

Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP)

Richard A. WILLIAMSON,*§ Fiona A. O. MARSTON,† Sarojani ANGAL,† Peter KOKLITIS,† Maria PANICO,‡ Howard R. MORRIS,‡ Alex F. CARNE,† Bryan J. SMITH,† Tim J. R. HARRIS†|| and Robert B. FREEDMAN*

* Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K., † Celltech Ltd., 216 Bath Road, Slough, Berks. SL1 4DY, U.K., and ‡ Department of Biochemistry, Imperial College of Science and Technology, South Kensington, London SW7 2AZ, U.K.

Disulphide bonds in human recombinant tissue inhibitor of metalloproteinases (TIMP) were assigned by resolving proteolytic digests of TIMP on reverse-phase h.p.l.c. and sequencing those peaks judged to contain disulphide bonds by virtue of a change in retention time on reduction. This procedure allowed the direct assignment of Cys-145–Cys-166 and the isolation of two other peptides containing two disulphide bonds each. Further peptide cleavage in conjunction with fast-atom-bombardment m.s. analysis permitted the assignments Cys-1–Cys-70, Cys-3–Cys-99, Cys-13–Cys-124 and Cys-127–Cys-174 from these peptides. The sixth bond Cys-132–Cys-137 was assigned by inference, as the native protein has no detectable free thiol groups.

INTRODUCTION

The tissue inhibitor of metalloproteinases (TIMP) is a small protein secreted by many cell types, including fibroblasts and macrophages in vertebrate connective tissue, and by connective-tissue-derived cells in culture [1–4]. TIMP is a stoichiometric inhibitor of collagenase and the other metalloproteinases (gelatinase and stromelysin) secreted into the extracellular matrix by connective-tissue cells [5–7]. These proteinases are together able to bring about the degradation of all the major extracellular-matrix components, and it appears that TIMP has an important function in the regulation of this connective-tissue turnover *in vivo* [8,9]. Understanding of this function, and the application of this understanding to therapy, requires a fuller knowledge of the structure and properties of TIMP. An important step towards the understanding of TIMP's activity at the molecular level was the cloning of TIMP cDNA, which permitted both the analysis of TIMP's amino acid sequence and the expression of active recombinant TIMP by mammalian cells in culture [10].

The cDNA sequence predicts a polypeptide of 184 residues, including 12 cysteine residues, and two asparagine residues in the consensus glycosylation sequence Asn-Xaa-Thr/Ser. We have confirmed (R. A. Williamson & R. B. Freedman, unpublished work) previous studies suggesting that TIMP contains no free thiol groups [6,11], and hence the native molecule contains six disulphide bonds and up to two oligosaccharide side chains. There are 10395 possible disulphide arrangements for a protein containing 12 intramolecularly linked half-cystine residues, and, clearly, establishment of the native bonding pattern is a prerequisite for structure-function analysis. No sequence similarities are detectable to other proteins of known disulphide linkages. Several authors have shown that the disulphide bonds of TIMP are essential for its biological activity [11–13].

The TIMP sequence contains several half-cystine residues close together (residues 1 and 3 and residues 124, 127, 132 and 137). Assignment of disulphide pairing by the isolation and characterization of peptides linked by single disulphide bonds requires cleavage between each half-cystine residue; methods of cleavage of such broad specificity are of limited use because of

the extreme complexity of the product mixture generated. To generate a large but manageable number of product peptides, we have selected peptic and tryptic digestion. To exclude the possibility of artifactual thiol-disulphide interchange reactions, all digestion and separation procedures were performed at pH ≤ 6.5. Pepsin is particularly suited to this regime, as the enzyme has maximal activity at low pH. In addition to conventional sequencing methods, we have analysed disulphide-bonded peptic peptides by fast-atom-bombardment m.s. (f.a.b.-m.s.), which in many cases can provide rapid disulphide assignment even in mixtures of peptides [14]. The work described in the present paper has led to the direct assignment of five of the six disulphide bonds of TIMP, allowing the final bond to be assigned by inference, as no reactive thiol groups could be detected in the native protein.

EXPERIMENTAL

TIMP expression, isolation and purification

The purification of TIMP employed a monoclonal antibody designated MAC 015. The antibody was developed in the hybridoma department of Celltech with the use of human amniotic-fluid TIMP purified and kindly provided by Dr. G. Murphy (Strangeways Research Laboratory, Cambridge, U.K.). Mice received three intraperitoneal injections of 25 µg each in Freund's complete adjuvant followed by a 50 µg intravenous booster 1 day before the fusion of their spleen cells with SP2/0 myeloma cells [15]. The resultant monoclonal antibodies were screened for their ability to bind TIMP. The selected antibody, MAC 015, was purified on Protein A–Sephacrose after large-scale cell culture in the manufacturing division of Celltech. Purified MAC 015 was immobilized on CNBr-activated Sepharose at 5 mg/ml by Pharmacia.

Recombinant human TIMP was expressed in a mouse fibroblast (C127) cell line [10]. Supernatants from the culture of these cells were concentrated 10-fold, and 8 litres containing 120 mg/l were applied to a column of MAC 015–Sephacrose (7.9 cm × 11 cm) equilibrated in 20 mM-sodium phosphate buffer,

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; f.a.b.-m.s., fast-atom-bombardment mass spectrometry.

§ To whom correspondence should be addressed.

|| Present address: Glaxo Group Research, Greenford Road, Greenford, Middx. UB6 0HE, U.K.

pH 7.0. Loosely bound proteins were washed off with 0.1 M-sodium acetate buffer, pH 5.5. Bound TIMP was eluted with 0.1 M-glycine/HCl buffer, pH 3, and immediately neutralized with 2 M-Tris/HCl buffer, pH 7.5. This immunopurified TIMP was further purified by gel-permeation chromatography on Sephacryl S200 (7 litres in a Pharmacia K100/100 column) in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.5. TIMP-containing fractions were pooled and stored at -70°C .

