FEBS 16915

FEBS Letters 384 (1996) 155–161

Folding and characterization of the amino-terminal domain of human tissue inhibitor of metalloproteinases-1 (TIMP-1) expressed at high yield in *E. coli*

Wen Huang[a], Ko Suzuki[b], Hideaki Nagase[b], S. Arumugam[c], Steven R. Van Doren[c], Keith Brew[a,*]

[a] *Department of Biochemistry and Molecular Biology, University of Miami, Miami, FL 33134, USA*
[b] *Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA*
[c] *Biochemistry Department, University of Missouri, Columbia, MO 65211, USA*

Received 15 February 1996; revised version received 11 March 1996

Abstract Methods are described for producing an active amino-terminal domain of tissue inhibitor of metalloproteinases-1 (N-TIMP-1) from inactive protein expressed as inclusion bodies in *E. coli*. Yields exceed 20 mg per litre of bacterial culture. Activity measurements, CD spectroscopy and NMR spectroscopy of the ^{15}N-labeled protein show that it is fully active, homogeneous in conformation and suitable for high-resolution structural analysis. The affinity of N-TIMP-1 for matrix metalloproteinases 1, 2 and 3 is 6–8-fold less than that of the recombinant full-length protein, indicating that deletion of the C-terminal domain reduces the free energy of interaction by <10%.

Key words: Bacterial expression; Enzyme inhibition; TIMP; Metalloproteinase; NMR

1. Introduction

Tissue inhibitors of metalloproteinases (TIMPs) play important roles in regulating the activities of matrix metalloproteinases (MMPs or matrixins) [1,2], a family of enzymes that are responsible for the breakdown of components of connective tissue. Matrix turnover is an important aspect of many normal and pathological processes, such as growth, tissue remodeling, wound repair, and tumor metastasis. TIMPs consist of two domains, an amino-terminal inhibitory domain of about 120 amino acids through which they bind to an active MMP to form a tight (K_i < 1 nM), 1:1, non-covalent complex and a carboxy-terminal domain which is involved in interactions with progelatinases [3]. In addition to their inhibitory activities towards MMPs, TIMPs have also been reported to possess cell growth promoting activity [4–6].

Three members of the family of TIMP have been identified to date: TIMP-1, TIMP-2 and TIMP-3 [3,7–11]. The level of overall amino acid sequence similarity between pairs of TIMPs is 28–40% and 12 cysteinyl residues known to form six disulfide bonds are conserved in all TIMPs, each domain being stabilized by three disulfide bonds [12]. TIMP-1 is a glycoprotein with heterogeneous N-linked complex glycan units attached to Asn-30 and Asn-78 [7,13], whereas TIMP-2 and chicken TIMP-3 are not glycosylated [8,9]. The recombinant human and mouse TIMP-3s expressed in NSO mouse myeloma cells, however, are secreted primarily as 27 kDa glycosylated forms [14]. A low resolution NMR solution structure of the inhibitory amino-terminal domain of TIMP-2 has been reported [12], which provides a basis for interpreting the results of structure–function relationships in members of the TIMP family.

Recombinant active forms of human TIMP-1 and TIMP-2 and of the amino-terminal domains of TIMP-1 and TIMP-2, have been expressed in mammalian cells [15–17] and in baculovirus-infected insect cells [18,19]. Eukaryotic expression systems are often preferred for producing correctly processed and folded recombinant forms of mammalian proteins but microbial expression systems have advantages in terms of yield, cost and they are more easily used for producing mutant proteins.

