preserves macromolecular structure. The negative staining method is based on the principle that there is no reaction between the stain and the specimen. This is accomplished by using a pH which minimises the attraction between the stain and protein. The specimen substructure is revealed by the penetration of the stain into holes and crevices, with the
stain drying into a relatively electron-opaque layer around the specimen. In transmission electron microscopy (TEM) the sample appears as a light image on a dark background. Uranyl acetate (UA) and phosphotungstic acid (PTA) are the most commonly used stains (Hayat, 1989).

For negative staining a suspension of collagen fibrils formed both in the absence and presence of TRAMP, was applied to carbon-colloidon-coated copper 400-mesh grids (Spiess et al., 1987). After 1 min the grids were blotted with filter paper and a drop of 1 % PTA in water (pH adjusted to 7.4 with NaOH) was added, then removed by blotting after a further 1 min, and the grid then air dried.

2.5.1.2 Rotary Shadowing

Although negative staining provides higher resolution than metal shadowing when viewing biological material under TEM. The contrast provided by negative staining when studying long, slender, rod-shaped proteins is inadequate. In this case, metal shadowing provides a good alternative. The specimen is shadowed in vacuo with heavy metal from a shallow angle, the angle being chosen to give optimal contrast of the structural features of interest. Rotation of the specimen during evaporation (rotary shadowing) gives an “all round” view. For a review of rotary shadowing see Furthmayr and Madri (1982).

To study the interaction between TRAMP and collagen during the lag phase of fibril formation, the mica sandwich technique for preparing molecules for rotary shadowing was used (Mould et al., 1985). Spraying is the usual method for depositing the sample onto the mica surface. However it has been shown that the shear forces induced by spraying can cause fragmentation of extended macromolecules. To reduce this risk a gentler spreading technique is used. The mica sandwich procedure involves forming a thin film of sample solution between 2 pieces of freshly cleaved mica.

Fibrils were reconstituted at 34 °C for 1-2 min, with and without TRAMP, and then diluted with fibril formation buffer to a final collagen concentration of 20 μg/ml. A small volume (5 μl) of the solution was then spread between 2 (20 x 20 x 0.125 mm)
pieces of freshly cleaved mica, and allowed to adsorb for 1 min. To ensure that the specimen was not obscured by any non-volatile salts, the mica sandwich was placed into 0.2 M ammonium acetate buffer (pH 7.2) and separated under the liquid surface. After agitation to remove the salts, the mica pieces were reapposed, removed from the solution, and blotted on the outside before plunging into liquid nitrogen. After separation of the mica pieces under liquid nitrogen, the pieces with sample uppermost, were placed onto a brass block submerged in liquid nitrogen. The block was immediately transferred into the chamber of an Edwards vacuum coating unit. The pressure in the unit was reduced to $10^{-4}$-$10^{-5}$ Torr, and the ice layer sublimed off after 10 min. A thermocouple monitored the temperature of the brass block, which showed the temperature of the sample was at least -90 °C during freeze drying. Although drying from glycerol gives good preservation of single extended molecules, it was shown by Fowler and Aebi (1983) that freeze drying had significant advantages over glycerol drying in preserving molecular structural integrity. After approximately 30 min when any frost on the block had cleared, the specimen was returned to room temperature by admitting dry nitrogen (evaporated from liquid N$_2$) into the chamber. The specimen was removed after a further 30 min.

An 8 cm length of platinum wire, 0.1 mm in diameter was coiled around a 1 mm diameter tungsten filament to occupy about 1 cm at the centre of the filament. Samples (on a rotating stage) were shadowed with platinum at a glancing angle of 5° by resistive heating of the tungsten filament, at about 10 cm from the source and at a pressure of $2 \times 10^{-5}$ Torr. The metal replicas were stabilised by coating with carbon. The carbon was evaporated by resistive heating of a carbon fibre. The replicas were then floated off onto a clean distilled water surface by inserting the mica slowly into the water at an angle of about 30°, and picked up from below on uncoated 400-mesh copper grids, which were blotted and left to dry before TEM.

2.5.1.3 Embedding and Sectioning

To assess whether TRAMP influenced the diameter of collagen fibrils, collagen
fibrils were reconstituted, in the presence and absence of TRAMP over 4 h at pH 7.4, 34 °C, and then pelleted at 15600 g for 1 min at room temperature. The pellets were fixed overnight in 4 % formaldehyde, 0.1 M sodium phosphate, pH 7.2. The formaldehyde arrests self assembly by stabilising existing aggregates without itself inducing further aggregation (Kadler & Chapman, 1986). After blotting the samples dry, 1-2 mm cubes were dehydrated in ascending grades of ethanol, from 30 % to 50 % (15 min each), and then transferred to 70 % (30 min), this step being repeated. The samples were then placed into a 2:1 LR-White/70 % ethanol mixture for 1 h (This solution should be clear, it is also important not to exceed the shelf life of LR-White). Following a further 1 h in 100 % LR-White, samples were blotted with filter paper and placed in fresh LR-White overnight. Finally the samples were blotted and sealed in gelatin capsules containing fresh LR-White. Polymerisation proceeded over 24 h at 50 °C.

100 nm sections were cut on an ultramicrotome, and then picked up on carbon-colloidon-coated copper grids (technical support was provided by staff at the Pathology department). Sections were stained with unbuffered 1 % PTA for 8 min followed by 1 % UA for 15 min. PTA binds to positive ions such as the e-amino groups of lysine and hydroxylysine. UA is a weak electrolyte in aqueous solution, and exists not only as uranyl ions but also as anionic and cationic complexes. By using a combination of PTA and UA, all charged residues should be bound by stain (Chapman & Hulmes, 1984).

2.5.1.4 Electron Microscopy

TEM was performed on a Phillips CM12 operating at 80 kV. Micrographs were taken and calibrated using a replica line grating with 2160 lines/mm.

2.5.1.5 Measurement of Fibril Diameters

Fibril diameters from embedded sections were measured directly from the negative using a calibrated eyepiece with 5 x magnification. The minimum width of each fibril was taken, and fibril diameters were calculated using the a calibration from a
replica line grating (2160 lines/mm). Around 300 fibril diameters were recorded for each sample, and the accuracy of measurement was within ± 5nm.

2.5.2 TRAMP Binding Assays

2.5.2.1 Cosedimentation

To study the interaction between collagen and TRAMP, fibrils were formed as before, but over a range of TRAMP concentrations, and with a fixed 200 µg/ml collagen. The mixture was incubated for 4 hours at 34 °C, then centrifuged at 15,600 g at room temperature for 1 min. Both supernatant and pellet were analysed by SDS-PAGE.

2.5.2.2 Fluorescence Polarisation

Several workers have used the technique of fluorescence polarisation (P) to study the binding properties of fibronectin (Forastieri & Ingham, 1983; Forastieri & Ingham, 1985; Bentley et al., 1985; Benecky et al., 1990). In this approach one member of a pair of interacting molecules is labelled with a fluorescent dye, whose fluorescence polarisation provides a measure of its rotational freedom. Hence P permits the observation of binding events by monitoring the decrease in the rotational diffusion coefficient of a relatively small labelled molecule upon its binding to a much larger molecule (Bentley et al., 1985).

In this study TRAMP was labelled with fluorescein isothiocyanate (FITC) and its fluid-phase interaction with collagen investigated (under non-fibrillogenic conditions).

For fluorescein labelling, the method of Forastieri and Ingham (1983) was used. TRAMP (1.5 mg/ml) was incubated for various times in a suspension containing 7.5 mg/ml FITC (isomer 1 bound to cellite; Sigma) in 0.05 M NaHCO₃ at pH 8.5 and 25 °C. Following centrifugation at 15600 g for 5 min, to pellet the cellite, excess dye was removed from the supernatant by gel filtration. For gel filtration a small amount
of Biogel P-6DG (Biorad) previously swollen and equilibrated in double strength fibril formation buffer (60 mM disodium phosphate, 60 mM TES, 270 mM NaCl, pH 7.4; Williams et al., 1978) was placed in an Eppendorf tube with a small hole punched in the base of the tube which was plugged with glass wool. A small volume (200 µl) of the sample was placed on top of the gel. The TRAMP-FITC conjugate was then collected from the bottom of the tube after centrifugation at 1000 g in a bench centrifuge (MSE) for 1 minute. The degree of labelling at 16 h was between 0.75 and 1.25 mol of fluorescein per mol of TRAMP as estimated from the relative absorbance at 495 and 280 nm based on $\varepsilon_{495} = 72,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the dye (Forastieri & Ingham, 1983). Labelled TRAMP was utilised immediately or stored at -20 °C.

Polarisation measurements were recorded on a Perkin Elmer LS-3B fluorescence spectrophotometer equipped with a thermostated cell holder and Perkin Elmer polarisation accessory 5212-3269. Excitation and emission wavelengths were set at 495 and 540 nm respectively, all slit widths were maintained at 10 nm and fluorescence was excited and viewed through rotatable polarisers. All measurements were made at 20 °C in single strength fibril formation buffer unless stated otherwise. Titrations of FITC-TRAMP were performed in 1 cm quartz cuvettes, small aliquots of concentrated collagen were added to 1 ml of FITC-TRAMP solutions and mixed. Any changes in polarisation were recorded upon addition of the collagen. Corrections for background were made using samples equivalent in all respects to the sample in which $P$ was being determined except for the absence of fluorophore.

The polarisation was computed using the expression:

$$P = \frac{I_{VV} - I_{VH} (I_{HV}/I_{HH})}{I_{VV} + I_{VH} (I_{HV}/I_{HH})}$$

where $I$ is the fluorescence intensity. The first subscript of each intensity is the orientation of the excitation polariser and the second subscript denotes the orientation of
the emission polariser. These orientations were either vertical (V) or horizontal (H) (Bentley et al., 1985).
Chapter 3

Characterisation of TRAMP and Lysyl Oxidase from Porcine Skin
3.1 Results

3.1.1 Purification of TRAMP and Lysyl Oxidase

TRAMP and lysyl oxidase were prepared from urea extracts of porcine skin by DEAE-Sepharose chromatography and selective interaction (at low ionic strength) with Sephacryl S-200, as described in section 2.2. The chromatogram in Figure 3.1 shows the 6 M PBU (pH 7.5) elution profile from the S-200 column. The ultrafiltration assay for lysyl oxidase (section 2.4.3) shows that enzyme activity was eluted with the major protein peak (Fig. 3.1). Substantial purification was achieved by these methods as evidenced by SDS-PAGE and Coomassie Blue staining (sections 2.3.4 & 2.4.8.1) of fractions at different stages of purification (Fig 3.2, lanes 2 and 3). The final preparation consisted almost entirely of two protein bands (Fig. 3.2, lane 4). The major band (TRAMP) often represented 60-90% of the total protein, and had an apparent molecular mass of 24 K under reducing conditions. Lysyl oxidase was a minor component which migrated with an apparent molecular mass of 34 K. It was observed that lysyl oxidase sometimes appeared as a doublet with a weak slowly migrating component (Fig. 3.2, lane 5).