Pepsin proteolysis

Purified TIMP, in 0.2 mg portions, was freeze-dried from water and redissolved in 200 μl of either 5% (v/v) acetic acid, pH 2.5, or 5% (v/v) acetic acid adjusted to pH 4.0 with NH_4HCO_3 . Pepsin (from pig gastric mucosa; Boehringer Corp.) was added to a concentration of 0.1 mg/ml from a 2 mg/ml stock solution in water, and the digests were incubated for 24 h at 35°C .

Trypsin proteolysis

Portions (0.2 mg) of TIMP freeze-dried from water were each redissolved in 200 μl of 50 mM-ammonium acetate buffer, pH 6.5. Trypsin (Worthington Diagnostics Systems; tosylphenylalanylchloromethane-treated; 247 units/mg) was added to a concentration of 0.05 mg/ml from a 1 mg/ml stock solution in water, and the digests were incubated for 24 h at 35°C . Insoluble material generated during the digestion was separated from the soluble fraction by centrifugation at 10000 g for 10 min.

Reverse-phase h.p.l.c.

Samples (20–140 μl) of the digests were resolved on a C_{18} reverse-phase column (Vydac 218TP54) at room temperature. Linear gradients of 0–50% (v/v) solvent A [0.05% (v/v) trifluoroacetic acid in acetonitrile] in solvent B [0.1% (v/v) trifluoroacetic acid in water] over 60 min were used for separation. The flow rate was kept constant at 1 ml/min, and the absorbance was measured at 220 or 225 nm. Peaks were collected manually. Samples of the collected peaks (a volume equivalent to that which would have derived from 10–20 μl of whole digest) were either diluted 1:1 with water (unless previously reduced) or evaporated to dryness (vacuum centrifuge) and redissolved in solvent A (100 μl) before re-analysis by reverse-phase h.p.l.c. The conditions of separation were identical with those used for the whole digests, except that linear gradients of 10–40% or 10–50% (v/v) solvent A in solvent B over 36 or 48 min were used for elution.

Disulphide reduction

Samples (20 μl) of the whole digests were each added to an equal volume of a 1% (w/v) NH_4HCO_3 solution, and the pH was adjusted to 8.0–8.5 with aq. NH_3 (if necessary). Dithiothreitol (Sigma Chemical Co.) was added from a freshly prepared 50 mM stock solution in water to a 600-fold molar excess over TIMP (430 nmol). The vials were purged with N_2 , sealed and incubated at 35°C for 1 h. After reduction, the samples were acidified (to approx. pH 5.0) with 10% (v/v) trifluoroacetic acid and placed on ice before analysis (within 1 h) by reverse-phase h.p.l.c. Samples of peak fractions (a volume equivalent to that which would have derived from 10–20 μl of whole digest) were either reduced directly by the above procedure or first evaporated to dryness (vacuum centrifuge) and redissolved in 75 μl of water.

Amino acid sequence analysis

The amino acid sequences of the various peptides generated were determined by use of an Applied Biosystems 470A peptide sequencer followed by identification of amino acid phenylthiohydantoin derivatives by h.p.l.c. on a C_{18} reverse-phase

column. Cysteine residues were not modified before sequencing, so they were not identified directly by this process. Individual peptides within mixtures were identified by comparison of results with the sequence determined previously for TIMP [10].

F.a.b.-m.s.

Samples for m.s. analysis were dissolved in 2–5 μl of 5% (v/v) acetic acid, and 0.1–1.0 nmol of each was transferred to the f.a.b. probe (2 μl) for analysis, with the use of a matrix of glycerol or glycerol/thioglycerol (3 μl) as appropriate. F.a.b. mass spectra were recorded on a VG ZAB High Field instrument with an M-SCAN Fast Ion Gun operating at 10 kV and 20 μA Xe-beam energy.

Staphylococcus aureus-V8-proteinase digestion

After initial m.s. analysis of peak P24 to establish the intact M_r values, and thus the identities, of the three component disulphide-bridged peptides, the sample was digested with *S. aureus* V8 proteinase (ICN Biomedicals) in order to determine the disulphide positions. Peak-P24 material was dissolved in 100 mM-ammonium bicarbonate buffer, pH 7.8, V8 proteinase was added (enzyme/substrate ratio 1:40, by wt.) and the digestion was carried out for 6 h at 40°C .

Manual Edman degradation

The *N*-terminal residues of peak-P21 peptides were cleaved with the use of one cycle of manual Edman degradation. The procedure used was essentially as described by Gray for Edman coupling and cleavage [16].

Peak-P21 material (collected from 130 μl of peptic digest at pH 4.0), peak-P24 material (collected from 135 μl of peptic digest at pH 4.0) and a control (water) were evaporated to dryness (vacuum centrifuge) in Pyrex vials (0.3 ml) fitted with Teflon seals. The peptides were redissolved in 50 μl of 50% (v/v) pyridine in water, the vials were flushed with N_2 (1 min), 25 μl of 10% (v/v) phenyl isothiocyanate (sequence grade; Beckman) in pyridine was added to each, and the vials were re-flushed with N_2 (1 min), sealed and then incubated at 50°C in an aluminium heating block. After 30 min the vials and heating block were transferred to a vacuum desiccator containing NaOH, and the samples were evaporated (under vacuum) to dryness. The pinkish crystalline residues seen after drying were redissolved in 25 μl of anhydrous trifluoroacetic acid, and the vials were sealed and incubated in a heating block at 50°C for 30 min. The samples were then dried down over NaOH in a vacuum desiccator as before. To each of the dried samples was added 50 μl of water followed by 250 μl of ethyl acetate. The vials were thoroughly mixed with a vortex mixer and the phases were then separated on a bench centrifuge (5 min). The upper organic phase of each was discarded and the ethyl acetate wash was repeated two more times. The remaining aqueous phase of each was stored frozen (-20°C) before separation on reverse-phase h.p.l.c.