With the goal of developing an expression system that is suitable for high yield protein production and mutagenesis studies of the inhibitory domain of human TIMP-1 (N-TIMP-1), we have chosen to use pET vectors and an *E. coli* host. The overexpression of proteins in *E. coli* leads to the formation of inclusion bodies, which are easily isolated but require treatment to generate native protein [20]. Since TIMP-1 does not require glycosylation to be active [21,22], the absence of glycosylation in an *E. coli* system was not perceived to be problematic. Previous work has shown that mouse TIMP-1 [23] human TIMP-1 [24,25] and human TIMP-2 [26] are expressed as inclusion bodies in *E. coli* and can be folded in vitro, but the bacterial expression of the amino-terminal domain of TIMP-1 has not been described. Here we describe the expression of the amino-terminal domain of human TIMP-1 (N-TIMP-1) as inclusion bodies, a modified high yield in vitro folding procedure that avoids the production of large volumes of very dilute solutions of protein, and the functional and physical properties of the protein determined by CD spectroscopy and 2D NMR spectroscopy of the ^{15}N-substituted protein. The small size of the isolated inhibitory domain, the high folding yield and conformational homogeneity of the product indicate that our *E. coli* expressed N-TIMP-1 is a suitable subject for structural analysis by multi-nuclear NMR and X-ray crystallography.

*Corresponding author. Fax: (1) (305) 243-3065.

Abbreviations: IPTG, isopropylthiogalactoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinases; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Mca, (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva, norvaline; MMP, matrix metalloproteinase; HMQC, heteronuclear multiple quantum coherence.

SSDI S0014-5793(96)00304-3

2. Materials and methods

2.1. Materials

MacroPrep 50Q anion exchange resin and Affi-Gel 10 were from BioRad Laboratories. CM-52 cation exchange resin was from Whatman and Sephacryl S-300 and S-200 were from Pharmacia. $[^{15}N]H_4Cl$ and deuterated reagents for NMR spectroscopy were purchased from Cambridge Isotope Laboratories, Andover, MA. The pET3a expression vector was from Novagen and restriction enzymes were from New England Biolabs. Taq DNA polymerase was from Promega. Bis(2-hydroxyethyl) disulfide is from Aldrich Chemical Co., Milwaukee, Wisconsin. The fluorogenic substrate Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 was a gift from Dr. G.B. Fields, University of Minnesota, Minneapolis, MN, while Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 was from Bachem Bioscience Inc., King of Prussia, PA. Oligonucleotides were synthesized by Dr. R. Werner, Univ. of Miami School of Medicine. Human TIMP-1 cDNA was kindly supplied by Dr. Masanobu Naruto of Toray Industries, Kamakura, Japan. The mammalian expression vector, pEE-14 was generously provided by Celltech Inc., Slough, UK. Specific polyclonal anti-(human TIMP-1) antibody was prepared as described previously [27]. ProMMP-1, proMMP-2 and proMMP-3 were isolated from the culture medium of human rheumatoid synovial cells stimulated with rabbit macrophage-conditioned medium and activated as described previously [28–30]. The catalytic domain of MMP-3 (MMP-3(ΔC)) was obtained by expressing proMMP-3(ΔC) in *E. coli* using a MMP-3 cDNA in which a stop codon is substituted for Pro-256 (Suzuki et al., in preparation). ProMMP-3(ΔC) was activated to MMP-3(ΔC) using 1.5 mM 4-aminophenylmercuric acetate.

2.2. Amplification of N-TIMP1 cDNA

The cDNA encoding N-TIMP-1 was amplified by PCR using three primers. The first amplification was carried out using the 5′ primer: 5′CTCACTACGTCATATGTGCACCTGTGTCCCCA3′ which contains an *Nde*I cloning site and Met-1, and a mutagenic primer: 5′GGATAAACAGGGAATTCCTGTTCATTCCTCACAGCC-3′ which introduces a stop codon at the position corresponding to residue 127 of TIMP-1 so that only the N-terminal domain of TIMP-1 will be expressed. The product of this reaction was gel purified and used in the second amplification as a megaprimer [31] with the 3′ primer: 5′GAATTGTCGCGGATCCTCAGGCTATCTGGA3′ which contains a *Bam*HI cloning site. PCR reactions were carried out using a Perkin Elmer/Cetus thermocycler for 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1.5 min. To circumvent a difficulty in cleaving the PCR product at the *Nde*I site, it was firstly ligated to a T-vector (pUC19 plasmid opened at polycloning site by *Sma*I with extra 'T' bases added using DNA polymerase), transformed into DH5α and amplified. The plasmid DNA containing the TIMP-1 cDNA insert was digested with *Bam*HI and *Nde*I and the TIMP-1 cDNA was cloned into the pET3a vector as follows.