Attempts were made to modify the purification procedure, to improve the separation between TRAMP and lysyl oxidase and reduce the time required. Omitting the DEAE-Sepharose step and applying the extract directly onto the Sephacryl S-200 column resulted in a preparation that was significantly contaminated with other proteins (not shown). It was not possible to separate TRAMP and lysyl oxidase by selective salt precipitation as both proteins precipitated at 30 % saturation of ammonium sulphate, although this did provide a means of concentrating the proteins from dilute solutions. However, it was found that although these two proteins co-purify when prepared at pH 7.8 (Cronshaw et al., 1993), lowering the pH to 6.0 for the DEAE-Sepharose step resulted in TRAMP that was uncontaminated with lysyl oxidase (Fig. 3.2, lane 6). Alternatively lysyl oxidase was almost completely separated from TRAMP when the
Figure 3.1 Sephacryl S-200 chromatography of porcine TRAMP and lysyl oxidase
The elution profile is shown after the addition of 6 M urea to the 10 mM phosphate buffer. Enzyme activity was completely inhibited by 0.2 mM BAPN. ——, Absorbance (280 nm); ——, enzyme activity.
Figure 3.2 SDS-PAGE of lysyl oxidase and TRAMP preparations
Proteins were electrophoresed on 12 % SDS-polyacrylamide gels and Coomassie Blue stained. 1, molecular weight marker proteins; 2, 6 M urea extract from porcine skin; 3, partially purified proteins after DEAE Sephacel chromatography; 4, lysyl oxidase (34 K) and TRAMP (24 K) purified by DEAE Sephacel and Sephacryl S-200 chromatography; 5, lysyl oxidase (showing that it also occurs as a doublet, 34-36 K); 6, TRAMP purified at pH 6.0 for the DEAE Sephacel step; 7, lysyl oxidase purified by DEAE Sepharose and Sephacryl S-400 chromatography (see Cronshaw et al., 1993); 8 & 9, TRAMP. All samples (except 9) were reduced with β-mercaptoethanol.
DEAE-Sephacel and Sephacryl S-200 columns were eluted at different urea concentrations (Shackleton, personal communication). Lysyl oxidase eluted from DEAE-Sephacel in the presence of 3 M PBU containing 0.3 M NaCl, while 6 M PBU was required to elute the enzyme from Sephacryl S-200 (Fig. 3.2, lane 7). TRAMP eluted from the DEAE-Sephacel column with 6 M PBU containing 0.5 M NaCl, and was subsequently eluted from the S-200 column with 1.5 M PBU. The electrophoretic mobility of TRAMP was found to depend on whether the protein was reduced prior to SDS-PAGE (Fig. 3.2, lanes 8 and 9), while the mobility of lysyl oxidase was not affected (not shown).

3.1.2 Identification of Lysyl Oxidase and TRAMP Variants

After further purification by anion exchange Mono Q FPLC (section 2.4.2), both proteins eluted in the form of several peaks, with some overlap between individual lysyl oxidase and TRAMP variants (Fig. 3.3). Lysyl oxidase purified using the method of Cronshaw et al. (1993) contained four peaks of lysyl oxidase activity when eluted from the Mono Q column with a salt gradient from 0 to 1 M NaCl in 6 M PBU (20 mM sodium phosphate, 6 M urea, pH 7.8; Fig. 3.3). Similar results have been reported for human umbilical cord lysyl oxidase (Burbelo et al., 1986). The specific activity of each enzyme variant appears to be the same (Cronshaw, personal communication). Peaks from the Mono Q column were analysed by SDS-PAGE and silver staining (sections 2.3.4 & 2.4.8.1), and the electrophoretic mobilities of the four lysyl oxidase variants were identical (Mr 34 K; Fig. 3.4(a)). It was found that chromatographic resolution between individual lysyl oxidase and TRAMP variants was further improved if a salt gradient from 0 to 1 M NaCl in 6 M TBU (20 mM Tris-HCl, 6 M urea, pH 7.5) was used (Fig. 3.4(b)). By the same criteria Mono Q FPLC of TRAMP (prepared at pH 6.0 for the DEAE-Sephacel step) identified five ionic variants (Fig. 3.5(a)). When analysed by SDS-PAGE and Coomassie Blue staining, the five variants of TRAMP (Mr 24 K; Fig. 3.5(b)) were found to be pure, with no trace of contaminating proteins including
Figure 3.3 Mono Q FPLC of porcine lysyl oxidase and TRAMP
Separation of lysyl oxidase and TRAMP variants by Mono Q FPLC using a linear gradient of 0-1 M NaCl in 6 M PBU, following partial purification by elution from DEAE-Sepharose and Sephacryl S-400 columns at different urea and salt concentrations (Cronshaw et al., 1993). Lysyl oxidase variants L1 to L4, with small amounts of TRAMP variants T1 to T5 are indicated.

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Figure 3.4 SDS-PAGE of lysyl oxidase variants
Protein peaks from the Mono Q FPLC column (Fig. 3.3) were analysed by SDS-PAGE (12 % separating gel) and silver staining (a). By the same criteria (but using Tris-HCl buffered urea instead of phosphate buffered urea to equilibrate and elute the column) a silver stained gel of protein peaks from the Mono Q FPLC column is shown for comparison (b). In both cases identical amounts of the same lysyl oxidase preparation were applied to the column. The positions of TRAMP (24 K) and lysyl oxidase variants (L1-L4, 34 K) are indicated. The two bands near the top of the gel are staining artefacts, which also appear when only reducing sample buffer is loaded (b, lane S).
Figure 3.5 SDS-PAGE of TRAMP variants following Mono Q FPLC
(a) Separation of TRAMP variants (T1 to T5) by Mono Q FPLC, following partial purification by DEAE-Sephacel (at pH 6.0) and Sephacryl S200 chromatography. (b) Analysis of TRAMP variants from (a) by SDS-PAGE. Samples are reduced with β-mercaptoethanol and the gel was stained with Coomassie Blue. Molecular weight markers and TRAMP variants T1 to T5 are indicated.
lysyl oxidase. Staining of proteins (separated by SDS-PAGE) with Stains-All suggested that TRAMP was not phosphorylated (section 2.4.8.3). All 5 variants of TRAMP stained red with Stains-All, whereas phosphoproteins are reported to stain blue (Green et al., 1973). Electrophoresis marker proteins (Pharmacia) also stained red, and the phosphoprotein phosvitin stained blue, indicating that the technique can differentiate between the proteins. No lysyl oxidase activity was associated with TRAMP.

3.1.3 Amino Acid Analysis and Sequencing

Variants of TRAMP and lysyl oxidase (separated in denaturing conditions by reverse phase HPLC; Cronshaw et al., 1993) were subjected to amino acid analysis. These analyses were carried out by Mr A D Cronshaw on an Applied Biosystems 420A Derivatiser with automatic hydrolysis and an on-line Model 120A phenylthiocarbamyl (PTC) analyser (Cronshaw et al., 1993). The amino acid composition of TRAMP (variant T3) is shown in Table 3.1. It is remarkably rich in tyrosine with a low level of lysine and a moderately high level of cysteine. No obvious differences were found in the compositions of the five TRAMP variants (not shown). Also shown in Table 3.1 is the amino acid composition of a 23 K contaminant found in lysyl oxidase preparations from human placenta (Kuivaniemi et al., 1984) and of a 22 K bovine protein which co-purifies with dermatan sulphate proteoglycans (Neame et al., 1989). The composition of TRAMP shows some similarities with the 23 K protein of Kuivaniemi et al. (1984) and is almost identical to that of the bovine protein. The amino acid composition of porcine lysyl oxidase (variant L3) is shown in Table 3.2. The composition is similar to that of the rat aorta (Trackman et al., 1990; Trackman et al., 1991) and human placental (Hamalainen et al., 1991) enzymes derived from the cDNA sequence, given certain assumptions as to the position of the pro-enzyme cleavage site (see Table 3.2). No obvious differences were found in the compositions of the four lysyl oxidase variants (not shown).
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Porcine skin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bovine skin&lt;sup&gt;b&lt;/sup&gt; (22 K protein)</th>
<th>Human placenta&lt;sup&gt;c&lt;/sup&gt; (Contaminant, pool II)</th>
</tr>
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<tr>
<td>Aspartic acid</td>
<td>19.6</td>
<td>20</td>
<td>14.1</td>
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<tr>
<td>Glutamic acid</td>
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<td>21.9</td>
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<tr>
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<tr>
<td>Tryptophan</td>
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</table>

ND = not determined

<sup>a</sup> variant T3 (see Fig. 3.5(a)). Values are expressed as residues per molecule, normalised to 7 alanine residues (Neame et al., 1989; Cronshaw et al., manuscript in preparation).

<sup>b</sup> from protein sequence data of Neame et al. (1989), expressed as residues per molecule

<sup>c</sup> from Kuivaniemi et al. (1984), re-expressed as residues per molecule, assuming 7 alanine residues.
Table 3.2 Amino acid composition of lysyl oxidase from various species

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Porcine skin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rat aorta&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Human placenta&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>Glutamic acid</td>
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<tr>
<td>Serine</td>
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<tr>
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<td>Lysine</td>
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<tr>
<td>Tryptophan</td>
<td>ND</td>
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</tr>
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</table>

ND = not determined.

<sup>a</sup> variant L3 (see Fig. 3.3. Values are expressed as residues per molecule, normalised on the assumption that the mature form of lysyl oxidase contains 16 lysine residues (see note b).

<sup>b</sup> from the rat lysyl oxidase precursor sequence derived from the cDNA (Trackman <i>et al.</i>, 1990; Trackman <i>et al.</i>, 1991), assuming the mature enzyme consists of the entire C-terminal region beginning at residue 161 (based on preliminary observations of porcine skin lysyl oxidase by mass spectrometry (Cronshaw, unpublished observations)).

<sup>c</sup> from the human lysyl oxidase precursor sequence derived from the cDNA (Hamalainen <i>et al.</i>, 1991), assuming the mature enzyme consists of the entire C-terminal region beginning at residue 167 (see note b).
Reverse phase HPLC analysis of CNBr peptides from TRAMP and lysyl oxidase variants were carried out by Mr A D Cronshaw according to the methods described by Cronshaw et al. (1993). All five TRAMP variants showed similar profiles (not shown), as did the four variants of lysyl oxidase (not shown). No similarities were observed between the CNBr profiles of TRAMP and lysyl oxidase (Cronshaw et al., 1993).

Amino acid sequence analysis was carried out on TRAMP and lysyl oxidase (separated in denaturing conditions by reverse phase HPLC) by Mr A D Cronshaw using an Applied Biosystems 477A pulsed-liquid microsequencer (Cronshaw et al., 1993). The entire amino acid sequence of TRAMP (variant T3) has been determined on peptides generated by cyanogen bromide or clostripain digestion (see appendix II). The sequence has a tyrosine-rich region at the N-terminus and is identical (differing in only 4 positions) to a 22 K dermatan sulphate proteoglycan-associated extracellular matrix protein from bovine skin (Neame et al., 1989). It is concluded that TRAMP is the same as the protein of Neame et al., (1989). The sequence of a 36 residue peptide from porcine lysyl oxidase (Cronshaw et al., 1993) is identical to residues 291-326 in the rat lysyl oxidase precursor (Trackman et al., 1990; Trackman et al., 1991) and differs in 4 positions from residues 299-334 in the human placental enzyme precursor (Hamalainen et al., 1991). The amino acid sequence of the 36 residue lysyl oxidase peptide was not found in TRAMP. From amino acid analysis, sequence data and CNBr peptide mapping it is concluded that TRAMP and lysyl oxidase are unrelated.