RESULTS

TIMP purification

The purification of recombinant human TIMP from cell-culture supernatants is illustrated in Fig. 1. Purity was assessed by microdensitometry of Coomassie Blue-stained gels. TIMP was 98% pure at the end of the purification procedure. TIMP migrates as a number of diffuse bands at M_r 30000–34000, which is thought to be due to heterogeneity in glycosylation, analogous to that observed for TIMP from natural sources [17]. The overall purification yield of TIMP was 60%, and the purified TIMP was fully active in metalloproteinase-inhibition assays (G. Murphy, personal communication).

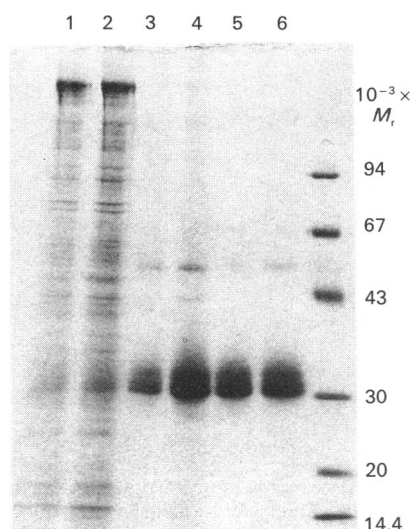


Fig. 1. SDS/7.5–15% polyacrylamide gradient-gel electrophoresis of human TIMP

The samples were reduced with 2-mercaptoethanol before being loaded on to the gel. M_r markers were from Pharmacia. The gel was stained with Coomassie Brilliant Blue G-250. Lanes 1 and 2, cell-culture supernatants (50 μ l loading) containing recombinant human TIMP; lanes 3 and 4, immunoaffinity-purified TIMP (10 μ g loading); lanes 5 and 6, pure TIMP after Sephacryl S-200 purification (10 μ g loading).

Analysis of peptic digests

Recombinant TIMP was subjected to digestion by pepsin and the digest was resolved by reverse-phase h.p.l.c. Preliminary work indicated that the profile of products obtained was dependent on the precise pH at which proteolysis occurred. Two separate batches of TIMP, one freeze-dried from water and the other evaporated to dryness from 1% (w/v) NH_4HCO_3 , gave rise to different product profiles, though with some peaks in common. The former digest was found to be at pH 2.5, whereas the latter was at pH 4.0, and an equivalent product profile could be obtained by freeze-drying TIMP from water, dissolving it in 5% (v/v) acetic acid and adjusting the solution to pH 4.0 with NH_4HCO_3 before digestion. In subsequent work peptic digestion was carried out either at pH 2.5 or 4.0 as described in the Experimental section.

The strategy for assignment of disulphide bonds was to identify disulphide-bonded peptides in the peptic digests by comparison of the h.p.l.c. profiles of digests analysed before and after reduction. Fig. 2 shows the corresponding profiles of reduced and non-reduced products obtained at both pH values. Attention was focused on major, symmetrical, well-resolved peaks in the profiles of non-reduced products that were absent from or displaced in the profiles of reduced products. Two candidate peaks (P24 and P26) satisfied these criteria in the profiles from both digests, and a third peak (P21) was found only in the digest at pH 4.0 but was similarly identified as a potential disulphide-bonded peptide. Evidence to suggest that the peaks labelled P24 and P26 were the same in both digests (Fig. 2) was obtained when equal amounts of each peak from both digests were mixed and re-resolved by h.p.l.c. In each case a sharp symmetrical peak was seen that was indistinguishable from either peak resolved separately.

Analysis of tryptic digest

Digestion of recombinant human TIMP with trypsin in 50 mM-

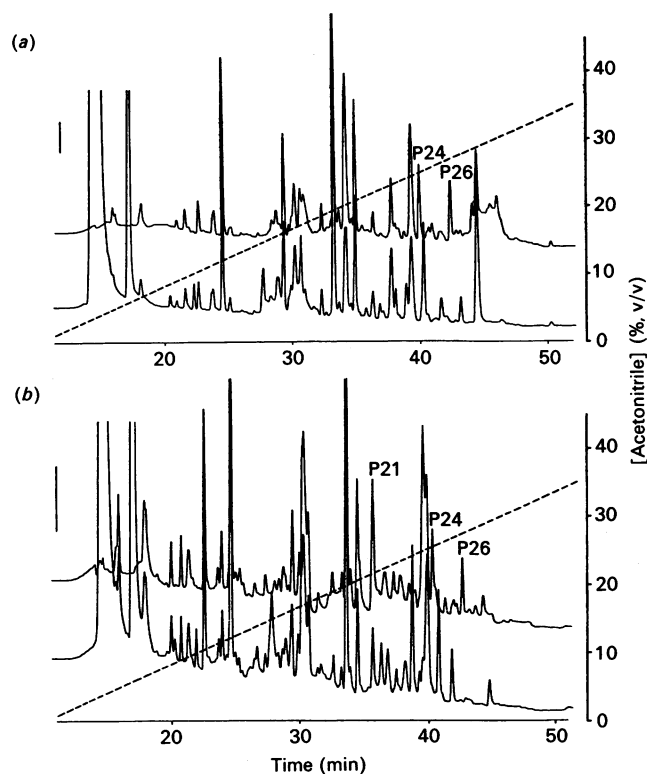


Fig. 2. Reverse-phase h.p.l.c. of peptic digests of TIMP

(a) Digestion at pH 2.5; (b) digestion at pH 4.0. In each case equal amounts of digest were resolved without disulphide reduction (upper trace) and after disulphide reduction (lower trace). —, A_{225} ; - - - -, acetonitrile gradient. The peaks P21, P24 and P26, judged to contain disulphide bond(s), are labelled. The vertical bars represent an A_{225} of 6.4×10^{-3} unit.