2.3. Construction of the expression vector

The *Bam*HI and *Nde*I sites in the pET3a vector are only 12 bases apart so that vector that has been cleaved at both restriction sites is not readily separable from partially cleaved vector. Thus, pET3a was first cleaved with *Pst*I and *Bam*HI, to generate the 1264 base fragment A and with *Pst*I and *Nde*I to generate the 3338 base fragment B. Fragments A and B were purified from the digests by gel electrophoresis. A ligation reaction was then carried out with fragments A and B and the *Bam*HI/*Nde*I-cleaved coding region from the TIMP-1 cDNA. This produced the pET3a-N-TIMP1 expression vector, pNT1, which was used to transform BL21(DE3) cells for N-TIMP1 expression.

2.4. Expression of N-TIMP-1

Host cells with the pNT1 vector were grown in LB medium (1–6 l) containing 200 μg/ml ampicillin at 37°C until the A_{600nm} reading reached 0.6–0.8. At this point expression was induced by the addition of IPTG to 0.4 mM. Growth was continued for an additional 3 h. Cells were harvested by centrifugation and lysed using lysozyme and deoxycholate following a standard procedure [32]. Inclusion bodies were collected by centrifugation and washed and the protein was extracted with 8 M urea containing 20 mM Tris-HCl, pH 8.5, and 10 mM DTT. SDS-PAGE indicates that N-TIMP-1 (M_r 14000) represents >90% of the protein in this extract. However, this material also contained a large amount of DNA which interferes with the folding process of N-TIMP-1 (pI ≈ 10.5) as they interact during removal of the urea by dialysis. To remove DNA, the extract was first passed through a column of MacroPrep 50Q anion exchange resin equilibrated with 20 mM Tris-HCl, pH 8.0, containing 8 M urea, to remove free anionic macromolecules. The flow-through fraction was then applied to a column (5.5×28 cm) of Sephacryl S-300 in 8 M urea, 20 mM Tris HCl, 0.5 M NaCl, pH 7.5, to separate N-TIMP-1 from high molecular weight contaminants.

2.5. Expression of $[^{15}N]$N-TIMP-1

E. coli BL21(DE3) cells, transformed with the pNT1 vector, were inoculated into LB medium and grown overnight at 37°C. The cells were collected by centrifugation, washed with M9 minimal medium [32] and then grown with shaking at 37°C in M9 minimal medium containing $[^{15}N]H_4Cl$. After approx. 12 h, N-TIMP-1 expression was induced by adding IPTG (0.4 mM) and the cells grown for another 12 h prior to harvesting. $[^{15}N]$N-TIMP-1 was isolated and folded as described below.

2.6. In vitro folding of N-TIMP-1

Fractions containing N-TIMP-1 from Sephacryl S-300 were pooled and diluted to <100 μg protein/ml with 20 mM Tris-HCl, pH 8.0, containing 8 M urea. This solution was treated with cystamine (50 mM) overnight at 4°C to form mixed disulfides between protein and cystamine. The solution was then dialyzed for against two changes (24 h each) of 15 vols. of folding buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$, 5 mM ß-mercaptoethanol and 1 mM bis(2-hydroxyethyl) disulfide), followed by two changes (4 h each) of 20 mM Tris-HCl, pH 7.0 [33]. The protein was finally concentrated and further purified by cation exchange chromatography with columns (2×10 cm) of CM-cellulose CM-52 equilibrated with 20 mM Tris-HCl, pH 7.0. After washing the CM-52 with 2 vols. of equilibration buffer, N-TIMP-1 was eluted with a linear gradient (500 ml) from 0 to 0.5 M NaCl in the same buffer at a flow rate of 2 ml/min.