3.1.4 Further Characterisation of TRAMP

Complete sequence data of TRAMP (Cronshaw et al., manuscript in preparation) agrees well with the amino acid analysis and shows it to be rich in acidic amino acids (Table 3.1). The calculated isoelectric point (pI) determined with the University of Wisconsin GCG software package, and allowing for pyroglutamate at the N-terminus is 4.53. To experimentally determine the pI, TRAMP variants in 4 M urea
were analysed by isoelectric focussing using a Biorad mini IEF cell (section 2.4.4). TRAMP variants T1 to T5 were found to be increasingly acidic, with pI's in the range 4.43 to 4.07 (Table 3.3). The observed pI's are less than the theoretical value and they decrease as expected from the Mono Q elution profile (Fig. 3.5(a)).

Precise mass determinations for TRAMP (carried out by staff at Finnigan Mat Ltd, Hemel Hempstead) were made by mass spectrometry using a LaserMat laser desorption ion source coupled to a time-of-flight mass analyser. TRAMP variants were prepared for mass analysis as described by Cronshaw et al. (1993), and the results are shown in Table 3.3. Based on the complete amino acid sequence for porcine TRAMP (Cronshaw et al., manuscript in preparation), the molecular mass found in non reducing conditions was 21989 daltons. The observed molecular mass of all TRAMP variants was greater than the calculated value, and the mass increase was linear between adjacent variants, with a concomitant decrease in isoelectric point (Table 3.3).

The differences between the observed and calculated isoelectric points suggested that some amino acids may have been modified at the post-translational level. The modifications would be acid labile to account for the absence of modified amino acids by sequence analysis, and would increase the net negative charge on the protein to account for the low pI. The presence of an acidic post-translational modification was suggested by the observation that TRAMP stains strongly with Alcian Blue following SDS-PAGE (section 2.4.8.4; Fig. 3.6). The staining was quantified by densitometry (section 2.3.5) and found to be linear up to 5 μg of TRAMP (Fig. 3.7). Alcian Blue is normally used to identify proteoglycans (Wall & Gyi, 1988), however TRAMP does not appear to be a proteoglycan as mass spectrometry of variant T1 (Table 3.3) indicates a molecular mass only about 160 daltons greater than that predicted on the basis of the amino acid sequence. Following SDS-PAGE, a protein with the electrophoretic migration of TRAMP was the only one to stain with Alcian Blue in the original 6 M urea extract from skin (Fig. 3.6). On the basis of Alcian Blue staining followed by densitometry of the stained gel (sections 2.4.8.4 & 2.3.5) it was possible to estimate
Table 3.3  Molecular masses and isoelectric points (pI) of TRAMP variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
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<tbody>
<tr>
<td>Mass(^a) (Da)</td>
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<td>22208 ± 22</td>
<td>22278 ± 22</td>
<td>22328 ± 22</td>
<td>22377 ± 22</td>
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<td>pI(^b)</td>
<td>4.43 ± 0.1</td>
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<td>4.27 ± 0.08</td>
<td>4.18 ± 0.08</td>
<td>4.07 ± 0.06</td>
</tr>
</tbody>
</table>

\(^a\) Errors are standard deviations based on standards (carbonic anhydrase, n = 8).

\(^b\) Errors are standard deviations based on standards (soyabean trypsin inhibitor, n = 6).
Figure 3.6 SDS-PAGE of porcine skin TRAMP and lysyl oxidase

TRAMP and lysyl oxidase were partially purified by elution from DEAE-Sepharose and Sephacryl S-400 columns at different urea and salt concentrations (Cronshaw et al., 1993). L = lysyl oxidase, T = TRAMP, E = initial 6 M PBU extract. Gels were run in duplicate and equal amounts of protein were loaded in corresponding lanes for staining with either Coomassie Blue or Alcian Blue. The diffuse bands in the lower part of the Alcian Blue gel are a staining artefact.
Figure 3.7 Linear relationship between the intensity of Alcian Blue staining and known amounts of TRAMP

Different amounts of TRAMP (determined by the bicinchoninic acid assay) were analysed by scanning densitometry after SDS-PAGE. Samples were reduced with β-mercaptoethanol and the gel was stained with Alcian Blue.
that 1 g of piglet skin (wet weight) contained approximately 4 μg of TRAMP.

Tyrosine sulphation represents one possible post-translational modification. There are five tyrosine residues in TRAMP that meet four of the five empirically determined rules for tyrosine sulphation (Hortin et al., 1986) involving the specific positioning of tyrosine residues in exposed protein domains adjacent to acidic amino acids, and relative paucity of hydrophobic residues, basic residues and cysteine. Residues 5, 15, 148, 149 and 176 in the TRAMP sequence represent these putative sulphation sites. The tyrosine sulphate bond is particularly labile to acid hydrolysis (Huttner, 1984). Following treatment of TRAMP with 1 M HCl (section 2.4.7) for 5 min at 100 °C, and prior to SDS-PAGE, Alcian Blue staining almost halved relative to Coomassie Blue staining (Fig. 3.8(a)), though there was evidence of partial hydrolysis. By the same criteria, TRAMP boiled in distilled water did not affect subsequent Alcian Blue staining relative to Coomassie Blue staining (not shown). To further investigate tyrosine sulphation, TRAMP was incubated with sulphatase (either type V from limpet or type VII from abalone; section 2.4.7) prior to SDS-PAGE. Following treatment with either enzyme, Alcian Blue staining decreased almost three fold with no change in Coomassie Blue staining (Fig. 3.8(a)). A small amount of β-glucuronidase activity was present as a contaminant in the sulphatases. To examine any effects of such contamination, separate experiments with purified β-glucuronidase (E. coli; Boehringer) were carried out at two times the level present in the sulphatase preparations. There was no effect on the electrophoretic migration of TRAMP or staining with Alcian Blue and Coomassie Blue (Fig. 3.8(a)). To further establish whether TRAMP contained sulphated tyrosine residues, the chromatographic behavior of a mixture of variants (T3, T4 & T5) was examined on Mono Q FPLC before and after sulphatase treatment. The results (Fig. 3.9) show that, while small protein peaks elute at the original positions of T3, T4 and T5, three prominent peaks appear earlier in the elution gradient. By SDS-PAGE analysis (not shown), all three peaks co-migrated with T3, T4 and T5. No protein peaks were observed on the Mono Q elution profile of
Figure 3.8 Effects of various deglycosylation and desulphation treatments on TRAMP staining by (a) Alcian blue and (b) periodic acid Schiff (PAS) reagent.

Staining of SDS-PAGE gels was quantified with a scanning densitometer and expressed relative to Coomassie Blue staining of equal amounts of protein treated in identical conditions. Error bars are standard deviations based on at least three measurements. TRAMP was analysed after purification by DEAE-Sephacel and Sephacryl S200 chromatography, without subsequent separation of ionic variants. Con = control; E/N = endoglycosidase F/N-glycosidase F; Neu = neuraminidase; O-G = O-glycosidase; Glu = β-glucuronidase; HCl = 1 M HCl; Sul = sulphatase (type V).
Figure 3.9 Chromatography of TRAMP variants following sulphatase treatment
Chromatography of TRAMP variants (T3, T4, & T5) on Mono Q FPLC, before (a) and after (b) treatment with sulphatase (type V).
sulphatase alone (when used at the same level used for the TRAMP incubations; data not shown).

Sequence analysis, mass spectrometry and susceptibility to mild acid hydrolysis and sulphatase treatment suggest that TRAMP contained two sulphate residues. However the chemical basis of the five variants of TRAMP is not understood (see discussion). Carbamylation at lysine residues (due to prolonged exposure in urea containing buffers) is a possible explanation, as conversion to more acidic forms would be expected. However, when separated by Mono Q FPLC, TRAMP variants (T2 or T3) did not interconvert on further exposure to 6 M urea for prolonged periods (up to 10 days) at 37 °C, as evidenced by an unaltered Mono Q elution profile following rechromatography of T2 and T3 (not shown). Similarly, isoelectric focussing data also confirmed that the pI of variant T3 was not changed on further exposure to urea (not shown).

Possible glycosylation of TRAMP was investigated by the following experiments. There are no hexosamines in the amino acid composition of TRAMP, as also observed by Neame et al. (1989). The presence of carbohydrate was suggested by periodic acid Schiff (PAS) staining (section 2.4.8.2; Fig. 3.8(b)) of TRAMP following SDS-PAGE. However, after digestion of TRAMP variants with endoglycosidase F/N-glycosidase F, neuraminidase, O-glycosidase and β-glucuronidase (section 2.4.5), there were no changes in the apparent molecular mass, band sharpness, PAS or Alcian Blue staining intensity when analysed by SDS-PAGE (Fig. 3.8). Control experiments with fetuin (which contains sialic acid residues, and N and O-linked carbohydrate; manufacturers literature) treated with the above enzymes (excluding β-glucuronidase) caused a decrease in the apparent molecular mass indicative of enzyme activity (not shown). Possible glycosylation of TRAMP was further investigated by incubating lectins (wheat germ agglutinin (WGA) and concanavalin A (Con A) with protein blotted onto nitrocellulose (sections 2.3.4.2 & 2.4.6.2). TRAMP was not detected with the WGA (cojugated to horseradish peroxidase; HRP), however when incubated with con
A and peroxidase, some staining of TRAMP was observed (Fig. 3.10), and indicated that the protein was glycosylated. To examine whether con A or HRP was binding directly to TRAMP the following experiments were carried out. By fluorography of proteins in nitrocellulose transfers after incubation with the iodinated con A, there was no binding of the lectin to TRAMP, Con A-I$_{125}$ did however detect the target glycoprotein (procollagen C-propeptide). By incubating protein electroblots with HRP alone, it was found that the enzyme bound to TRAMP (Fig. 3.10). These results taken together indicate that TRAMP is not glycosylated, and that TRAMP may have an affinity for the enzyme glycoprotein HRP.

Polyclonal antibodies to Mono Q purified TRAMP were raised in rabbits (section 2.4.9). The specificity of the antiserum was tested by immunoblotting experiments after SDS-PAGE of the denatured proteins (section 2.3.4). Using the ECL detection system for localisation of the antigen (section 2.4.9.1), TRAMP was the only protein to be stained in the original 6 M urea crude extract from porcine skin (Fig. 3.11), no staining was observed with purified lysyl oxidase (not shown). Similarly, TRAMP was the only protein to be stained in urea extracts of several porcine tissues (Fig. 3.11). Control experiments with serum taken before the TRAMP immunisations had began did not detect any protein in immunoblots.

Because lysyl oxidase and TRAMP frequently copurify, possible interactions between them were examined. The activity of lysyl oxidase on an elastin substrate (section 2.4.3) was not affected in the presence of increasing concentrations of TRAMP, up to a 40 : 1 molar ratio of TRAMP to lysyl oxidase (Shackleton, personal communication).