ammonium acetate buffer, pH 6.5, led to the formation of a precipitate within 3 h, which did not redissolve on prolonged treatment; h.p.l.c. analysis of the supernatant, after centrifugation, revealed the presence of many soluble peptides, and this supernatant was then analysed both before and after reduction, in order to identify disulphide-bonded soluble peptides. Fig. 3 demonstrates that a single peptide (T26) in the profile of non-reduced products is absent from the profile of reduced products.

Isolation of disulphide-bonded peptides

Each of the candidate peaks was collected manually from h.p.l.c. analysis of 0.10–0.14 ml of digest, and samples were re-resolved by h.p.l.c. to check for purity and altered retention on reduction. Fig. 4 shows representative results for peptide T26. The peaks derived from reduction of peptide T26 are also evident in the profile of the reduced mixture of soluble peptides and are indicated in Fig. 3. For each of the candidate peptic-digest peptides the isolated peak material ran as a single peak in non-reducing conditions, and in each case disappeared and was replaced by two or more smaller peaks when the sample was reduced before h.p.l.c. analysis (results not shown).

Sequence analysis of disulphide-bonded peptides

The four isolated disulphide-bonded peptides were characterized by automated Edman degradation, and in the case of the peptic-digest peptides by f.a.b.-m.s. The automated Edman sequencing data are summarized in Table 1.

Edman-degradation data for peptide T26 indicated the pres-

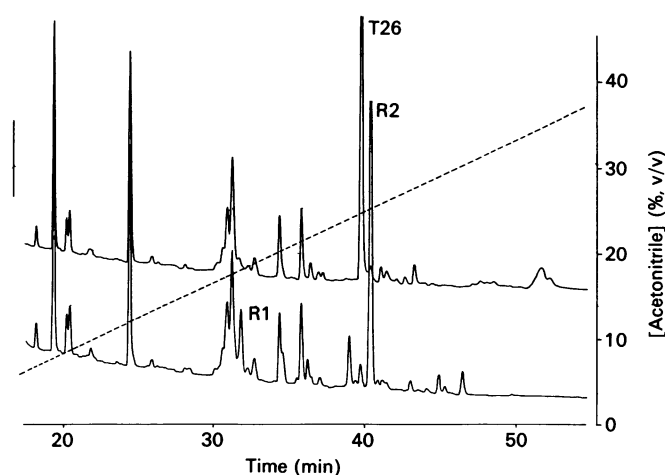


Fig. 3. Soluble peptides from a tryptic digest of TIMP analysed by reverse-phase h.p.l.c.

Equal amounts of material were resolved without disulphide reduction (upper trace) and after disulphide reduction (lower trace). —, A_{225} ; - - - - -, acetonitrile gradient. The reduced products from peptide T26 are labelled 'R1' and 'R2'. The vertical bar represents an A_{225} of 13×10^{-3} unit.

ence of two sequences corresponding exactly to residues Leu-139–Lys-157 and His-163–Arg-169 in the protein sequence inferred from the complete cDNA sequence of human TIMP [10]. Each of these sequences corresponds to a predicted tryptic-digest peptide, and contains a single cysteine residue, Cys-145 and Cys-166 respectively. The characterization of peptide T26 establishes the presence of a disulphide bond between these residues in native TIMP.

The data for the peptic-digest peptides show, first, that peptides P24 and P26 have identical *N*-terminal sequences, and, secondly, that each of the isolated peptic-digest peptides comprises three

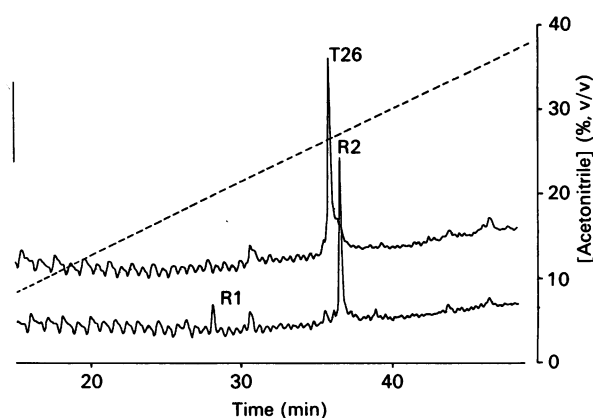


Fig. 4. Reverse-phase h.p.l.c. of isolated peptide T26

Equal amounts of material were analysed before disulphide reduction (upper trace) and after disulphide reduction (lower trace). —, A_{225} ; - - - - -, acetonitrile gradient. The reduced products from peptide T26 are labelled 'R1' and 'R2'. The vertical bar represents an A_{225} of 4.0×10^{-3} unit.

peptide sequences linked by disulphide bonds. The Edman-degradation data for peptide P21 are those expected for peptides beginning at Cys-1, Ser-68 and His-95, and linked by disulphide bonds between Cys-1, Cys-3, Cys-70 and Cys-99, and those from peptides P24 and P26 are those expected for peptides beginning at Phe-12, Tyr-120 and Pro-168 linked by disulphide bonds between Cys-13, Cys-124, Cys-127 and Cys-174. In contrast with peptide T26, the Edman-degradation sequence data on the peptic-digest peptides do not permit immediate unambiguous disulphide assignment.

