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Synthesis of the *C*-terminal Domain of the Tissue Inhibitor of Metalloproteinases-1(TIMP-1)

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Abstract: According to recent investigations, the *C*-terminal domain of the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) is responsible for some biological effects that are independent of the enzyme-inhibiting effect of the *N*-terminal domain of the molecule. The *C*-terminal domain has been prepared for structure–biological activity investigations. After the chemical synthesis and the folding of the linear peptide, LC-MS and MALDI-MS analysis revealed that two isomers with different disulphide bond arrangements were formed. Since more than 30 folding experiments resulted in products with a very similar HPLC-profile, it was concluded that in the absence of the TIMP-1 *N*-terminal domain no entirely correct folding of the *C*-terminal domain occurred. Furthermore, it was observed that, in spite of several purification steps, mercury(II) ions were bound to the 6SH-linear peptide; it was demonstrated — using disulphide bonded TIMP-1(Cys^{145}-Cys^{166}) as a model — that mercury(II) ions can cause peptide degradation at pH 7.8 as well as in 0.1% trifluoroacetic acid. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: TIMP-1; *C*-terminal domain; synthesis; structural isomers; mass spectrometry; disulphide-bridge; mercury(II) ions; degradation

INTRODUCTION

In the turnover of extracellular matrix (ECM), the balance of matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinases (TIMP) plays a pivotal role[1]. The disturbance of this balance leads to various pathological events causing diseases, i.e. rheumatoid arthritis, periodontosis, tumor metastasis etc.

Four members of the TIMP family are known. All of them are capable of inhibiting all active MMPs by forming a strong non-covalent complex of 1 : 1 stoichiometry. All members of the TIMP family feature an *N*- and a *C*-terminal domain, each containing three loops held together by three disulphide bonds. There are two special MMPs, MMP-2 and MMP-9, also known as gelatinases, which can form complex proenzymes, proMMP-2 and proMMP-9 with TIMP-2 and TIMP-1, respectively. The *C*-terminal domain of the TIMPs is implicated in these complexes [1].

The most common member of the TIMP family is TIMP-1, which is produced by almost all kinds of cells and is found in every human body fluid. It has been reported that the recombinant

N-terminal domain of this polypeptide possesses enzyme-inhibiting potency similar to that of the full-length structure [2]. This observation initiated further structure-enzyme-inhibiting-activity relationship investigations in this region [3] and in the complete molecule [4].

Besides the enzyme inhibiting potency, TIMP-1 exerts other biological effects, such as growth factor-like activity, apoptosis and angiogenesis inhibition [5]. Interestingly, growth promotion and apoptosis inhibition are independent of enzyme inhibition, as reduction and alkylation of TIMP-1 do not abolish these two activities, but eliminate the enzyme inhibiting ability [6,7]. Furthermore, the growth factor-like activity significantly decreases on complex formation with MMP-9, indicating the role of TIMP-1 *C*-terminal domain in this biological effect.

It is thus of interest to study structure–biological activity relationships in the *C*-terminal domain. Neither the chemical synthesis nor the expression of the recombinant *C*-terminal domain of TIMP-1 has been reported. In this paper the chemical synthesis of the linear sequence is described, as well as the folding experiments, and the disulphide bond assignment of the end product. In addition, a side-reaction is reported triggered by mercury(II) ions remaining from the deprotection of Cys side-chains.

MATERIALS AND METHODS

Chemicals and Equipment

The chloroform used for the fragment condensation was *for synthesis* grade, purchased from Merck (Darmstadt, Germany). Other solvents and reagents, unless otherwise stated, were of analytical grade, obtained from Reanal Fine Chemical Works (Budapest, Hungary). Fmoc amino acids were obtained from Novabiochem (Calbiochem-Novabiochem GmbH, Germany), Boc-amino acids were also purchased from Reanal, Fmoc-Trp(Hoc)-OH was synthesized in our laboratory according to the literature [8]. 2-Chlorotrityl chloride resin, HBTU and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt) were purchased from Chemimpex International (Wood Dale, IL, USA). EDC was a product of Fluka Chem. AG (Budapest, Hungary). For the digestions, side-chain protected porcine trypsin (Promega, Madison, WI), and immobilized pepsin (Pierce, Rockford, IL) were used. For reduction dithiothreitol (DTT) was used, and for alkylation iodoacetamide (IAA) (Sigma-Aldrich Kft.

Budapest, Hungary). Solvents used in the MS experiments were all HPLC grade.

TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60 F_{254} precoated plates and column chromatography was performed on Merck Kieselgel 60 (40–63 µm). Analytical RP-HPLC, unless otherwise mentioned, was performed on a Knauer model using a YMC-Pak ODS C18, 120Å, 5 µm, (4.6mm × 150 mm) column at a flow rate of 1 ml/min. Preparative RP-HPLC was performed on a Pharmacia instrument using a Waters Delta Pak C18, 300Å, 15 µm, (19 × 300 mm) column at a flow rate of 14 ml/min. Detection was in both cases at 220 nm. Solvent A was 0.1% TFA in water, solvent B was 0.07% TFA in MeCN. Average molecular masses were measured by ESIMS, performed on an API-2000 quadrupole mass spectrometer (MDS Sciex, Toronto, CA).