3.1.5 Further Characterisation of Lysyl Oxidase

SDS-PAGE analysis of lysyl oxidase showed that the protein often occurred as a doublet (M$_r$ 34-36 K; Fig. 3.2, lane 5) with a weak slowly migrating component. Preliminary data indicated that there were glycosylated and non glycosylated forms of
Figure 3.10 Detection of glycoprotein in lysyl oxidase and TRAMP preparations
Proteins were partially purified by DEAE Sephacel and Sephacryl S-200 chromatography (Fig. 3.2, lane 4), electrophoresed on a reducing 12 % SDS-polyacrylamide gel and then transferred to nitrocellulose. 1, TRAMP (24 K) and lysyl oxidase (34-36 K) stained with the concanavalin A-peroxidase procedure; 2, stained with peroxidase only.
Figure 3.11 Immunoblotting of porcine TRAMP from different tissues
Samples were electrophoresed on a reducing 12 % SDS-polyacrylamide gel, blotted onto nitrocellulose and incubated with polyclonal antibodies. All porcine tissue samples are crude 6 M urea extracts, and equal amounts of each protein were loaded. TRAMP (lane 6) was purified by DEAE Sephacel and Sephacryl S-200 chromatography. 1, heart; 2, lung; 3, liver; 4, skeletal muscle; 5, skin; 6, purified TRAMP; 7, umbilical cord.
the enzyme. By concanavalin A affinity chromatography (section 2.4.6.1), the minor component (36 K) was retained, and was subsequently eluted with 1 M α-D-methylmannoside. The 34 K component which was not retained by the column had enzyme activity, as did the 36 K component; both activities were inhibited with 0.2 mM BAPN, a specific inhibitor of lysyl oxidase (Tang et al., 1983). Further experiments using lectins incubated with protein electroblots (section 2.4.6.2) demonstrated binding of con A to the 36 K protein (Fig. 3.10), which was abolished in the presence of α-D-methylmannoside. Control experiments with the procollagen C-propeptide (which has carbohydrate groups mannose and N-acetylglucosamine present) also showed binding to con A. The binding of both proteins to con A appears to be specific as molecular weight standards (Pharmacia) were not detected by the lectin on nitrocellulose blots.

3.2 Discussion

The interaction that occurs between lysyl oxidase and TRAMP with Sephacryl S-200, and recently Sephacryl S-400, in conditions of low ionic strength, is not understood (Cronshaw et al., 1993). Kagan and Trackman (1991) suggested that removing urea from the extracted preparation induces polymerisation of the enzyme. The aggregated enzyme is retained at the top of the S-200 gel filtration column, which, after washing with urea-free buffer, is disaggregated and eluted with urea-supplemented buffer. However, the observation that both lysyl oxidase and TRAMP do not interact with either the polyacrylamide based Biogel or agarose based Sepharose 4B gel filtration media under the same conditions (MacBeath, unpublished observations) suggests that there is a more specific interaction with S-200.

Amino acid sequence data from peptide fragments confirm that the enzyme (purified from urea extracts of porcine skin, followed by DEAE ion exchange chromatography and selective interaction with Sephacryl S-200; Shackleton & Hulmes, 1990a; Cronshaw et al., 1993) corresponds to both rat aorta (Trackman et al., 1990; Trackman et al., 1991) and human placenta (Hamalainen et al., 1991) lysyl oxidase.
It was surprising to find that TRAMP was unrelated to lysyl oxidase, as several workers had reported the presence of a degradation product in enzyme preparations with approximately the same molecular mass as TRAMP (Sullivan & Kagan, 1982; Kuivaniemi et al., 1984; Burbelo et al., 1986). The identity between TRAMP and the 22 K extracellular matrix protein from bovine skin (Neame et al., 1989) became apparent only after searching a protein sequence database (Collins, personal communication). By urea extraction followed by DEAE ion exchange chromatography and then gel filtration on Sepharose CL-6B, the 22 K protein from bovine skin copurified with dermatan sulphate proteoglycans (Choi et al., 1989; Neame et al., 1989). A similar procedure was used to purify lysyl oxidase from porcine skin, but with TRAMP copurifying using a specific interaction with Sephacryl S-200 as the final step. It is possible that TRAMP is also related to the 23 K protein contaminant found in preparations of human placental lysyl oxidase (Table 3.1; Kuivaniemi et al., 1984), although, in the absence of sequence data, it is impossible to confirm this. TRAMP does not appear to be present in urea extracts of bovine cartilage (Choi et al., 1989), although preliminary data from immunoblots indicate that porcine TRAMP has a widespread tissue distribution (MacBeath & Forbes, unpublished observations). Using polyclonal antisera, TRAMP was the only protein to be detected in extracts from several porcine tissues (Fig. 3.11); this appears to suggest that TRAMP is not derived from a precursor form.

From the data, TRAMP is clearly not a degradation product of lysyl oxidase, although a distinct protein of similar molecular mass (22-24 K) exists which from peptide mapping (Sullivan & Kagan, 1982) or immunoblotting (Kuivaniemi et al., 1984; Burbelo et al., 1986) does appear to be a genuine degradation product of the enzyme.

It is not clear why both TRAMP and lysyl oxidase occur as several variant forms. The existence of multiple ionic forms of lysyl oxidase has been observed in enzyme preparations from chick cartilage (Stassen, 1976), bovine aorta (Sullivan &
Kagan, 1982), human placenta (Kuivaniemi et al., 1984) and umbilical cord (Burbelo et al., 1986). As only one gene has been found in rat genomic DNA (Trackman et al., 1990), molecular heterogeneity of lysyl oxidase may stem from post-translational modifications. Another possibility is carbamylation of amino groups on the enzyme due to cyanate derived from urea (Meens & Feeney, 1971). Both TRAMP (Neame et al., 1989) and lysyl oxidase (Trackman et al., 1990; Trackman et al., 1991; Hamalainen et al., 1991) contain only a few lysine residues (Tables 3.1 & 3.2). The decreasing isoelectric point for TRAMP variants T1 to T5 are consistent with an increased extent of carbamylation since, from complete amino acid sequence data (Cronshaw et al., manuscript in preparation), the theoretical pI's would be 4.53, 4.44, 4.36, 4.28 and 4.20 (with pyroglutamate at the N-terminus and 0, 1, 2, 3, or 4 lysines carbamylated, respectively; cf. Table 3.1). Mass spectrometry data is also consistent with carbamylation, since the additional mass for each lysine residue carbamylated (43 Da) is within the experimental range of the average mass increment between consecutive TRAMP variants (32-76 Da). However carbamylation is probably not the basis for multiple forms of TRAMP, since Mono-Q-separated variants T2 and T3 retain their original chromatographic behaviour when further exposed to urea for prolonged periods. Isoelectric focussing also confirmed that TRAMP was not converted to more acidic forms, following prolonged storage in urea-containing buffers. Furthermore, amino acid analysis (Cronshaw, personal communication) of acid hydrolysates from the five variants of TRAMP did not contain detectable amounts of homocitrulline, the carbamylated derivative of lysine (Kagan et al., 1979). Similar observations were reported by Kuivaniemi et al. (1984) for human placenta lysyl oxidase variants.

Although lysyl oxidase does not appear to be glycosylated (Sullivan & Kagan, 1982), preliminary data indicates that a small proportion of porcine lysyl oxidase (or an enzyme precursor) appears to contain N-linked sugars. This observation is consistent with the finding of possible N and O-linked glycosylation sites in the enzyme sequence derived from the cDNA (Trackman et al., 1990; Trackman et al., 1991; Hamalainen et
Staining of proteins (separated by SDS-PAGE) with Stains-All indicate that TRAMP is not phosphorylated. However, several observations indicate the presence of tyrosine sulphation in TRAMP. Sequence data analysis indicates five possible sites. All five TRAMP variants stain strongly with Alcian Blue, which is relatively specific for sulphated biopolymers in 50 mM MgCl₂ (Scott, 1973), and susceptibility to mild acid hydrolysis and sulphatase treatment are also consistent with sulphation at tyrosine residues. The difference of around 154 Da between the observed and calculated molecular mass is consistent with sulphation at two tyrosine residues (i.e. additional mass 2 x 79 Da). The presence of two sulphated tyrosines would be expected to lower the isoelectric point of each TRAMP variant by about 0.17 pH unit, which is also in agreement with the difference between the observed and calculated pI's. Re-chromatography of TRAMP variants following sulphatase treatment demonstrates that there is a shift to less acidic forms of the protein and hence earlier elution from the column. Even more interesting was the observation that sulphatase treatment caused the variants to elute closer together, indicating that they have been converted to more similar ionic species. Although the results are consistent with the presence of tyrosine sulphation in TRAMP, in the absence of mass spectrometry data (following desulphation of TRAMP), it is not yet possible to conclude that variants T1 to T5 differ in their extents of sulphation. Tyrosine sulphation is, however, a widespread post-translational modification in extracellular matrix proteins, e.g. nidogen/entactin, fibronectin, dermatan sulphate proteoglycans (Huttner, 1988), Procollagens III and V (Jukkola et al., 1990), bone sialoprotein II (Ecarot-Charrier et al., 1989; Midura et al., 1990) and fibromodulin (Antonsson et al., 1991). In types III and V procollagen, the anion is located at the N-terminus (Jukkola et al., 1990), and in fibromodulin tyrosine sulphation is also in the N-terminal region, in a tyrosine-rich sequence where tyrosine occurs every second or third residue (Antonsson et al., 1991). This feature is also present in TRAMP (Neame et al., 1989), though the sequences are not homologous. Multiple variants of fibromodulin have also been observed (Lauder, R.M., et al., 1991).
Nieduszynski, I.A. & Huckerby, T.N., personal communication). The role of tyrosine sulphation has not been extensively investigated for extracellular matrix proteins, although desulphation of fibronectin affects its binding affinities to several ligands (Suiko & Liu, 1988).

The biological properties of TRAMP are not fully understood. The primary structure contains three repeat regions, each having a similar pattern of looped disulphide bonds (Neame et al., 1989). A six amino acid consensus sequence Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr is found in each loop. This domain may be involved in association of the molecule with other matrix components (Neame et al., 1989). The equivalent 22 K protein from bovine skin, appears to show binding activity to cell surface receptors, possibly of the integrin class (Lewandowska et al., 1991). TRAMP has no lysyl oxidase activity on an elastin substrate, nor does it affect the activity of added lysyl oxidase. The apparent lack of interaction with elastin is consistent with the absence of TRAMP in lysyl oxidase purified from rat skin by an elastin affinity procedure (Romero-Chapman et al., 1991). It has been shown, however, that TRAMP binds to collagen and accelerates the formation of fibrils in vitro (Chapter 4). Since it is already known that the activity of lysyl oxidase on a collagen substrate is markedly increased by prior assembly of the molecules into fibrils (Siegel, 1974), it is possible that TRAMP may influence enzyme activity indirectly, via aggregation of the substrate. The observations that the bovine 22 K protein interacts with dermatan sulphate proteoglycans (Neame et al., 1989; Lewandowska et al., 1991) and that DSPG II (decorin) is known to inhibit collagen fibril formation (Brown & Vogel, 1989) suggest a complex regulatory role for TRAMP interactions with other components of the extracellular matrix (see Chapter 4).
Chapter 4

Influence of TRAMP on Collagen I Fibrillogenesis
4.1 Results

4.1.1 Characterisation of TRAMP and Collagen

The five variants of TRAMP (separated by Mono Q FPLC; Fig. 3.5(a)) were analysed for purity by SDS-PAGE (see Chapter 3, Fig. 3.5(b)). No trace of contaminating proteins, including lysyl oxidase, was present in these preparations. Further SDS-PAGE analysis (Fig. 4.1) revealed that the purified lathyritic rat skin collagen (section 2.1) used for fibril formation consisted almost entirely of α-chains, with the remaining protein bands (approximately 5% of the total) in the form of multimeric cross-linked, collagen β and γ components. Scanning densitometry of the gel stained with Coomassie Blue showed that the α1 and α2 chains were in a 2:1 ratio, indicative of the absence of type III collagen which is also found in skin and migrates under reducing conditions with a similar electrophoretic mobility to the α1 chain of collagen I. Identical gels were obtained under both reducing and non-reducing conditions (not shown). This also indicated the absence of collagen III, which contains reducable disulphide bonds. Collagen preparations were further characterised by SDS-PAGE after CNBr cleavage (section 2.3.2). The peptide pattern (Fig. 4.1) was as expected for pure type I collagen (Bornstein & Sage, 1980). Further to this, no appreciable 280 or 260 nm absorbing material was found, suggesting low levels of contaminating non-collageneous proteins and/or nucleic acids, respectively. Amino acid analysis (section 2.3.1) of the purified collagen indicated 330 residues of glycine/1000 (Table 4.1) which was also consistent with the preparation being 100% collagenous. The amino acid composition for the skin collagen is also in agreement with that obtained by other workers (Table 4.1). The tyrosine content was 3.2 ± 0.64 (SD, N = 3) per 1000 residues. Since all the tyrosine residues are located in the terminal, non-helical telopeptide regions of the collagen molecule (approximately 4/1000 residues) which are susceptible to proteolytic cleavage, it was concluded that the telopeptides were intact following extraction and purification (Payne et al., 1986).
Figure 4.1 SDS-PAGE of collagen I and peptides produced after CNBr cleavage
Collagen I was electrophoresed on a 6 % SDS-PAGE gel (track 1). CNBr peptides of collagen I were separated by SDS-PAGE on a 12 % gel (track 2). The CNBr peptide pattern produced from collagen III is shown for comparison (track 3). Samples were reduced with β-mercaptoethanol and the gels were stained with Coomassie Blue.
Table 4.1 Amino acid composition of rat skin collagen

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ND = not determined.