It is difficult to be precise about the *C*-terminal residues of the individual component peptic-digest peptides from Edman-degradation data, but the m.s. data allowed determination of the M_r values of the intact P21, P24 and P26 samples and thus

Table 1. Sequence analysis of isolated disulphide-bonded peptides

Where two or three residues are shown at one cycle, they represent stoichiometric quantities. Thus for peptide P21 at cycle 2 Thr = 145 pmol, Ile = 120 pmol and Val = 120 pmol, for peptide P24 at cycle 1 Phe = 171 pmol, Pro = 184 pmol and Tyr = 180 pmol, for peptide P26 at cycle 1 Phe = 52 pmol, Pro = 69 pmol and Tyr = 46 pmol, and for peptide T26 at cycle 1 His = 54 pmol and Leu = 48 pmol. The sequence data for each peptide have been obtained reproducibly from samples collected from two or more independent digests.

Cycle	Peptide...	Amino acid(s) released			
		P21	P24	P26	T26
1		His, Ser	Phe, Pro, Tyr	Phe, Pro, Tyr	His, Leu
2		Ile, Thr, Val	Arg, Thr	Arg, Thr	Leu, Gln
3		Thr	Glu, Asn, Val	Glu, Asn, Val	Ala, Ser
4		Gly, Val	Gly, Pro, Ser	Gly, Pro, Ser	Gly
5		Pro, Tyr	Asp, Gly	Asp, Gly	Leu, Thr
6		Phe, Pro, Ser	Glu, Leu	Glu, Leu	His, Pro
7		His	Glu	Glu	Arg
8		Pro	Thr	—	Leu
9		Gln	Thr	—	Trp
10		Thr	Val	—	Thr
11		Ala	Phe	—	Asp
12		—	—	—	Gln
13		—	—	—	Leu
14		—	—	—	Leu
15		—	—	—	Gln
16		—	—	—	Gly
17		—	—	—	—
18		—	—	—	Glu

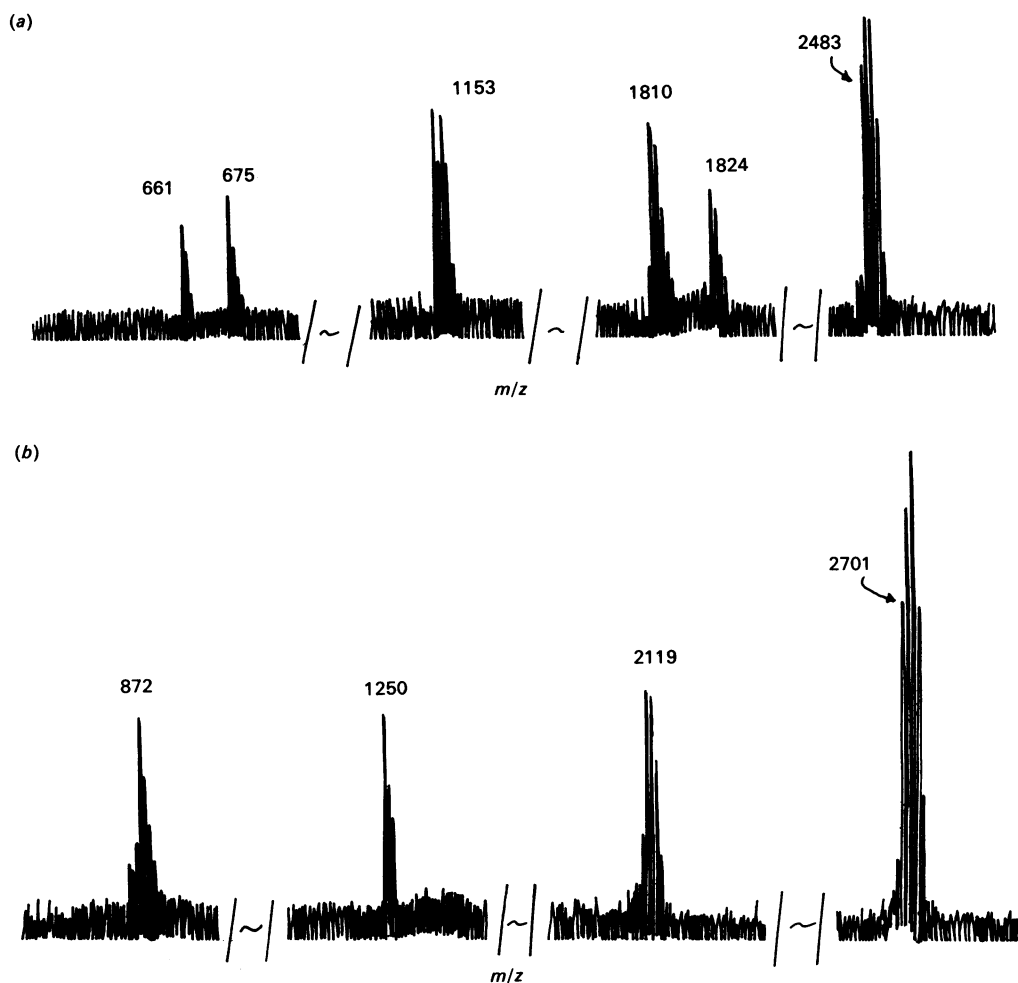


Fig. 5. Partial f.a.b. mass spectra of (a) peptide P21 and (b) peptide P24 showing respective quasimolecular ions at m/z 2701 and 2483, and some of the partially reduced component peptides at lower mass when thioglycerol is added to the matrix

the precise nature of the component peptides. For example, the quasimolecular ion initially predicted for peptide P24 on the basis of pepsin substrate preference and preliminary Edman-degradation results was either m/z 2667, corresponding to peptides Phe-12–Leu-17, Tyr-120–Val-129 and Pro-168–Thr-175, or m/z 2853, incorporating Trp-176 in the third peptide component. The observed quasimolecular ion for peptide P24 was at m/z 2701 (an additional 34 atomic mass units on m/z 2667), which can be reached by subtracting Leu-17 and adding Phe-130, thus defining the components as Phe-12–Asp-16, Tyr-120–Phe-130 and Pro-168–Thr-175 linked by two disulphide bonds. Comparable analysis of peptide P26 showed an intact quasimolecular ion at m/z 2814, an addition of 113 atomic mass units compared with peptide P24, consistent with the presence of an additional leucine or isoleucine residue, thus defining the components as Phe-12–Leu-17, Tyr-120–Phe-130 and Pro-168–Thr-175.