Synthesis of Segments

The fragments F1–F6 shown in Figure 1 were synthesized manually, starting from 0.75 g of 2-chlorotrityl chloride resin (substitution 0.68 mmol/g), using HBTU, HOBt and DIEA in DMF. The functional side chains of Fmoc-amino acids were protected by the following groups: Acm for Cys, Bzl for Ser and Thr, OcHex for Asp and Glu, Trt for Asn, Tos for Arg, Bom for His, Z(2Cl) for Lys and cyclohexyloxycarbonyl (Hoc) for Trp. In the case of peptides containing Trp, 20% morpholine in DMF [9] was used for the cleavage of Fmoc groups instead of 30% piperidine in DMF. The last amino acid of the fragments was coupled as the Boc derivative, with the exception of F1, where the last amino acid was added as an Fmoc derivative; after removal of the Fmoc group acetylation was performed with acetic anhydride in the presence of DIEA in DMF. After completing the elongation reactions, the protected segments were removed from the resin by treatment with chloroform ($CHCl_3$)–methanol (MeOH)–acetic acid (AcOH) 8:1:1 for 2 h at room temperature and were purified by repeated precipitation from an appropriate solvent. Protected F3 was purified by flash chromatography using the solvent system $CHCl_3$/MeOH/AcOH 80:5:5. A small sample of F3 (after the removal of Boc and Trt groups) was treated with HF for 1 h in the presence of 10% anisole, the product was characterized by HPLC and MS. Boc-Gln^{182}-Ile-Ala^{184}-OBzl was prepared stepwise in solution starting from H-Ala-OBzl, using 1.05 eq of each Boc-amino acid derivative, 1.05 eq of DIC and 1.05 eq of HOBt in DMF at each stage. The homogeneity of the fragments was proved by
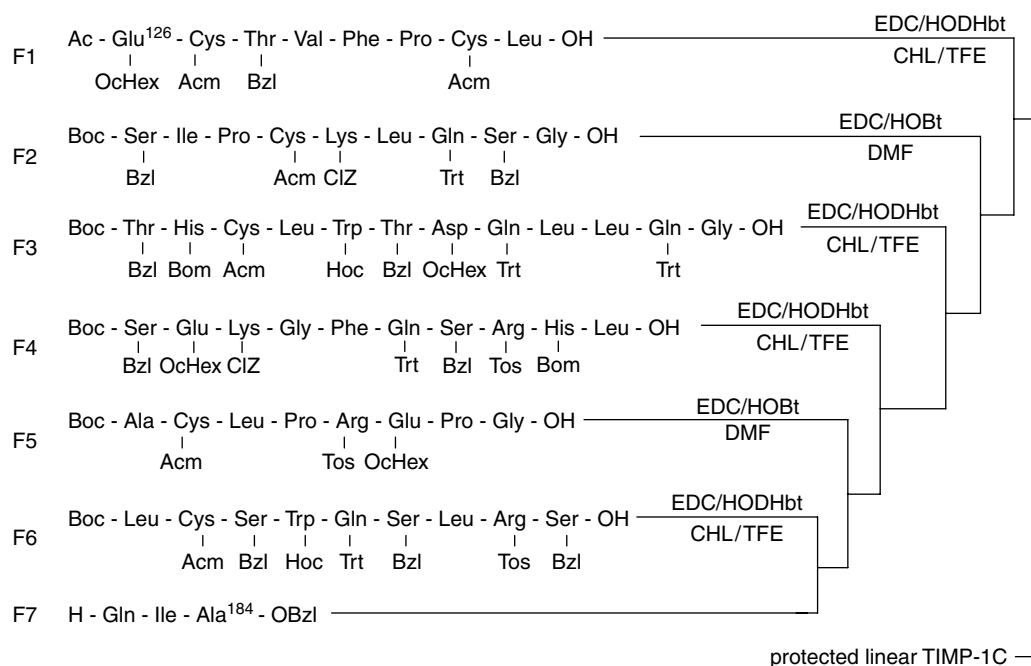
Figure 1 Synthesis scheme of the fully protected AC-TIMP-1C.

TLC and RP-HPLC, and they were identified by MS (Table 1).

Cleavage of the Boc Group

TFA cooled to −10 °C (or 0.1% triisopropylsilane (TIS)/TFA in the presence of Trt group) was added to the protected peptide under a N_2 atmosphere; after 20 min stirring at room temperature the TFA was evaporated, and the resulting TFA salt was converted to the corresponding hydrochloride by adding 1.1 eq HCl in dioxane. The dioxane was evaporated and the residue was triturated with cooled ether. The product was filtered off and dried over KOH *in vacuo*.

Assembly of Segments

The synthetic scheme is shown in Figure 1; experimental details are given in Table 2. The carboxyl component (1.05 eq) and additive (HODhbt or HOBt) (1.05 eq) were added to the solution of the deprotected amino component in an appropriate solvent. The mixture was chilled to 0 °C and EDC (1.05 eq) was added. If necessary, the excess of HCl bound to the amino component was neutralized by adding a further small amount of EDC. The reaction mixture was stirred at room temperature and monitored by TLC (CHCl$_3$–MeOH–AcOH

95 : 5 : 3 or 85 : 15 : 5). After complete reaction, the product was precipitated from the reaction mixture by adding appropriate precipitating solvent (see Table 2) and filtered off. The crude intermediates were further purified by trituration or/and reprecipitation, filtered and washed on the filter with ether and then with *n*-hexane. In this way 350 mg (0.017 mmol) fully protected linear Ac-TIMP-1C was prepared. The overall yield of the fragment condensations was 23% based on the starting *C*-terminal Boc-Gln^{182}-Ile-Ala^{184}-OBzl fragment.

Removal of the Protecting Groups

The fully protected linear peptide (330 mg) was treated with 10 ml HF in the presence of 10% anisole at −5 °C for 1h. After removal of HF *in vacuo*, the residue was triturated with ether. The precipitate was collected by filtration and washed with ether, then dissolved in 10% aqueous acetic acid and purified immediately by FPLC, resulting in the 6Acm-protected linear TIMP-1C. Yield: 125 mg (54%).