Values are residues per 1000

<sup>a</sup> The average of triplicate analyses are presented. The error was approximately 5%, values lower than 10 residues/1000 tended to have larger errors (10-20%).
4.1.2 Kinetics of Fibril Assembly and Disassembly

Collagen fibril assembly was initiated by the "warm start" technique (section 2.5; Holmes et al., 1986), in which collagen in 5 mM acetic acid and double-strength fibril formation buffer (60 mM disodium phosphate, 60 mM TES, 270 mM NaCl, pH 7.4) are pre-incubated separately at 34 °C before mixing 1:1. Fibril formation was monitoring by changes in turbidity at 313 nm. Prior to this, collagen solutions were centrifuged (100,000 g for 4 hours) to remove large aggregates, if present. All other solutions were filtered with 0.22 μm Millipore filters. When TRAMP was present it was dialysed against double-strength fibril formation buffer at 4 °C for 24 hours with 2 changes. In the absence of TRAMP and with a final collagen concentration of 0.2 mg/ml, fibrils were reconstituted with a typical sigmoidal turbidity curve (Fig. 4.2). (Collagen concentrations were determined by the hydroxyproline or BCA assay; sections 2.3.3 & 2.4.1, respectively). The curve shown in Figure 4.2 is characterised by a lag phase (t_{lag}; measured by extrapolating the linear region of the growth phase to the time axis) of about 20 minutes during which there is no detectable change in turbidity, a growth phase during which turbidity changes rapidly, and finally a plateau region after about 150 minutes where turbidity again remains constant. When TRAMP was present, however, fibril formation was accelerated, as also shown in Figure 4.2 by a decrease in t_{lag} and an increase in growth rate (i.e. maximum slope of growth phase). These changes were concentration dependent, with progressive acceleration of fibril formation in the presence of increasing amounts of TRAMP (Fig. 4.2).

Acceleration of fibril formation was observed at very low concentrations of TRAMP, down to a 1 to 20 molar ratio of TRAMP to collagen. Table 4.2 represents data collected from several experiments, and shows that the kinetics (t_{lag}, maximum slope of growth phase and t_{1/2} (time required to reach the middle point of the final fibril opacity)) of collagen reconstitution and the total turbidity change (Δh) are consistently increased in the presence of TRAMP. All five TRAMP variants were found to be equally effective in accelerating fibril formation. TRAMP also stimulated collagen
Figure 4.2 Turbidity-time curve showing the acceleration of collagen fibril formation by TRAMP
Initial assembly at 34 °C with a fixed concentration of collagen (200 μg/ml) and increasing concentrations (μg/ml) of TRAMP variant T5.
Table 4.2 Effect of TRAMP on the kinetics of collagen fibril formation

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<th>$t_{\text{lag}}$ (min)</th>
<th>$t_{1/2}$ (min)</th>
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<td>(3.31)</td>
<td>(0.0033)</td>
<td>(0.015)</td>
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<td>(2.60)</td>
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</tbody>
</table>

Kinetic parameters are indicated as means, with standard errors in parentheses, where the number of experiments in each group was 24. Significant differences at the $p<0.001$ level (Student's $t$ test) are indicated (**). $t_{\text{lag}}$ = lag time, determined by the intercept of the line of maximum slope in the turbidity-time curve with the time axis. $t_{1/2}$ = time to reach half maximum turbidity. Data for TRAMP variants T4 and T5 were combined as the results obtained with each variant were indistinguishable (not shown).
fibrillogenesis when fibril formation was initiated by a "cold start" procedure (Holmes et al., 1986; not shown). In this approach cold neutral collagen solutions (4 °C; pH 7.4), were centrifuged (100,000 g for 4 hours) to remove any large aggregates, followed by warming the solution to 34 °C to allow fibril assembly to proceed. Fibrillogenesis experiments also showed that the presence of 1 mg/ml bovine serum albumin had no effect on collagen fibril formation or on the ability of TRAMP to accelerate the rate of fibril formation (not shown).

Taking $t_{1/2}$ as a measure of the rate of fibril assembly, the dependence of $t_{1/2}$ on collagen concentration was determined in the range 0.05 to 0.4 mg/ml. It was found that the rate of fibril assembly in the absence and presence of TRAMP expressed as a reciprocal of $t_{1/2}$ was directly proportional to collagen concentration. The slope of the line plotted as $\log \left( \frac{1}{t_{1/2}} \right)$ as a function of log concentration fitted with a least squares straight line was 1, when fibrils were formed in the presence and absence of TRAMP (Fig. 4.3). This finding suggests that fibrils formed in the absence or presence of TRAMP assemble by a similar mechanism (Williams et al., 1978).

The maximum turbidity values reached for collagen fibrils formed in the absence of TRAMP was less than that when TRAMP was present (Table 4.2). The final turbidity level for collagen formed in the presence of 20 μg/ml TRAMP (i.e. approximately equimolar concentrations of collagen to TRAMP) was about 20 % higher than that seen for collagen alone (Fig. 4.2). As the final turbidity is a measure of fibril diameter and/or the amount of collagen participating in fibril formation, it may be that TRAMP either enhances the efficiency of the incorporation of collagen into those fibrils and/or increases the final fibril diameter. In order to ascertain whether TRAMP increased the extent of fibril formation, fibrils were sedimented by centrifugation (15,600 g, 2 min) at room temperature 1-2 hours after the turbidity plateau was reached, and hydroxyproline in the supernatants was determined and compared to the total hydroxyproline present in the experiments. It was found that 7.5 % of the total collagen remained in the clear supernatant of fibrils formed in the presence of 20 μg/ml
Figure 4.3 Dependence of $t_{1/2}$ of fibril formation on initial concentration of collagen

Fibrils were formed at 34 °C in the presence (■) and absence (○) of TRAMP (variant T4, 5 µg/ml). The least squares line through both sets of points has a slope of 1.
TRAMP (Fig. 4.4). Increasing amounts of collagen were detected in the supernatants after fibrillogenesis in the presence of decreasing amounts of TRAMP (Fig. 4.4). In the absence of TRAMP, only 75% of total collagen was incorporated into the fibrils, as ascertained by the amount of sedimentable collagen (Fig. 4.4).

The kinetics of fibril disassembly were examined by a temperature jump method (Brennan & Davison, 1980). TRAMP was found to partially stabilise fibrils against dissociation following a temperature drop from 34 °C to 4 °C (Fig. 4.5). The rate and extent of dissociation were progressively smaller when fibrils were initially formed in the presence of increasing amounts of TRAMP. The disassembly reaction was initially very rapid and was complete within 40 minutes. This was followed by a much slower rate of dissociation which was complete within 16 hours. After this period fibrils formed without TRAMP had completely solubilised. This complete reversibility of the fibril assembly reaction demonstrates the absence of lysine-derived aldehydes which are known to form covalent inter-molecular cross-links, thus stabilising the fibrillar aggregate against low temperature solubilisation (Na, 1989). However, for fibrils formed in the range of TRAMP concentrations used, dissociation was only 85-95% complete after 16 hours, and no further changes were observed on further storage (up to 72 hours) at 4 °C (Fig. 4.5). Again, all TRAMP variants were found to stabilise fibrils in this way.

As lysyl oxidase was also present in the early stages of TRAMP purification, and as lysyl oxidase is known to bind to collagen (Cronlund et al., 1985), it was possible that the apparent effect of TRAMP on the kinetics of fibril assembly and disassembly may have been due to trace amounts of lysyl oxidase. Several observations indicated that this was not the case. First, when large amounts (5-10 μg) of each TRAMP isoform were subjected to SDS-PAGE followed by Coomassie Blue, no trace of lysyl oxidase was found (Chapter 3; Fig. 3.5(b)), and subsequent silver staining of the Coomassie stained gel also showed the absence of contaminating enzyme. Second, no enzyme activity (as judged by the ultrafiltration assay using an elastin substrate) was
Figure 4.4 The binding of TRAMP to collagen fibrils and its effect on increasing the amount of fibrillar collagen

Amounts of collagen in the supernatant (determined by hydroxyproline assay) and TRAMP in the pellet (determined by scanning densitometry) after centrifugation of fibrils formed at 34 °C from a fixed concentration of collagen (200 μg/ml) and increasing concentrations of TRAMP (variant T5). The volume of the incubation mixture was 1 ml.
Figure 4.5 Turbidity-time curve showing the acceleration of collagen fibril formation by TRAMP and partial stabilisation of fibrils against low temperature dissociation.

Assembly at 34 °C as in Figure 4.2 followed by a rapid temperature drop to 4 °C. Turbidity values (A<sub>313 nm</sub>) after 16 h and 72 h at 4 °C are indicated. TRAMP variant T5 was used.
associated with the TRAMP preparations in fibril formation buffer. Third, when preparations containing lysyl oxidase and TRAMP elute from a Mono Q FPLC column, TRAMP variants (T4 & T5) are completely resolved from the enzyme (Chapter 3; Fig. 3.4(b)). Therefore, because all TRAMP variants have a similar effect on fibril assembly and disassembly, it is unlikely that these effects are due to contaminating enzyme. Fourth, when fibril formation was carried out in the presence of 0.2 mM β-aminopropionitrile, a specific inhibitor of lysyl oxidase (Tang et al., 1983), TRAMP (pre-incubated in 0.2 mM β-aminopropionitrile for 30 minutes) continued to accelerate fibril formation and inhibit subsequent low temperature dissolution of the formed fibrils (not shown). Fifth, SDS-PAGE analysis showed that there was no increase in β and γ components (cross-linked aggregates of collagen I) in fibrils formed in the presence of 20 µg TRAMP. Finally, preliminary observations with 3-5 µg of purified lysyl oxidase variant L1 (Chapter 3; Fig. 3.4(b)), at concentrations comparable to that of TRAMP used in the above experiments, showed no effect on the kinetics of fibril assembly (Fig. 4.6).