The f.a.b. mass spectra for peptides P21 and P24 are shown in Fig. 5. The quasimolecular ion for peptide P21 at m/z 2483, taken together with the Edman-degradation data, calculates for Cys-1–Ala-11, Ser-68–Phe-73 and His-95–Ser-100 linked by two disulphide bonds. Note also that under mild reducing conditions, with thioglycerol as matrix, it is often possible to observe some of the reduced components, thus confirming the assignments made from the intact M_r value, e.g. for peptide P21 m/z 661, 675

and 1153 correspond to the fully reduced components and m/z 1810 and 1842 correspond to Cys-1–Ala-11 disulphide-linked to His-95–Ser-100 and to Cys-1–Ala-11 disulphide-linked to Ser-68–Phe-73 respectively. Analysis of peptide P26 in thioglycerol showed reduced components at m/z 689, 872 and 1250, confirming that peptide P26 differs from peptide P24 in retaining Leu-17. The Edman-degradation and f.a.b.-m.s. data for both the peptic-digest and the tryptic-digest fragments are summarized in Fig. 6.

Disulphide assignment in peptide P24

Although there are three possible ways in which four cysteine residues can be linked, the presence of a Cys-124–Cys-127 bond in peptide P24 can be excluded; such a situation would imply that the P24 sample was a mixture of peptide Tyr-120–Phe-130 (with an intramolecular disulphide bond) and peptides Phe-12–Asp-16 and Pro-168–Thr-175 linked by the interchain Cys-13–Cys-174 bond. This can be ruled out both by the homogeneity of the material (seen as a well-resolved single peak when re-analysed on reverse-phase h.p.l.c.) but in particular by the f.a.b.-m.s. M_r data, which show that the three peptides are bound together. Hence the possible arrangements are Cys-13–Cys-124 and Cys-127–Cys-174 or Cys-13–Cys-127 and Cys-124–Cys-174. Provided that cleavage between cysteine residues in the same peptide chain can be achieved (e.g. between residues Cys-124 and

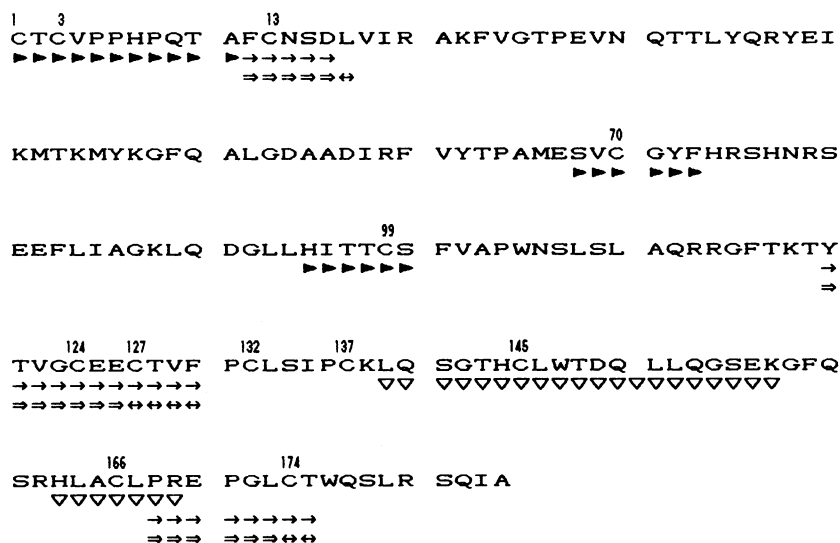


Fig. 6. Complete primary structure of TIMP as predicted from its cDNA sequence [10]

The numbers above the cysteine residues denote their relative positions in the peptide chain. Residues identified by automated Edman degradation are shown: ▶ for peptide P21, → for peptide P24, ⇒ for peptide P26 and ∇ for peptide T26. C-Terminal residues assigned by f.a.b.-m.s. analysis are denoted ↔.

Cys-127 in peptide Tyr-120–Phe-130), then an analysis of the f.a.b.-m.s. map created [18] can be used in an m.s. strategy for determining the disulphide bridge present [14]. This cleavage was achieved for the P24 sample by subjecting it to further digestion with *S. aureus* V8 proteinase. The resultant f.a.b.-m.s. map of the V8-proteinase-treated material is shown in Fig. 7. This shows signals at m/z 1338 and m/z 1382, which correspond to cleavage following Glu-126 only, giving rise to two disulphide-bridged peptides linking Cys-13 and Cys-124 (Phe-12–Asp-16 linked to Tyr-120–Glu-126 at m/z 1382) and Cys-127 and Cys-174 (Cys-127–Phe-130 linked to Pro-168–Thr-175 at m/z 1338).