Removal of the Acm Groups

A solution of the above 6Acm-protected peptide (123 mg, 0.017 mmol) in 10 ml of 50% AcOH was

Table 1   Characteristics of the Protected Fragments for the Synthesis of the Ac-TIMP-1 *C*-terminal Domain

	Protected fragment	TLC[a] R_f	Yield	HPLC[b] Gradient	t_R/min	ESI-MS Calculated (average)	Measured MH$^+$
F1	Ac-^{126}E(OcHex)C(Acm)T(Bzl)VFPC(Acm)L^{133}-OH	a 0.47 b 0.39	0.56 mg (88%)	d	23.5	1266.6	1267.6
F2	Boc-^{134}S(Bzl)IPC(Acm)K(ClZ)LG(Trt)S(Bzl)G^{142}-OH	a 0.64 b 0.41	0.75 mg (86%)	d	31.1	1693.3	1694.3
F3	Boc-^{143}T(Bzl)H(Bom)C(Acm)LW(Hoc)T(Bzl)D(OcHex)G(Trt)LLG(Trt)G^{154}-OH	a 0.62 b 0.52	0.69 mg (64%)	d	23.9	2577.2	n.d.^{c}
F4	Boc-^{155}S(Bzl)E(OcHex)K(ClZ)GFG(Trt)S(Bzl)R(Tos)H(Bom)L^{164}-OH	a 0.49 b 0.41	0.81 mg (71%)	e	19.7	2236.2	2237.4
F5	Boc-^{165}AC(Acm)LPR(Tos)E(OcHex)PG^{172}-OH	c 0.46	0.43 mg (67%)	e	9.9	1248.6	MK$^+$ 1287.6
F6	Boc-^{173}LC(Acm)S(Bzl)W(Hoc)G(Trt)S(Bzl)LR(Tos)S(Bzl)^{181}-OH	b 0.72	0.76 mg (61%)	e	25.5	2056.0	2057.8

[a] TLC eluents: a: EtOAc-Py-AcOH-H$_2$O 120:20:6:11; b: CHCl$_3$-MeOH-AcOH 85:10:5, c: EtOAc-Py-AcOH-H$_2$O 60:20:6:11.
[b] HPLC gradients: d: 40–95% B in 40 min; e: 40–95% B in 25 min (eluent A and B see Materials and methods).
[c] MH$^+$:1485.6 for H-THC(Acm)LWTDG LLG G^{154}-OH (see syntheses of segments).

Table 2   Reaction Conditions of the Fragment Condensation Steps Carried Out in the Synthesis of Ac-TIMP-1 C-terminal Domain

	Carboxyl component	Amino component	EDC	Additive	Solvent	TLC	Precipitation	Purification Removing the Boc group	Yield product
1.	F6 412 mg (0.2 mmol)	F7 101 mg (0.22 mmol)	41 µl (0.22 mmol)	33 mg HODhbt (0.20 mmol)	2.0 ml CHCl$_3$-TFE	0.77[a]	Et$_2$O	Trituration, H$_2$O Precipitation, CHCl$_3$—MeOH TFA cleavage	380 mg (78%) F6-7
2.	F5 225 mg (0.18 mmol)	F6-7 355 mg (0.16 mmol)	31 µl (0.180 mmol)	25 mg HOBt (0.18 mmol)	4.5 ml DMF	0.51[b]	H$_2$O	Precipitation, DMF—H$_2$O TFA cleavage	435 mg (81%) F5-6-7
3.	F4 309 mg (0.138 mmol)	F5-6-7 385 mg (0.12 mmol)	24.2 µl (0.138 mmol)	22.4 mg HODhbt (0.138 mmol)	4.0 ml CHCl$_3$-TFE	0.39[b]	Et$_2$O	Precipitation, DMF—MeOH TFA cleavage	389 mg (60%) F4-5-6-7
4.	F3 211 mg (0.08 mmol)	F4-5-6-7 360 mg (0.07 1mmol)	14.5 µl (0.08 mmol)	13.3 mg HODhbt (0.080 mmol)	5.0 ml DMF	0.29[b]	H$_2$O	Trituration, MeOH—H$_2$O Precipitation, DMF TFA cleavage	413 mg (76%) F3-4-5-6-7
5.	F2 82 mg (0.052 mmol)	F3-4-5-6-7 340 mg (0.048 mmol)	9.1 µl (0.08 mmol)	7.3 mg HODhbt (0.080 mmol)	4.0 ml CHCl$_3$-TFE	0.37[b]	MeCN	Trituration, MeOH—H$_2$O TFA cleavage	389 mg (91%) F2-3-4-5-6-7
6.	F1 59.7 mg (0.047 mmol)	F2-3-4-5-6-7 350 mg (0.041 mmol)	8.2 µl (0.08 mmol)	7.7 mg HODhbt (0.080 mmol)	4.0 ml CHCl$_3$-TFE	0.40[b]	MeCN	Trituration, MeCN	350 mg (88%) F1-2-3-4-5-6-7

Solvent systems for TLC: [a]CHCl$_3$-MeOH-AcOH 95:5:3; [b]CHCl$_3$-MeOH-AcOH 85:15:5.

treated with mercury(II) acetate (6.6 eq, 3.7 mg) at room temperature for 2 h. After adding 400 μl of 2-mercaptoethanol, the solution was stirred for another 2 h, and then applied to a Sephadex G-25 column and eluted with 5% aqueous AcOH. The fractions containing the peptide were combined and lyophilized. 85.5 mg (74%) of crude linear 6SH-Ac-TIMP-1C was obtained.