4.1.3 Morphological Studies

To examine any effects of TRAMP on fibril morphology, the following experiments were done. Fibrillar collagen samples were obtained by centrifugation at room temperature 1-2 hours after the turbidity plateau was reached (approximately 3-4 h after the initiation of fibril formation). When analysed by electron microscopy after negative staining (sections 2.5.1.1 & 2.5.1.4) fibrils reconstituted in the presence and absence of TRAMP appeared to be well formed with no apparent differences in their D-periodic banding pattern (Figs. 4.7(a) & 4.7(b)). The banding pattern consisted of a fine polarised band pattern superimposed on alternate light and dark regions. When viewed in the electron microscope at lower magnification, the fibrils were of undetermined length and fibril ends were seen only occasionally. When the ends were seen they had highly tapered tips (Figs. 4.7(c) & 4.7(d)). However, measurements of
Figure 4.6 Effect of lysyl oxidase on the kinetics of collagen fibril formation
Turbidity-time curve showing the effect of TRAMP (T, variant T5, 4 μg/ml) and lysyl oxidase (L, variant L1, 4 μg/ml) on fibril formation at 34 °C from a fixed concentration of collagen (200 μg/ml). Assembly in standard buffer conditions in the absence of TRAMP or lysyl oxidase (B) is shown for comparison.
Figure 4.7 Morphology of collagen fibrils formed in the absence and presence of TRAMP

Electron micrographs of fibrils assembled from collagen (200 μg/ml) in the absence (a & c) and presence (b & d) of TRAMP (variant T5, 10 μg/ml). Specimens were negatively stained with 1 % (w/v) phosphotungstic acid at pH 7.4. Panels a and b are at a magnification of 28,000X and panels c and d at a magnification of 169,000X. The D-periodicity is 65 nm.
fibril diameter obtained from micrographs of ultra-thin sections (sections 2.5.1.3, 2.5.1.4 & 2.5.1.5) revealed consistent differences (Fig. 4.8). The mean diameter of collagen fibrils formed in the absence of TRAMP was 116 ± 29.7 nm (SD; n = 659). In the presence of TRAMP, the fibrils were thinner, and had a mean diameter of 85.5 ± 21.3 nm (SD; n = 705). The maximum diameter observed was 200 nm for collagen alone, and 149 nm for fibrils formed with TRAMP. Thus in the presence of TRAMP, both the mean diameter and the maximum diameter are decreased by a factor of approximately 0.75.

4.1.4 TRAMP-Collagen Interactions

The possible binding of TRAMP to collagen was investigated by SDS-PAGE analysis of pellets and supernatents after fibril formation and centrifugation as above (section 2.5.2.1). Following electrophoresis and subsequent Coomassie Blue staining of the gel, TRAMP remained in the supernatant in the absence of collagen. (The staining of TRAMP previously quantified by densitometry was linear up to 5 μg protein; Fig. 4.9). When collagen was present however, increasing amounts of TRAMP were found in the pellet as the total TRAMP concentration increased (Fig. 4.4). The amounts of TRAMP in the pellet approached saturation at 8 μg TRAMP to 200 μg collagen. Taking 22 kDa and 300 kDa as the respective molecular masses of TRAMP and collagen, this corresponds to a stoichiometric ratio of 1 mol TRAMP to 2 mol collagen. The results shown in Figure 4.4 also demonstrate that the amount of TRAMP bound was appreciably higher than that due to simple entrapment of the protein, as explained below. A constant proportion of total TRAMP would be expected to appear in the pellet by trapping alone (Carr & Hermans, 1978). Thus specific incorporation was evaluated by adding bovine serum albumin (BSA) in the place of TRAMP and it was shown that only 3% of the total BSA occurred in the pellet when fibrils were assembled at 200 μg/ml collagen and up to 200 μg/ml BSA. In contrast, when fibrils were formed in a total concentration of 20 μg/ml TRAMP, the amount
Figure 4.8 Diameter distributions of fibrils formed in the presence and absence of TRAMP

Diameters of fibrils (determined by electron microscopy of sectioned pellets) formed at 34 °C in the presence (a) and absence (b) of TRAMP (variant T5, 5 µg/ml). The collagen concentration was 200 µg/ml. Both distributions are significantly different at p<0.001 (Student's t test).
Different amounts of TRAMP (determined by the bicinchoninic acid assay) were analysed by scanning densitometry after SDS-PAGE. Samples were reduced with β-mercaptoethanol and the gel was stained with Coomassie Blue.
bound was 8 µg/ml, or 42% of the total.

The results from the kinetics of fibril assembly revealed that TRAMP had a pronounced effect during the lag phase. Thus attempts were made to study possible interactions between TRAMP and collagen during the early stages of fibril assembly. Samples taken 2-3 minutes after the initiation of fibril formation were examined by electron microscopy, after rapid freeze drying and rotary shadowing (sections 2.5.1.2 & 2.5.1.4). The results (Fig. 4.10) show representative electron micrographs of the first formed aggregates in the assembly process. In the presence and absence of TRAMP, a similar meshwork of filamentous microfibrils were observed. However, it was found that when TRAMP was present, collagen monomers occasionally had a globular region bound to one end of the molecule. These molecules were often seen projecting from the surface of the microfibrils (Fig. 4.10). By comparison with electron micrographs of rotary shadowed procollagen molecules, the globular region was similar in size to the C-propeptide (i.e. approximately 100 kDa). This was much larger than expected for TRAMP (22 kDa), though gel filtration of TRAMP in phosphate buffered saline (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.8) indicated that TRAMP forms high molecular weight complexes in physiological buffers (Shackleton, personal communication). In order to determine whether TRAMP was bound to the N or C-terminal part of the the collagen molecule, similar rotary shadowing experiments were carried out but using procollagen (a gift from Mr A. Choglay) in place of collagen. However the results (not shown) were difficult to interpret as the procollagen appeared to be highly contaminated with other small molecules, therefore the location of the binding site(s) could not be confirmed.

The binding of TRAMP to collagen was further investigated by the following fluorescence polarisation experiments (section 2.5.2.2). Since an increase in polarisation over the polarisation of free (Pf) fluorescein isothiocyanate-labeled TRAMP (FITC-TRAMP) indicates an increase in bound TRAMP, binding here is reported in terms of measured polarisation values. In an attempt to study TRAMP binding to
Collagen (50 μg/ml) in the presence of TRAMP (variant T4, 5 μg/ml) were rotary shadowed with platinum and examined by transmission electron microscopy. Samples were examined immediately (a) and 3 min (b) after the initiation of fibril formation at 34 °C. A small percentage of the molecules have a terminal globular region (arrows, a & b). Collagen I in 5 mM acetic acid at 20 μg/ml is shown for comparison (c). Bar, 300 nm. Magnification, 53,000x.
collagen molecules, non-fibrillogenic conditions (i.e. 20 °C) were used. Only a small increase (0.02-0.03) in the polarisation of the FITC-TRAMP/collagen mixture was observed upon addition of collagen (Fig. 4.11), indicating that the majority of TRAMP was not bound. When collagen was added to FITC-TRAMP and examined approximately 1 min after the addition, an immediate increase in the measured polarisation ($P_m$) was observed over the polarisation of free FITC-TRAMP ($P_f$; FITC-TRAMP = 0.095), and which remained constant for at least 1 hour (Fig. 4.11). As the collagen concentration was increased from 50 µg/ml to 100 µg/ml the polarisation increased (relative to $P_f$) from $0.023 \pm 0.004$ (SD; n=3) to $0.038 \pm 0.003$ (SD; n=3) in the presence of a fixed amount (1 µg) of FITC-TRAMP. These results suggest that FITC-TRAMP binds to monomeric collagen at 20 °C, and that the binding is rapid. However it is not known if the binding affinity of FITC-TRAMP is similar to that of the unlabelled protein.

4.1.5 Nature of the TRAMP-Collagen Interaction

To further study the effect of TRAMP on fibril formation, the kinetics of assembly were modified in the presence of increased ionic strength (Williams et al., 1978), urea (Fessler & Tandberg, 1975) or glucose (Rathi et al., 1989) in the fibril formation buffer. The ionic strength was varied by changing the NaCl concentration. From 0.135 M to 0.27 M NaCl, and in the absence of TRAMP there was an approximately three fold increase in $t_{1/2}$ (Fig. 4.12(a)). Similar results have been reported by Williams et al. (1978), though this effect was partially reversed by the presence of TRAMP (Fig. 4.12(a)). There were no obvious differences in the final turbidity. A delay in fibril formation was also observed in the presence of 25 mM urea, as observed elsewhere (Fessler & Tandberg, 1975), though again TRAMP accelerated the assembly process (Fig. 4.12(b)). The final turbidity in the presence of TRAMP also appeared to be slightly greater. D-glucose at 0.05 M also reduced the rate of fibrillogenesis (Fig. 4.12(c)) as also observed elsewhere (Rathi et al., 1989). The final
Figure 4.11 Time course of TRAMP binding to collagen
TRAMP binding was monitored as the increase in polarisation, using 50 µg/ml collagen in the presence of 1 µg/ml of fluorescently labeled TRAMP at 20 °C. \( P_F \) the polarisation of fluorescein-labeled TRAMP in the absence of collagen, did not change during the course of the experiment.
Figure 4.12 The effect of ionic strength, urea and glucose on Collagen I self-assembly in the presence of TRAMP

Turbidity-time curves showing the effect of TRAMP (T, variant T4, 5 μg/ml) on fibril formation at 34 °C from a fixed concentration of collagen (200 μg/ml) at (a) increased ionic strength (S, 0.27 M NaCl), or in the presence of (b) 25 mM urea (U) or (c) 50 mM D-glucose (G). Assembly in standard buffer conditions in the absence of TRAMP (B) is shown for comparison.
opacity in the presence of 0.05 M glucose was also reduced by approximately 30 %, with respect to fibrils formed in the absence of glucose. However these effects were not reversed by TRAMP (Fig. 4.12(c)).

In an attempt to determine whether a native protein conformation was required for TRAMP to exert its effects on fibril assembly, fibrils were reconstituted in the presence of TRAMP which had been denatured by heating for 3 minutes at 100 °C. The results (not shown) demonstrate that denatured TRAMP still accelerated fibril assembly, and to the same extent as native TRAMP.

4.2 Discussion

In recent years, several collagen binding proteins have been identified, including fibronectin, laminin and link protein. Fibronectin and laminin bind to one or more collagen types and also to cell surfaces and thus link cells to the ECM (Albelda & Buck, 1990; Yamada, 1991b; Fox et al., 1991). These collagen binding proteins may also influence the organisation of extracellular matrices. Fibronectin, a large glycoprotein, binds to collagen I at a distinct region on the a1(I) chain (Dessau et al., 1978). The presence of fibronectin affects the rate of collagen fibril formation in vitro (Kleinman et al., 1981; Speranza et al., 1987). Link protein, although primarily involved in the stabilisation of cartilage proteoglycan aggregates (Smith et al., 1987), appear in combination with proteoglycans to modulate collagen fibril formation in vitro (Chandrasekhar et al., 1984). A 54 K collagen II binding protein isolated from Swarm rat chondrosarcoma (Chandrasekhar et al., 1986), is also capable of regulating collagen fibril formation, but only in the presence of proteoglycans. More recently fibromodulin, a 59 K protein with the ability to bind collagens I and II, has been isolated from bovine articular cartilage (Heddob & Heinegard, 1989; Antonsson et al., 1991). In the presence of this protein, fibril formation is markedly delayed, even when present at small concentrations. The ECM contains many constituents many of which remain uncharacterised (Neame et al., 1989). This study begins the functional characterisation
of TRAMP, a 24 K protein isolated from porcine skin that co-purifies with lysyl oxidase.