Disulphide assignment in peptide P21

The presence of an intrachain Cys-1–Cys-3 bond in peptide P21 can be excluded on the same grounds as those cited for peptide P24. The remaining possibilities are Cys-1–Cys-70 and Cys-3–Cys-99 or Cys-1–Cys-99 and Cys-3–Cys-70. To distinguish between these alternatives, isolated peptide P21 was subjected to one round of manual Edman degradation, in order to cleave between Cys-1 and Cys-3, as described in the Experimental section. Recovered material was then resolved by

h.p.l.c. as before, and the major peak was isolated. This was subjected to automated Edman analysis, and gave isoleucine and threonine (in equimolar amounts) at the first round, and threonine at the second round of automated degradation. This result was found in automated Edman analysis of two distinct samples obtained after one round of manual Edman degradation. This result indicates that the manual Edman step has removed Cys-1 and His-95 to give, as isolated product, peptides extending from Thr-2 and Ile-96, linked by the Cys-3–Cys-99 disulphide bond. The remaining 100 pmol of material, derived from one step of Edman degradation followed by purification, was then examined by f.a.b.-m.s., and the spectrum is shown in Fig. 8. The signal observed at m/z 1571 corresponds to the expected disulphide-bridged peptide linking Cys-3 and Cys-99 (Thr-2–Ala-11 linked to Ile-96–Ser-100, released by the Edman removal of Cys-1 phenylthiohydantoin derivative linked to Val-69–Phe-73 via Cys-70).

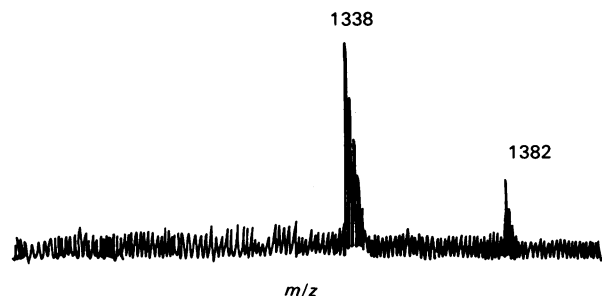


Fig. 7. Partial f.a.b. mass spectrum of V8-proteinase-treated peptide P24, showing disulphide-bridged peptides at m/z 1338 and 1382, which allow the assignment of the disulphide bridges given in the text

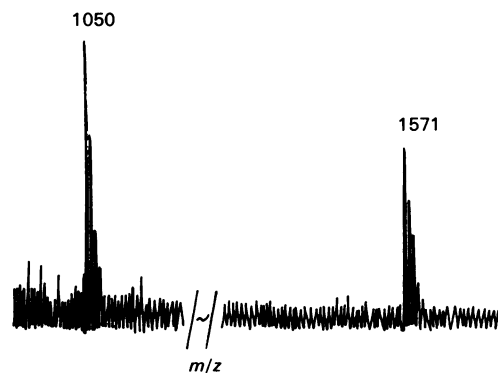


Fig. 8. Partial f.a.b. mass spectrum of 100 pmol of Edman-degraded (one cycle) peptide P21 showing a signal at m/z 1571 corresponding to Cys-3–Cys-99-linked peptides, and a partial reduction signal at m/z 1050 when thioglycerol is added to the matrix

DISCUSSION

The disulphide-bonded peptides readily identifiable in peptic digests of TIMP contained four of the six of the disulphide bonds of the molecule. The fact that the other two disulphide bonds were not found reflects the basis on which peptides were selected for study. Disulphide-bonded peptides would not have been selected for study if they were very small, and so gave low absorbance at 225 nm, if they were produced in low yield, because of non-quantitative proteolysis, if they were eluted close to a major peak given by a non-disulphide-bonded peptide, or if they showed little change in h.p.l.c. elution position following reduction, as might occur with peptides containing vicinal disulphide groups generating a small intrachain loop. The specificity of pepsin is not sufficiently predictable to allow speculation about the peptide products in which the Cys-132, Cys-137, Cys-145 and Cys-166 residues would be found, but they could well fall into the above categories. Disulphide-bonded products were tentatively identified in material at the end of the elution profile (Fig. 2a), but the quantity was insufficient and the peaks too poorly resolved to warrant further study.

In the disulphide-bonded peptides that were isolated, peptic cleavage had occurred at the sites -Ala-Phe-, -Asp-Leu-, -Glu-Ser-, -Phe-His-, -Phe-Pro-, -Leu-His-, -Leu-Pro-, -Leu-Val-, -Ser-Phe-, -Thr-Trp- and -Thr-Tyr-, confirming the unpredictable specificity of this proteinase. Two further points about the peptic hydrolysis are worth noting. First, the pattern of products obtained varied with pH; some major products were only obtained at pH 2.5, others only at pH 4.0 and others at both pH values. The difference was not dependent on the length of time of peptic digestion; at each pH the same profile was obtained after 24 h and after 48 h, implying an absolute difference in pepsin selectivity towards this substrate at the two pH values rather than a kinetic selectivity. Secondly, the peptic digestions at both pH values generated two major disulphide-bonded products (peptides P24 and P26), which gave identical *N*-terminal sequences but which were reproducibly eluted at different positions. The structural basis of this difference in behaviour was found to be peptic cleavage on the *C*-terminal side of Leu-17 in peptide P26 rather than the *N*-terminal side found in peptide P24.

The isolated disulphide-bonded peptides presented a problem in disulphide assignment in that each contained one sequence with two neighbouring cysteine residues, Cys-Thr-Cys- in peptide P21 and Cys-Glu-Glu-Cys- in peptides P24/P26. The methods selected to cleave between neighbouring cysteine residues in these products were successful. In peptide P24, *S. aureus* V8 proteinase cleaved quantitatively following Glu-126, and hence did not act at the Glu-125-Glu-126 bond or at the Glu-170-Pro-171 bond in the linked peptide; the result accords with the known specificity of the proteinase [19]. In peptide P21, the manual Edman method cleaved after at least two of the three *N*-terminal residues, including Cys-1. The product comprising Cys-1 phenylthiohydantoin derivative disulphide-linked to Val-69-Phe-73 (or Ser-

68-Phe-73) via Cys-70 was not recovered during the subsequent reverse-phase h.p.l.c. separation; this peptide is particularly hydrophobic and may have been retained by the column throughout the acetonitrile elution (linear gradient of 0-40%).