Oxidative Folding of 6SH-TIMP-1C

The crude linear 6SH-Ac-TIMP-1C was treated with 2-mercaptoethanol prior to the oxidation step to remove the traces of mercury(II) ions, and to reduce the accidentally oxidized SH-groups: 85 mg was dissolved in 10 ml of 0.1 M Tris buffer (pH 8.4) containing 6 M guanidine hydrochloride (GnHCl) and 1 mM EDTA. 1.2 ml of 2-mercaptoethanol was added and the solution was stirred under a N_2 stream for 2 h. The solution was acidified to pH 3 by adding TFA, then desalted and purified on a semipreparative RP-HPLC column (gradient: 10–40% B in 60 min). The reduced and purified linear 6SH-TIMP-1C peptide (69 mg) was folded in two different media. In the first experiment half of the peptide was dissolved in 0.1 M NH$_4$OAc buffer (pH 7.8) containing 1M GnHCl and 1 mM EDTA to give a peptide concentration of 10^{-5} M. The ratio of peptide/oxidized glutathione (GSSG)/reduced glutathione (GSH) was adjusted to be 1 : 10 : 100, and the solution was gently stirred for 24 h at room temperature. In the second experiment, 0.1 M Tris buffer (pH 7.8) containing 20% *i*-PrOH and 1 mM EDTA was used with the same ratio of peptide and GSH-GSSG system. In both cases the solution was acidified to pH 3 with TFA after the folding reaction, and then applied to an RP-HPLC column for desalting and purification using a linear gradient (15–40% B in 60 min). The yields of Ac-TIMP-1C were 5.4 mg and 5.2 mg, respectively (~8%). The monoisotopic MH$^+$ was determined by ESIMS as 6622.66 Da ($C_{289}H_{450}N_{82}O_{85}S_6$).

Synthesis of H-^{145} CLWTDQLLQGSEKGFQSRHLAC ^{166}-NH$_2$

The fully protected linear sequence was synthesized manually on 300 mg of 4-methylbenzhydrylamine resin (loading: 0.86 mmol/g) by the stepwise Boc technique using the above mentioned coupling method and side chain protecting groups except for the Cys which was protected with 4-methylbenzyl (Meb) group. The cleavage of the peptide from the resin and the removal of the protecting groups were achieved simultaneously by treatment with 10 ml HF in the presence of 10% anisole and 100 mg EDT at −5 °C for 1 h. The crude product (342 mg) was reduced with 324 mg of DTT (15 eq) in 20 cm^3 of 0.1 M NH$_4$OAc buffer, pH 7.8 under N_2 atmosphere, then the peptide was applied to an RP-HPLC column and purified using a linear gradient of MeCN in 0.1% aqueous TFA. Yield: 225 mg. For oxidation the 2SH-peptide was dissolved in 300 cm^3 of 0.1 M NH$_4$OAc buffer, pH 7.8 at room temperature and the mixture was gently stirred for 1 day. Then it was acidified to pH 3 by adding TFA, and the oxidized peptide was purified by semipreparative RP-HPLC using a linear gradient of MeCN in 0.1% aqueous TFA to obtain 109 mg of product. Yield: 29%. M($C_{109}H_{169}N_{33}O_{32}S_2$): 2516.2; MH$^+$ (average): 2517.8.

Decomposition Experiments in the Presence of Mercury(II) Ions

1 mg of the above prepared disulphide bridged TIMP-1 [145–166] was dissolved either in 1 ml of 0.1 M NH$_4$OAc buffer (pH 7.8) or in 1 ml 0.1% aqueous TFA. 5 eq of mercury(II) acetate in 50% acetic acid (~40 μl) was added, and the solution was allowed to stand for 24 h at room temperature. Blank experiments were also performed without the mercury(II) ions. The mixtures were analysed by RP-HPLC.

MS Analysis of the Full-length Peptide

The molecular mass measurement was performed on a QSTAR quadrupole-orthogonal-acceleration-time-of-flight mass spectrometer (MDS Sciex, Toronto, Canada) equipped with a nanospray source (Protana, Odense, Denmark). The peptide mixture was dissolved in 33% EtOH : PrOH 5 : 2 and 0.1% aqueous formic acid.

The LC/MS experiment was performed on a Mariner ESI orthogonal-acceleration-time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). The HPLC separation was carried out using an ABI (Foster City, CA) 140B dual syringe pump with a microbore column (C18, 1 × 150 mm) at a flow rate of 50 μl/min. Solvent A was 0.1% formic acid in water, solvent B was 0.05% formic acid in EtOH : PrOH 5 : 2. The peptides were separated by gradient elution: the percentage of solvent B was linearly increased from 5% to 50% over 65 min. The eluent was split at a ratio of approximately 1 : 10

prior to the MS detection, the flow rate to the mass spectrometer was ~5 µl/min.

Both mass spectrometers afforded high enough resolution for monoisotopic mass determination.

Disulphide Bridge Assignments

Trypsin digestion. 50 pmol Ac-TIMP-1C was incubated with 5 ng side-chain protected porcine trypsin in 10 µl 25 mM NH$_4$HCO$_3$ buffer in 20% MeCN/water (pH 6.5), for 2 h at 37 °C. The digestion was terminated with 2 µl 2% formic acid in 50% aqueous MeCN.

Pepsin digestion. Immobilized pepsin was washed twice with the digestion buffer (20 mM NaOAc, pH 4.0) and resuspended in the same buffer prior to use (0.13 µl gel/1 µl suspension). Peptide (50 pmol) was incubated in ~75 µl of the pepsin suspension for 4 h at room temperature.

Reduction and alkylation. The digests (5 pmol/µl) in 50 mM NH$_4$HCO$_3$ were mixed with a 200-fold excess of DTT. Reduction was allowed to proceed at 56 °C for 60 min. The alkylation was performed by 220-fold excess of iodoacetamide over 30 min, at room temperature, in the dark.