In the present study it was shown that reconstituted collagen fibrils which are similar to fibrils formed \textit{in vivo} can be obtained reproducibly from purified lathyritic (i.e non-cross-linked) rat skin collagen I. The results also show that the kinetics of the assembly reaction are markedly accelerated in the presence of highly purified TRAMP. Acceleration of collagen fibrillogenesis was observed at low, sub-stoichiometric molar ratios of TRAMP to collagen. This observation suggests that the effect of TRAMP may be on the early, nucleation stage of fibril assembly. The size and nature of the nuclei has been the subject of several investigations (Veis & Payne, 1988). One of the first interactions to be detected \textit{in vitro} is the assembly of 4 D staggered dimers, where D is the axial periodicity of collagen fibrils (Silver \textit{et al.}, 1979; Silver & Trelstad, 1980; Ward \textit{et al.}, 1986). It is possible that TRAMP may facilitate and/or stabilise the formation of such dimers and therefore hasten fibril formation by increasing the number of nuclei (Fig. 4.13).

The shape of the turbidity curve is characteristic of a nucleation-growth mechanism for collagen fibril assembly (Na \textit{et al.}, 1986a). However a power dependence of $t_{1/2}$ on collagen concentration of 1 apparently refutes the cooperative nucleation growth mechanism as this value is half the number of monomer units in the nucleus, and lends support to an accretion mechanism for collagen fibril formation (Williams \textit{et al.}, 1978). In juxtaposition with the kinetic studies, electron microscopy after rotary shadowing was used to examine the morphology of collagen aggregates shortly after the initiation of fibrillogenesis. The electron micrographs shown in Figure 4.10 confirm the results reported by Gelman \textit{et al.} (1979), indicating that collagen forms long thin microfibrils during the lag phase of fibril assembly. However the interpretation of the results presented here is based entirely on EM data, thus it is very difficult to assure that no additional aggregation occurred during specimen preparation for electron microscopy.
Figure 4.13 Model of the proposed binding sites for TRAMP on the collagen molecule, and the possible role of TRAMP in stabilising the 4D stagger association between collagen molecules.
TRAMP was shown to bind to newly formed collagen fibrils by cosedimentation. From the kinetic results, fluorescence polarisation and electron microscopy after rotary shadowing, it seems likely that TRAMP also binds to collagen during the early nucleation stages of fibril assembly. By co-sedimentation TRAMP and collagen demonstrate a binding stoichiometry of 1 mol TRAMP to 2 mol collagen. The large amount of TRAMP found bound to the fibril further suggested that the protein may be stably integrated into the fibril aggregate rather than restricted to the fibril surface. However the suggestion that TRAMP may self-aggregate under physiological conditions to form high molecular weight complexes, and that these complexes may also bind to collagen is not ruled out. The location of the TRAMP binding site (or sites) has not been firmly established. Electron microscopy after rotary shadowing indicates that the collagen telopeptides are involved. A further clue to the binding site(s) was provided by fibrillogenesis experiments in the presence of glucose. Unlike the effects of increased ionic strength or urea which inhibited fibril assembly but were partially reversed by TRAMP, inhibition of fibril formation in the presence of glucose was unaffected by TRAMP. It has been suggested that hydrophobic interactions in the telopeptide are a prerequisite for the initiation of fibril formation, but that glucose delays fibrillogenesis by binding to the C-telopeptide of collagen (Lien et al., 1984; Rathi et al., 1989) preventing the formation of 4 D staggered dimers. Thus it is possible that TRAMP binds to the C-telopeptide and therefore facilitates the 4 D association of collagen molecules, but in the presence of excess glucose (50 mM) binding of TRAMP is prevented. Alternatively TRAMP may bind to glucose, thereby preventing its interaction with collagen. The possibility that TRAMP may also bind to other regions on the collagen molecule is not excluded by the present study. The observation that TRAMP stabilises fibrils against low-temperature solubilisation suggests that TRAMP may bind to regions on the collagen triple helix. Therefore it is the provision of multiple contacts between TRAMP with neighbouring collagen molecules which stabilise the fibrillar assembly.
The final turbidity in the presence of TRAMP was consistently increased relative to the control, and hydroxyproline analysis indicated that the amount of fibrillar collagen that could be pelleted by centrifugation also increased with TRAMP concentration. The presence of TRAMP during fibril formation also reduced the fibril diameter compared to controls, while the protein did not alter the D-periodic banding pattern of the fibril. Turbidity is determined by both the total mass of assembled polymers and also there average mass per unit length, and therefore turbidity is not necessarily a direct measure of the amount of sedimentable collagen (Vogel et al., 1984). The results show that any reduction in light scattering due to decreased mass per unit length (i.e. smaller diameter) was outweighed by the increased amount of fibrillar collagen.

There are several ways in which TRAMP could cause a decrease in fibril diameter. First, TRAMP may increase the number of nucleation sites. The presence of more nucleation sites for a given quantity of collagen I might result in smaller diameters, than fewer nucleation sites for the same amount of collagen. Second, TRAMP may alter the nature of the nuclei, such that the rate of axial growth is increased relative to lateral growth. Third, TRAMP may form a circumferential layer around the fibril once a “critical” diameter is reached, and which blocks further addition of monomers by steric exclusion.

The effect of TRAMP on collagen fibrillogenesis can be compared with that of other components of the ECM, particularly glycosaminoglycans (GAGs) and proteoglycans. A number of studies have examined the effects of GAGs (Wood, 1960; Mathews & Decker, 1968; Hayashi & Nagai, 1972; Obrink, 1973a; Snowden & Swann, 1980; Vogel et al., 1984; Gavriel & Kagan, 1988). In most cases chondroitin sulphate has been shown to accelerate fibril formation (Wood, 1960; Hayashi & Nagai, 1972; Obrink, 1973a; Snowden & Swann, 1980), while accelerating, neutral or inhibitory effects have been reported with heparin and dermatan sulphate (Wood, 1960; Obrink, 1973a; Snowden & Swann, 1980; Vogel et al., 1984; Gavriel & Kagan, 1988).
The accelerating effect of chondroitin sulphate appears to be greatest when present at the start of fibril formation (Wood, 1960; Obrink, 1973a), and the effect is enhanced when fibrillogenesis is initiated by the “warm start” procedure (Holmes et al., 1986), rather than the “cold start” procedure in which chondroitin sulphate retards fibril assembly (Wood, 1960). These observations suggest that the effects of chondroitin sulphate are on the early, nucleation stage of fibril assembly. The effect of low concentrations of SDS in promoting collagen fibril formation is of interest (Hayashi & Nagai, 1972; Dombi & Halsall, 1985). There is evidence that some tyrosine residues in TRAMP may be sulphated (Chapter 3), but it has yet to be established whether this post-translational modification is involved in accelerating fibrillogenesis.

There have been a number of reports showing that proteoglycans modulate collagen fibril assembly in vitro (Toole & Lowther, 1968a, b; Toole, 1969; Obrink, 1973a; Oegema et al., 1975; Snowden & Swann, 1980; Birk & Lande, 1981; Vogel et al., 1984; Vogel & Trotter, 1987; Kuijer et al., 1988; Hedbom & Heinegard, 1989; Karvonen et al., 1992). Complex results were obtained in some of the early studies (Toole & Lowther, 1968a, b; Toole, 1969) where the presence of chondroitin sulphate and dermatan sulphate proteoglycans in the fibrillogenesis assay led to a rapid precipitation of fibrillar collagen. These proteoglycans also stabilised fibrils against low temperature solubilisation (Toole, 1969), similar to the effect of TRAMP discussed here. A consistent feature in most of the subsequent reports have shown that the addition of proteoglycans results in retardation of fibril assembly (Oegema et al., 1975; Snowden & Swann, 1980; Birk & Lande, 1981; Vogel et al., 1984; Kuijer et al., 1988; Hedbom & Heinegard, 1989; Karvonen et al., 1992), and therefore it seems likely that some of the early collagen and proteoglycan preparations were contaminated with other matrix components (Veis & Payne, 1988). It is quite possible that TRAMP was present in some of the early preparations of dermatan sulphate proteoglycans from skin, since the bovine equivalent of TRAMP is found to co-purify with dermatan sulphate proteoglycans (Choi et al., 1989; Neame et al., 1989). TRAMP may also be related to
an acidic protein, identified in rat skin, that has been reported to accelerate collagen fibrillogenesis (Adelmann et al., 1966).

It has been demonstrated that TRAMP from porcine skin interacts with collagen I in an in vitro self-assembly assay, and that the interaction results in acceleration of collagen fibril assembly and eventual formation of collagen fibrils of thinner diameter. These findings imply that TRAMP may be a regulatory element in fibril assembly and the control of fibril structure in vivo, though it is likely to be integrated with other regulatory mechanisms in collagen fibrillogenesis. Several potentially important domains have been recognised in the equivalent 22 K protein from bovine skin (Neame et al., 1989; Lewandowska et al., 1991), that have been proposed to interact with several components of the ECM and with the cell surface. The small dermanatan sulphate proteoglycan decorin (PG-II or PG-S2) from bovine tendon and bovine cartilage specifically inhibit fibril formation of collagens I and II (Vogel et al., 1984), and using morphological techniques Scott (1991) concluded that DSPG bind to specific regions on the collagen fibril. The 22 K bovine equivalent of TRAMP co-purifies with DSPGs (Choi et al., 1989; Neame et al., 1989), and direct binding of DSPG (mediated by DS chains) to the 22 K protein has also been observed (Lewandowska et al., 1991). The complementary effects of decorin and TRAMP on fibril formation may suggest potential interactions between these two molecules and with collagen, in the control of fibril assembly in the ECM. The ability of TRAMP to bind native collagen is physiologically significant in that the resultant matrix may support cell adhesion (Lewandowska et al., 1991) and provide tissue integrity. Cell adhesion to TRAMP (Lewandowska et al., 1991) further suggests that this protein may regulate cellular control of fibril deposition. Interestingly osteoarthritic eroded cartilage has a high level of DSPG compared to normal cartilage (Karvonen et al., 1992). Preliminary data indicates that TRAMP is also present in cartilage, therefore it is possible that in vivo an ideal combination of DSPG and TRAMP supports collagen fibrillogenesis in normal cartilage. Thus qualitative or quantitative changes in DSPGs from osteoarthritic
cartilage could contribute or result in abnormal collagen fibrillogenesis. Fibril formation is also a critical step in collagen cross-linking. TRAMP also co-purifies with lysyl oxidase (Cronshaw et al., 1993), and lysyl oxidase activity is greatly enhanced by the assembly of collagen into fibrils (Siegel, 1974). Therefore decorin and TRAMP through their different effects on collagen fibril formation, may modulate assembly, cross-linking and hence the biomechanical stability and metabolism of collagen in the ECM. If the scenario (described above) for regulating collagen cross-linking operates in vivo, the biological function of the sugar derived cross-links (Reiser et al., 1992) takes on an added significance. For example, in diabetic subjects, the high levels of tissue glucose may inhibit fibril formation, as has already been observed in vitro (Rathi et al., 1989). The effect of TRAMP on stimulating collagen fibrillogenesis is also inhibited by glucose, hence the level of enzyme-derived cross-links may also be reduced in diabetic patients. Therefore it is possible that the presence of increased amounts of sugar derived cross-links serve to maintain tissue integrity. The collagen binding domain in TRAMP appears to be remarkably heat stable, and probably reflects the high level of disulphide bonds in the protein (Neame et al., 1989). By heating TRAMP at 100 °C for 3 minutes, the protein could still accelerate collagen fibrillogenesis, and to the same extent as native TRAMP. Thus in a wound or inflamed tissue where TRAMP may be partially degraded, its collagen binding domain might remain functional, and hence influence the repair process by maintaining a collagen scaffold.
Chapter 5

Final Discussion
The present study is an investigation of the interaction of TRAMP with collagen I in the context of fibril formation. It was demonstrated that low, sub-stoichiometric amounts of TRAMP increased both the rate of assembly and the amount of fibrils precipitating from purified solutions of collagen. Precipitates formed in the presence and absence of TRAMP consisted almost entirely of fibrils with a 64 nm periodicity, though electron microscopy revealed that fibrils reconstituted in the presence of TRAMP were significantly thinner. This last observation suggests that TRAMP accelerates the early nucleation stages of fibril assembly, perhaps by promoting the formation of and/or stabilising the initial aggregates. The provision of more nucleation centres for a given amount of collagen would produce larger numbers of smaller diameter fibrils (Wood & Keech, 1960).