The usefulness of the disulphide-bridge assignment strategies based on m.s. is clearly demonstrated here, in work on unknown structures. The method is based on comparisons of mass data from the f.a.b.-m.s. maps taken before and after chemical or enzymic digests or subdigests of the disulphide-bridged peptides or protein. Reduction (or oxidation) to yield the component peptides provides confirmation of the assignment of the structures deduced from the mass data and makes them definitive. The method can be used alone or to complement classical data, and it is applicable to peptide mixtures such as derived from protein or glycoprotein digests, sometimes without fractionation. In current work these m.s. strategies are being applied to the study of disulphide assignment during denaturation and refolding of proteins [20].

Analysis of the tryptic digest revealed only one soluble disulphide-bonded peptide (T26) and a precipitate that was soluble on reduction (results not shown). The soluble tryptic-digest peptide contained one of the two disulphide bonds not previously isolated in the peptic-digest peptides. Other workers have detected no free thiol groups in either human or bovine TIMP [6,11], and we have confirmed this finding by reaction of human recombinant TIMP (denatured in 6 M-guanidinium chloride) with 5,5'-dithiobis-(2-nitrobenzoic acid) and found less than 0.1 mol of SH/mol of TIMP polypeptide. The absence of reactive thiol groups implies the presence of six disulphide bonds in native TIMP and so establishes that the remaining disulphide bond is Cys-132-Cys-137.

It is of interest to consider why a precipitate is formed on tryptic digestion of TIMP, and why only a single soluble disulphide-bonded peptide is detected. Fig. 9 shows the complete disulphide bond assignment for human TIMP as inferred above. Predicted tryptic cleavages following lysine and arginine residues are also indicated. This analysis shows that complete tryptic digestion of TIMP would be expected to generate a very complicated disulphide-bonded peptide comprising residues 1-20, 60-75, 89-113, 119-138 and 170-180, containing more than half the total sequence, and ten of the 12 cysteine residues. This tryptic-digest 'core' peptide might be expected to be poorly soluble and to comprise the bulk of the observed precipitate; this hypothesis is confirmed by our further sequencing studies on the remaining soluble tryptic-digest peptides (results not shown), which account for almost all of the remaining sequence of the protein.

The pattern of disulphide bonds inferred in native TIMP provides some insights into the structural organization of the protein. Two cysteine-rich stretches (residue 1-13 and 124-137) are disulphide-bonded to each other and to other regions of the molecule to generate a highly cross-linked structure, which may account for the marked stability of TIMP [5,6,13]. There is no

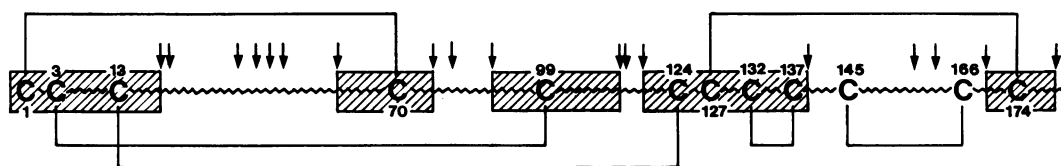


Fig. 9. Linear map of TIMP as predicted from its cDNA sequence [10]

Half-cysteine residues are numbered according to their relative positions in the peptide chain. Disulphide bonds are denoted —, predicted tryptic-cleavage sites are denoted ↓, and the insoluble tryptic 'core' is denoted ▨.

linkage between the first six half-cystine residues and the last six, so that the molecule consists of two distinct disulphide-linked units. As a result, a 'planar' representation of the disulphide-bonded structure is possible. Klapper & Klapper [21] have analysed possible disulphide-bond arrangements in proteins by graph theory, and shown that 30% of the possible disulphide pairings in a protein with six disulphide bridges are non-planar; such non-planarity has already been observed for co-lipase (with four disulphide bonds) and *Androctonus* neurotoxin II (with five disulphide bonds). All pairings for three disulphide bonds are planar, so that the arrangement of the six disulphide bonds in TIMP into two subsets of three each precludes non-planarity. The two sites of *N*-linked glycosylation (Asn-30 and Asn-78) are in the largest loops, and analysis of the soluble tryptic-digest peptides reveals that the potential sites in the long loop from Cys-13 to Cys-70 are all accessible to proteolysis (R. A. Williamson, A. F. Carne, B. J. Smith & R. B. Freedman, unpublished work). As yet there is no information on those parts of TIMP that interact directly with the metalloproteinase. When such data become available a knowledge of the disulphide-bond pattern will be invaluable in formulating structural models of the metalloproteinase-TIMP interaction.

Recently, a second tissue inhibitor of metalloproteinases (TIMP-2) has been described [22]. This protein is similar in size to TIMP, with which it shows a 39% amino acid sequence identity. The 12 half-cystine residues of TIMP are all conserved, which strongly suggests that TIMP-2 will display the same disulphide-bond arrangement. The identification of a second TIMP-related protein suggests that a family of such inhibitors may exist. If individual members of this family possess selective affinities for individual members of the matrix metalloproteinase family, a system enabling precise control of connective-tissue catabolism could be envisaged. The conservation of the 12 half-cysteine residues provides further evidence of the importance of the six disulphide bonds for a functional inhibitor.

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