Sample preparation for MALDI-TOF analysis. Crude digested or reduced and subsequently alkylated samples were ZipTip$_{C18}$ (Millipore)-purified, then mixed with the matrix solution and spotted onto the sample plate.

Matrices. α-Cyano-4-hydroxycinnamic acid solution (made for MALDI experiments) was purchased from Agilent Technologies (Wilmington, DE). A saturated solution of 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich Kft, Budapest, Hungary) was prepared in 0.1% aqueous TFA. Equal volumes of matrix and sample solutions (0.5 : 0.5 µl) were mixed and spotted onto the stainless steel sample plate. The mixture was allowed to dry before introduction into the mass spectrometer.

MALDI-TOF-MS. Experiments were performed on a Bruker Reflex III instrument (Bruker Saxonia Analytik GmbH, Leipzig, Germany) equipped with a reflectron and N$_2$ laser (337 nm) in reflectron mode, with external calibration.

MALDI-PSD (post-source decay) measurement. Precursor ion gating was employed to transmit an individual peptide and its metastable fragment ions selectively to the reflectron for PSD sequencing. Data

acquisition was performed in 11–14 segments; at each step the reflectron voltage was reduced by 25%. Then the segments were united to produce the complete spectrum. The spectra were smoothed so that partially resolved isotope peaks appear as single average mass peaks.

RESULTS

Synthesis

In order to synthesize the acetylated linear TIMP-1 *C*-terminal domain (TIMP-1C), Boc-protected peptide fragments were prepared and then, according to our previous convergent peptide synthetic approach [10], coupled sequentially in solution (Figure 1). Six orthogonally protected fragments (F1–F6) were synthesized by the Fmoc strategy on a 2-chlorotrityl chloride resin using HBTU/DIEA; fragment F7 was synthesized in solution by Boc chemistry. The *N*-terminal amino acids of fragments F2–F6 were coupled as Boc derivatives. The *N*-terminal amino acid of fragment F1 was coupled as an Fmoc derivative, then the Fmoc group was cleaved with piperidine/DMF, and the *N*-terminal amino group was finally acetylated with acetic anhydride. The protected segments were removed from the resin and purified by repeated precipitation. Condensation reactions were carried out starting from the carboxyl terminus using water-soluble EDC as coupling reagent in the presence of HOBt in DMF, or HODhbt in CHCl$_3$-TFE 3 : 1. The intermediate fragments were purified by precipitation using appropriate solvent mixtures, i.e. DMF-MeOH, DMF-MeCN or flash chromatography. The Boc protecting group was removed by TFA (0.1% TIS was used with TFA for Trt deprotection) at 0 °C, and the TFA salt was converted into the corresponding hydrochloride. The fully protected linear TIMP-1C sequence was treated with anhydrous HF and after purification by RP-HPLC, the 6Acm-peptide was treated with mercury(II) acetate in 50% AcOH for 2 h under a N$_2$ atmosphere to remove the Acm groups. In order to remove the mercury(II) ions, the product was treated with 2-mercaptoethanol and subjected to gel filtration on Sephadex G-25 and HPLC purification.

To find the optimal conditions for the oxidative folding reaction, a series of model experiments was carried out followed by HPLC monitoring. First the effect of the temperature and the oxidant was tested, and the GSH-GSSG system found to be more efficient than oxidation with air. As temperature

did not seem to influence the folding reaction, the next series of probes were performed at room temperature. In our further experiments two buffer solutions, 0.1 M NH$_4$OAc/1 mM EDTA and 0.1 M Tris/1 mM EDTA, two denaturants, 0.5 M (NH$_4$)$_2$SO$_4$ and 1.0 M GnHCl, and two detergents, *i*-PrOH and glycerine (20%) were used, in different combinations, at pH 7.8 or 8.5. The peptide concentration was always 10^{-5} M with a 1 : 10 : 100 ratio of peptide/GSSG/GSH. No significant difference of the HPLC profiles from the different experiments was observed, with one exception: the peptide precipitated when (NH$_4$)$_2$SO$_4$ was used. Eventually, two sets of folding conditions were selected on the basis of the peak heights and widths. Thus, the linear peptide was divided into two portions and the folding was performed both in 0.1 M NH$_4$OAc, 1 M GnHCl and 1 mM EDTA at pH 7.8 and in 0.1 M Tris, 1 mM EDTA and 20% *i*-PrOH at pH 7.8. Three different HPLC chromatograms of the main purified product are shown in Figure 2.

Mass Spectrometry

The monoisotopic molecular mass of the synthesized Ac-TIMP-1C was found by ESIMS to be 6622.66 Da. This mass clearly indicated the presence of the fully oxidized peptide, i.e. three disulphide bridges. In addition, this experiment revealed the presence of an impurity with a monoisotopic MH$^+$ of 4177.65 Da. An LC/ESIMS experiment was performed in order to estimate the relative amounts of the components more accurately. Ion extraction profiles identified two very closely eluting disulphide bonded isomeric structures for the main product; the above impurity, which proved to be a minor component, eluted at a slightly longer retention time (data not shown). Its structure was assigned as Ac-TIMP-1 [126–162] (its calculated monoisotopic MH$^+$ is 4177.96).