Analysis of the primary structure of TRAMP shows that the protein contains five disulphide bonds that define three loops (Neame et al., 1989), each loop containing a six-residue consensus sequence of Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr. That the ability of TRAMP to accelerate fibril formation was not altered by heating the protein to 100 °C suggests that the collagen binding domain(s) within TRAMP is (are) either resistant to or unaffected by denaturation. That TRAMP also stabilises fibrils against low temperature solubilisation suggests that it may form multiple contacts within the fibril aggregate. These observations support the idea that the repeating domains within TRAMP have collagen binding activity, and that these domains could be orientated to promote binding between collagen molecules arranged in D-staggered array. The disulphide bonds that define these domains may also be expected to stabilise them against thermal denaturation and it would therefore be of interest to reduce the disulphide bonds within TRAMP and examine the effect of this on fibrillogenesis. Monoclonal antibodies to various domains of TRAMP and/or synthetic peptides could also be used to further examine which regions within the TRAMP molecule are important for its interaction with collagen. It also remains to be determined whether TRAMP can interact with other components of the ECM, and in particular whether it
can influence the kinetics of fibril formation for other collagen types. It also remains to be established whether the short repeating domains within TRAMP represent another connective tissue sub-domain such as the RGD sequence identified in several ECM molecules (Ruoslaiti, 1991b).

The 22 K bovine equivalent of TRAMP (Neame et al., 1989) has been implicated in mediating cell adhesion (Lewandowska et al., 1991). It was also found that dermatan sulphate proteoglycans (DSPGs) inhibited cell adhesion to TRAMP. Further to this, a synthetic Arg-Gly-Asp-Ser (RGDS) containing peptide also inhibited cellular adhesion to the 22 K protein, thus implicating a cell-surface integrin as a possible receptor for TRAMP. Examination of the TRAMP primary structure (appendix II) reveals a candidate sequence (Arg-Gly-Ala-Thr (RGAT)) at residues 151-154 for potential reactivity with such an integrin (Lewandowska et al., 1991). The tetrapeptide sequence varies from the conventional RGDS sequence at the last two positions. It has been shown however that threonine effectively substitutes for serine in the fourth position (Hautanen et al., 1989), and an alanine substitution at the third position may also provide a relatively small side chain.

How size and form are regulated in the growth of supra-molecular assemblies is poorly understood. Although indeterminate in length and having diameters that vary over a large range (10-500 nm, depending on age and tissue), individual collagen fibrils tend to be uniform in diameter (except at their ends). In young tissues however, the fibrils often occur in bundles, and they exhibit sharp diameter distributions (Parry & Craig, 1984). As diameter distributions of fibrils formed in vitro are much broader, the additional information required for diameter limitation here does not reside within the collagen molecule alone. The present study demonstrates that in the presence of TRAMP there is more stringent control over fibril diameter. It would be of interest to compare the diameters of fibrils from collagen reconstituted in the presence and absence of TRAMP, with those from the original tissue in which the collagen was extracted.

Although it has been suggested that TRAMP may limit the lateral growth of
fibrils, the role of TRAMP in vivo is not understood. TRAMP has been found to co-purify with lysyl oxidase (Cronshaw et al., 1993) and with DSPGs (Neame et al., 1989). Indirect evidence suggests binding of DSPG (mediated through the DS chains) to TRAMP (Lewandowska et al., 1991). TRAMP does not affect the activity of lysyl oxidase on an elastin substrate, although this does not mean that no physical interaction exists between these two proteins. From rotary shadowing electron microscopy, TRAMP appears to bind mainly to the telopeptide regions of the collagen molecule, therefore it may be that TRAMP is involved in localising lysyl oxidase to this part of the molecule in preparation for cross-linking. Alternatively TRAMP and lysyl oxidase may exist under physiological conditions, either as a complex or as separate molecules with TRAMP acting to aggregate the collagen substrate prior to lysyl oxidase initiated cross-linking.

Much remains to be done to examine the nature and reason for the various ionic species of TRAMP, the physiological significance of TRAMP with regard to collagen fibril formation and the nature of the interactions that exist between TRAMP, lysyl oxidase, DSPGs and the cell surface. To further examine the nature of the TRAMP isoforms, dermal fibroblasts could be cultured in media containing [35S] sulphate. Secreted proteins could then be immuno-precipitated with anti-sera to TRAMP. Examination by SDS-PAGE and fluorography would then show whether the radiolabel was incorporated into TRAMP. Enzymatic digestion of radiolabeled TRAMP would make it possible to localise [35S] sulphate to specific peptide fragments within TRAMP, and hence assess whether the position of the radiolabel agrees with the position of the putative tyrosine sulphation sites (Hortin et al., 1986) predicted from the TRAMP sequence (see appendix II). Lasermat mass analysis following treatment of each of the TRAMP isoforms with sulphatase may also help establish whether the reason for the various isoforms is due to different extents of tyrosine sulphation. Despite the considerable observations made in the identification of tyrosine sulphated proteins (Huttner, 1984), little is known about the functional relevance of this type of covalent
modification in ECM proteins. The effect of desulphation of TRAMP in the collagen fibrillogenesis assay may provide some insight as to whether this post-translational modification is important for its interaction with collagen.

The nature of the TRAMP-collagen interaction needs further investigation. By pelleting fibrils formed in the presence of TRAMP and then washing the pellet with salt or non-ionic detergent solutions, it could be determined whether ionic or hydrophobic interactions were involved in TRAMP binding. In Chapter 4 it was proposed that the telopeptide regions of the collagen molecule were involved in TRAMP binding. To further investigate this possibility, the interaction of TRAMP with pepsin-treated collagen (collagen with telopeptides removed) could be studied in the fibrillogenesis assay.

Future studies should also be directed toward determining the cell source of TRAMP and in evaluating its possible derivation from a precursor. As TRAMP contains a large number of tyrosine residues, it should be possible to label TRAMP in cell culture with radiolabelled tyrosine and use TRAMP antisera to immuno-precipitate proteins either from cell lysates or secreted into the media.

Although preliminary data show TRAMP to have a widespread tissue distribution (Chapter 4), the polyclonal antisera may also be used to examine the tissue localisation of TRAMP. Using gold labelled second antibody it should be possible to immunolocalise TRAMP, by electron microscopy, to specific areas on and around collagen fibrils. The extent to which the gold label appears over fibrils from different tissues or at various stages in growth and development could be examined, as could the preferential association of TRAMP with thin fibrils. Therefore, by examining various tissues, it may be possible to correlate the amount of TRAMP in a given tissue with the gross morphology of the fibrils in that tissue.

Preliminary data by Alcian Blue staining (Chapter 3) showed that TRAMP was present at very low concentrations in skin. This may not be unexpected if the physiological role of TRAMP is only to influence the initiation of fibril assembly.
Cellular control of the stoichiometry of the different ECM components, as well as their vectorial discharge, are important in the determination of matrix structure. A developmental study would therefore be useful to examine any correlation between the tissue concentrations of TRAMP with the level of newly secreted collagen. It is my view that TRAMP may be important during periods of active fibrillogenesis, and hence it may play a vital role during tissue remodelling. Finally, a study of TRAMP and its effect on lysyl oxidase should be carried out, to establish whether TRAMP has a role in modulating collagen cross-linking, thus influencing both the mechanical properties of tissues and collagen turnover.
Appendix I - SDS-PAGE

Large format (16 x 14 x 0.15 cm) gels

(To make 2 gels)

<table>
<thead>
<tr>
<th></th>
<th>6%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Separating gel (Sg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>37.3 ml</td>
<td>22.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>18.75 ml</td>
<td>18.75 ml</td>
</tr>
<tr>
<td>2% (w/v) ammonium persulfate</td>
<td>1.85 ml</td>
<td>1.85 ml</td>
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<tr>
<td>10% (w/v) SDS</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Acrylamide : Bis (30 : 0.8% (w/v))</td>
<td>15.0 ml</td>
<td>30.0 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>50 μl</td>
<td>120 μl</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Focusing gel (Fg)</strong></td>
<td>3% for a 6% Sg</td>
<td>4.5% for a 12% Sg</td>
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<tr>
<td>Water</td>
<td>15.1 ml</td>
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<td>0.5 M Tris-HCl (pH 6.8)</td>
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<td>6.25 ml</td>
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<tr>
<td>2% (w/v) ammonium persulfate</td>
<td>0.65 ml</td>
<td>0.65 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Acrylamide : Bis (30 : 0.8% (w/v))</td>
<td>2.5 ml</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>25 μl</td>
<td>25 μl</td>
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</table>
Mini gels (7.5 x 8.5 cm)

(To make 5 x 1.5 mm gels)

<table>
<thead>
<tr>
<th>Separating gel</th>
<th>6%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>28.25 ml</td>
<td>16.9 ml</td>
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<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>14.2 ml</td>
<td>14.2 ml</td>
</tr>
<tr>
<td>2% (w/v) ammonium persulfate</td>
<td>1.4 ml</td>
<td>1.4 ml</td>
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<tr>
<td>10% (w/v) SDS</td>
<td>1.15 ml</td>
<td>1.15 ml</td>
</tr>
<tr>
<td>Acrylamide : Bis (30 : 0.8% (w/v))</td>
<td>11.35 ml</td>
<td>22.7 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>35 µl</td>
<td>70 µl</td>
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</table>

<table>
<thead>
<tr>
<th>Focusing gel</th>
<th>3% for a 6% Sg</th>
<th>4.5% for a 12% Sg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>9.5 ml</td>
<td>8.70 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>3.9 ml</td>
<td>3.9 ml</td>
</tr>
<tr>
<td>2% (w/v) ammonium persulfate</td>
<td>0.4 ml</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Acrylamide : Bis (30 : 0.8% (w/v))</td>
<td>1.56 ml</td>
<td>2.35 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>16 µl</td>
<td>16 µl</td>
</tr>
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</table>

Water saturated butanol was overlaid on the separating gel during polymerisation, and was removed before applying the focusing gel.
The primary structure of TRAMP variant T3 is shown using the single-letter code for amino acids (from Cronshaw, with permission). Putative tyrosine sulphation sites are denoted by *. The one-letter notation for amino acids is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, Glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
References


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Dombrowski, K.E., Vogel, B.E. & Prockop, D.J. (1989) Mutations that alter the
primary structure of type I procollagen have long-range effects on its cleavage by procollagen N-proteinase. *Biochemistry* 28, 7107-7112.


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