Determination of the Position of the Disulphide Bonds

In order to assign the disulphide bridges the polypeptide had to be cleaved to separate the Cys-residues. Trypsin and pepsin were used for this purpose. Trypsin displays high specificity, so the digestion products can be assigned easily. However, the first tryptic peptide Ac^{126}Glu-Lys^{138} contains three Cys residues. This situation makes the determination of the disulphide bonds involving these three Cys residues ambiguous. To overcome
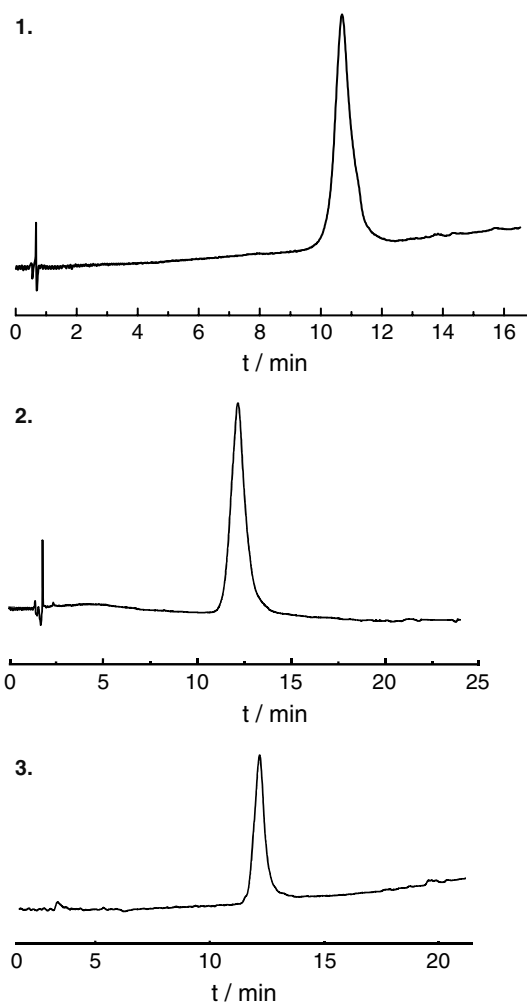

Figure 2 HPLC chromatograms of Ac-TIMP-1C: 1. YMC-ODS C18, 4.6 × 150 mm, 5 μm, 300 Å; isocratic elution: 33% B (A: 0.1% TFA in water, B: 0.07% TFA in MeCN); 2. YMC-ODS-AQ, 4 × 50 mm, 3 μm, 300 Å, gradient: 5%–35% B in 25 min (A: 0.1 M TEAP, pH 4, B: MeCN); 3. Vydac C18, 4.6 × 150 mm, 5 μm, 300 Å, gradient: 10%–40% B in 30 min. (A: 0.1% TFA in water, B: 0.07% TFA in MeCN). Flow rate: ml/min, λ = 220 nm.

this problem, pepsin, a non-specific protease, was also used.

The tryptic hydrolysis was carried out at pH 6.5 to prevent rearrangement of the disulphide bonds. The unfractionated digest was analysed by MALDI-MS (Table 3). Peptides were assigned from the masses observed, considering the TIMP-1C sequence as well as the cleavage specificity of trypsin. The identity of the disulphide-linked components was verified by reduction and alkylation followed by MALDI-MS (data not shown) or by PSD analysis. From these data, two disulphide bonds, Cys^{166}-Cys^{174} (Figure 3)

Table 3   Monoisotopic MH$^+$ Values of Ac-TIMP-1C Tryptic Peptides

Theoretical m/z [Da]	Experimental m/z [Da]	Residue	Disulphide bond
594.30	594.30	[158–162]	
809.45	809.48	[163–169]	free SH@166
1275.62	1275.65	[170–180]	free SH@174
2064.03	2064.02	[163–180]	166–174
2082.04	2082.08	[163–169]-[170–180]	166–174
2752.26	2752.37	Ac[126–138]-[170–180]	(127,132,137)-174 2S-S
2950.47	2950.57	[139–157]-[163–169]	145–166
3602.68	3602.60	Ac[126–157]	127,132,137,145 2S-S
3620.69	3620.61	Ac[126–138]-[139–157]	(127,132,137)-145 2S-S
4177.96	4177.29	Ac[126–162]	127,132,137,145 2S-S
5664.69	5665.15	Ac[126–157]-[163–180]	(127,132,137,145)-(166,174) 3S-S
5682.71	5683.16	Ac[126–157]-[163–169]-[170–180]	166-(127,132,137,145)-174 3S-S

The masses were determined from the unfractionated digest by MALDI-TOF-MS.

MALDI ionization may trigger prompt fragmentation of the disulphide-bridges [11,12] that accounts for the presence of free SH–containing peptides *prior* to the reduction.

When the linkages could not be assigned, the Cys residues involved and the number of the disulphide bridges are listed.
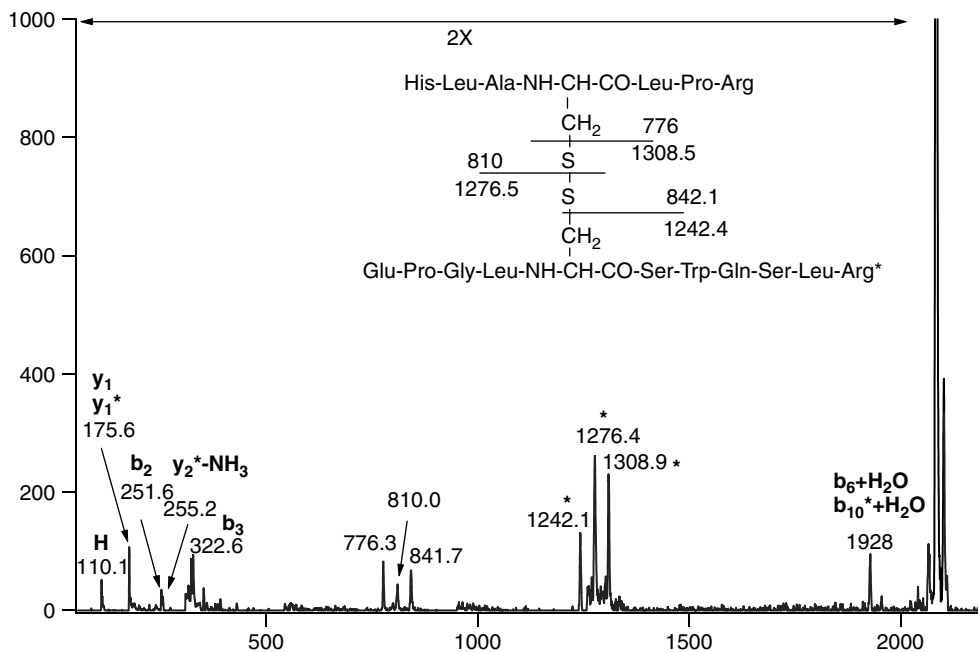

Figure 3   MALDI-PSD spectrum of the m/z 2082.08 fragment from the tryptic digest of Ac-TIMP 1C. Cleavage across the disulphide-bridge is characteristic for such peptides in PSD [13] as well as in CID [14] analysis. The other fragments are labelled according to the nomenclature of reference 15. Ions labelled with asterisks derived from the longer peptide.

and Cys^{145}-Cys^{166} were located unambiguously. The Cys^{145}-Cys^{166} linkage represents the expected (correct) **A** isomer of Ac-TIMP-1C (Figure 4). From the peptic digest, the presence of a Cys^{127}-Cys^{174}

bridge and thus, indirectly the Cys^{132}-Cys^{137} bridge were established (Table 4) as a further evidence for the structure of the **A** isomer of Ac-TIMP-1C. However, Cys^{137}-Cys^{145} and thus, Cys^{127}-Cys^{132}

(A)

Ac-C^{127}—C^{132}—C^{137}———C^{145}————R^{162}H^{163}—C^{166}—C^{174}————-OH

(B)

Ac-C^{127}—C^{132}—C^{137}———C^{145}————R^{162}H^{163}—C^{166}—C^{174}————-OH

(C)

Ac-C^{127}—C^{132}—C^{137}———C^{145}————R^{162}H^{163}—C^{166}—C^{174}————-OH

(D)

Ac-C^{127}—C^{132}—C^{137}———C^{145}————R^{162}H^{163}—C^{166}—C^{174}————-OH

(E)

Ac-C^{127}—C^{132}—C^{137}———C^{145}————R^{162}H^{163}—C^{166}—C^{174}————-OH
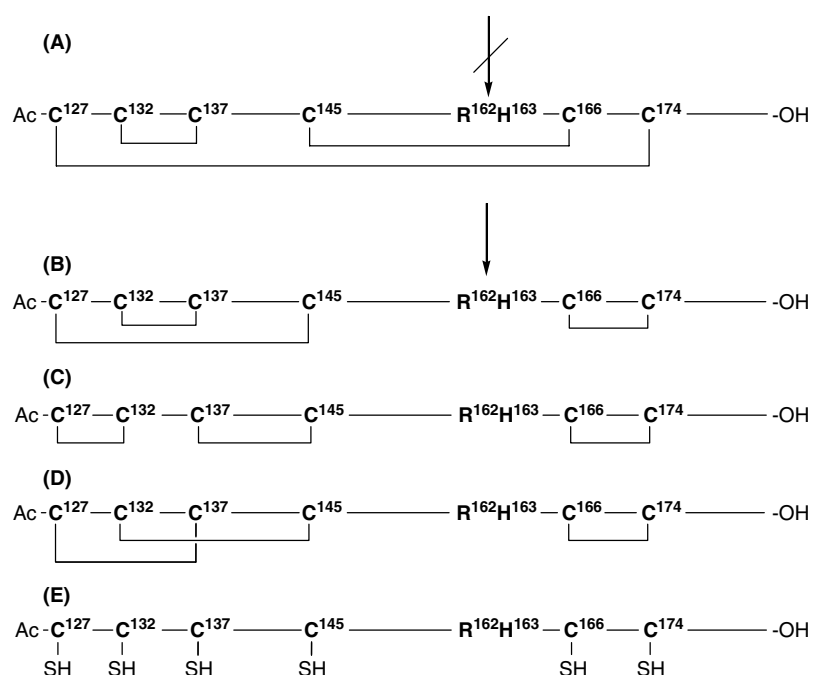   SH   SH   SH   SH   SH   SH

Figure 4 (A) Correct disulphide bond arrangement in Ac-TIMP-1 [126–184]; (B), (C), (D) incorrect disulphide bond arrangements and (E) linear Ac-TIMP-1C. The formation of Ac-TIMP-1 [126–162] is possible from B, C, D, E after cleavage of the Arg^{162}-His^{163} peptide bond.

Table 4   Monoisotopic MH$^+$ Values of Ac-TIMP-1C of the Disulphide-bridged Peptic Peptides

Theoretical *m/z* [Da]	Experimental *m/z* [Da]	Residue	Disulphide bond
1256.61	1256.66	[164–175]	166–174
1329.61	1329.68	[165–176]	166–174
1347.62	1347.69	[165–176] cleaved[a]	166–174
1442.69	1442.81	[164–176]	166–174
1460.70	1460.64	[164–176] cleaved[a]	166–174
1495.66	1495.59	Ac[126–130]-[168–175]	127–174
1570.75	1570.71	[164–177]	166–174
1671.80	1671.76	[134–148]	137–145
1681.74	1681.71	Ac[126–130]-[168–176]	127–174
1770.87	1770.86	[164–179]	166–174
1786.82	1786.87	[134–149]	137–145
2027.97	2028.00	[134–151]	137–145
2098.01	2098.03	[159–176]	166–174
2317.04	2317.10	Ac[126–146]	(127,132,137,145) 2S-S
2604.17	2604.20	Ac[126–148]	(127,132,137,145) 2S-S
2847.26	2847.28	Ac[126–150]	(127,132,137,145) 2S-S
2960.34	2960.36	Ac[126–151]	(127,132,137,145) 2S-S
3125.54	3125.55	[152–179]	166–174

The masses were determined from the unfractionated digest by MALDI-TOF-MS.
When the linkages could not be assigned, the Cys residues involved and the number of the disulphide bridges are listed.
[a] The exact cleavage site was not determined.

disulphide bonds were also identified that pointed to the presence of the **C** isomer of Ac-TIMP-1C.

Peptide Decomposition in the Presence of Mercury(II) Acetate

The appearance of the Ac-TIMP-1 [126–162] sequence, as an impurity in the mass spectrum of TIMP-1C needed an explanation. Considering the synthetic scheme in Figure 1, this peptide cannot be the result of miscoupling during the fragment-condensation procedure. Thus, it was suspected that in spite of thorough purification steps some mercury(II) ions had remained associated with the peptide. Indeed, atom absorption spectroscopy (AAS) showed the presence of 0.38% mercury(II) in the final product, which meant about 0.1 eq mercury(II) content referred to the peptide.

To study the role of mercury(II) ions in the cleavage of the peptide bond between Arg^{162} and His^{163} in the Ac-TIMP-1C, the [145–166] loop of the *C*-terminal domain of TIMP-1 containing the incriminated amino acid-pair was synthesized and its stability investigated in the presence of mercury(II) ions. According to HPLC analysis, the starting peptide disappeared after 24 h in 0.1% TFA solution, as well as at pH 7.8 in Tris buffer in the presence of 5 eq mercury(II) acetate (Figure 5). However, incubation without the mercury(II) ions did not result in the decomposition of the peptide (data not shown).
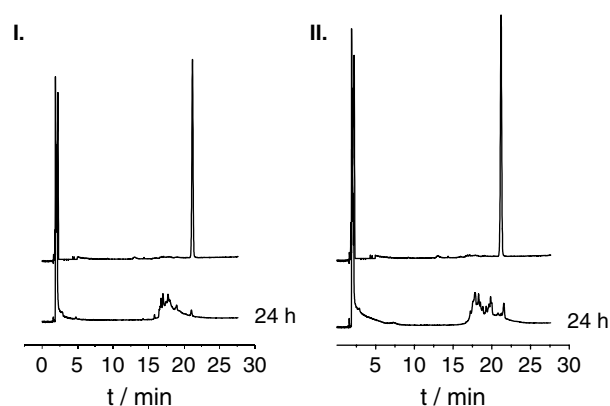

Figure 5   Decomposition of
H-^{145} CLWTDQLLQGSEKGFQS**RH**LAC ^{166}-NH$_2$ in the presence of 5 eq mercury(II) acetate: I. In 0.1% TFA; II. In 0.1 M Tris buffer, pH 7.8. YMC ODS-A, 5 µm, 120 Å, 150 × 4.6 mm column, gradient: 10%–50% B in 30 min. eluent: A: 1% TFA in water, B: 0.07% TFA in MeCN.

DISCUSSION

Structure–biological activity relationship investigations of the TIMP-1 *C*-terminal domain are desirable for the understanding of a series of physiological and pathological processes. The linear peptide was successfully synthesized and folded. The conditions for oxidative folding were extensively studied. However, in more than 30 oxidative folding experiments no significant differences in the outcome were detected. In addition, in spite of the many efforts made (for illustration see three representative HPLC chromatograms in Figure 2), no separation of the isomers was achieved. The disulphide bond assignment by MALDI-MS proved our assumption, that beside the desired isomer, the synthetic product contained a structural isomer as an impurity that corresponded to structure **C** in Figure 4.

Thus, our results strongly suggest that contrary to the TIMP-1 *N*-terminal domain, which already has been successfully prepared by gene technology, entirely correct folding of the *C*-terminal domain does not occur in the absence of the *N*-terminal part of the protein. This may be the reason why no successful genetic engineering of this domain has yet been reported.

It is worthy of mention that the simple mass determination step was informative with regard to the presence of a possible structural isomer as an impurity. The appearance of an unexpected side-product with a monoisotopic MH$^+$ of 4177.65 Da corresponding to Ac-TIMP-1 [126–162] drew our attention to the possibility of simultaneous incorrect folding. It was obvious that this peptide could not be the result of a synthetic failure (see Figure 1), and nor could it have arisen from the native *C*-terminal domain (see **A** in Figure 4). Thus, even before the enzymatic digestions, we were able to postulate that this impurity was formed from one of the structural isomers (**B, C, D**) or from the linear peptide (**E**) after the cleavage of the Arg^{162}-His^{163} bond (Figure 4). It is well known that histidine, and peptides containing this amino acid, readily form complexes with transition metal ions [16] and these complexes may catalyse peptide bond hydrolysis. Despite the several purification steps performed after the cleavage of the Acm groups with mercury(II) acetate, AAS showed the presence of mercury(II) ions. Our investigations on the effect of mercury(II) ions on the disulphide bonded Ac-TIMP-1 [126–162] sequence proved that indeed the metal ions were responsible for the bond cleavage. Interestingly, full decomposition occurred in 24 h both at the pH of the

folding experiment (7.8) and in 0.1% TFA solution, i.e. at the pH of the HPLC purification procedure.

This observation draws attention to the danger of degradation under the influence of tightly bond metal ions which cannot be fully removed by the usual purification procedures.

Acknowledgements

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