2.7. Expression of TIMP-1 in mammalian cells

Human TIMP-1 cDNA was cloned into the expression vector, pEE-14, between the human CMV promoter and the SV 40 early polyadenylation signal. The pEE-14 vector contains the glutamine synthetase gene which was used as an amplifiable marker in Chinese hamster ovary (CHO K-1) cells. Cells producing high levels of TIMP-1 were selected in glutamine-free medium supplemented with methionine sulfoximine as described by Cockett et al. [34]. The recombinant TIMP-1 secreted into the culture medium was purified with an immunoabsorbant column of Affi-Gel 10 coupled with sheep anti-(human TIMP-1) IgG followed by gel filtration with Sephacryl S-200. Recombinant TIMP-1 was homogeneous on SDS-PAGE.

Table 1
Inhibition constants (K_i values) of recombinant TIMP-1 and N-TIMP-1 for MMP-1, MMP-2 and full-length and truncated forms of MMP-3

Metalloproteinase	Inhibitor		Ratio (N-TIMP-1/TIMP-1)
	TIMP-1 (K_i, nM)	N-TIMP-1 (K_i, nM)	
MMP-1	0.25 ± 0.03	1.48 ± 0.35	5.9
MMP-2	0.14 ± 0.002	1.11 ± 0.13	7.9
MMP-3	0.24 ± 0.02	1.40 ± 0.02	6.3
MMP-3(ΔC)	0.25 ± 0.04	1.92 ± 0.08	7.8

K_i values are ± S.D.

2.8. Assay of inhibitory activity

The inhibitory activity of recombinant TIMP-1 and N-TIMP-1 preparations were assayed by preincubating with MMP-3 to form proteinase-inhibitor complexes. The residual MMP-3 activity in these samples was assayed using *S*-[^{3}H]carboxymethylated human transferrin as described by Nagase [35].

2.9. CD spectrum of TIMP-1

Near-and far-UV CD spectra of TIMP-1 and recombinant N-TIMP-1 were determined with a JASCO J-710/720 spectropolarimeter. 20 spectra were scanned for each sample at a speed of 100 nm/min which were subsequently averaged and smoothed. Near-UV CD spectra (250–320 nm) were determined using a cell with a path length of 1 cm, and far-UV spectra (200–250 nm) using a cell with a path length of 0.1 cm. Protein samples were dissolved in 20 mM Tris-HCl, pH 7.0, containing 0.2 M NaCl.

2.10. NMR spectra of [^{15}N]N-TIMP-1

NMR spectra of ^{15}N-enriched N-TIMP-1 were acquired using AMX-500 or DRX-500 spectrometers (Bruker Analytische Messtechnik GMBH, Karlsruhe, Germany) using either a 5 mm Bruker inverse detection probe or a 8 mm triple resonance probe (Nalorac Cryogenics Corporation, Martinez, CA).

2.11. Kinetic studies

Synthetic fluorogenic peptide substrates, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 for MMP-3 [36] and Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, for MMP-1 and MMP-2 [37] were used to assay TIMP activity as described previously [36,37]. Assays of the inhibition of each of the above MMPs by both recombinant full-length TIMP-1 and N-TIMP-1 was carried out by preincubating the MMP (0.1 to 1 nM) and TIMP (0–50 nM) in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$ and 0.02% Brij at 37°C for 1 h. The time required for equilibration was determined by following the progress of inhibition after mixing MMPs and TIMP at concentrations in the range used in the assay. An aliquot (60 μl) of substrate (15 μM) was then added to 540 μl of the preincubated MMP-TIMP mixture and activity was measured at 37°C by following product release with time. Inhibited rates were obtained from the initial 20 min of the reaction profile where product release was linear with time. Fluoresence was measured using a Perkin Elmer LS50 fluorimeter. K_i values were calculated using the treatment of data for slow tight-binding inhibitors described by Morrison and Walsh [38]. The values used for the total concentration of TIMP were those of the preincubation mixture. Because of the slow dissociation rate for TIMP from complexes with MMPs, and the low substrate concentration (1.5 μM) relative to the K_m, no attempt was made to correct for the 10% dilution between preincubation and assay or for competition between TIMP and substrate for binding to MMPs.

3. Results

N-TIMP-1 was expressed as inclusion bodies to a level of 20% of the total wet weight of the *E. coli*. After extraction,

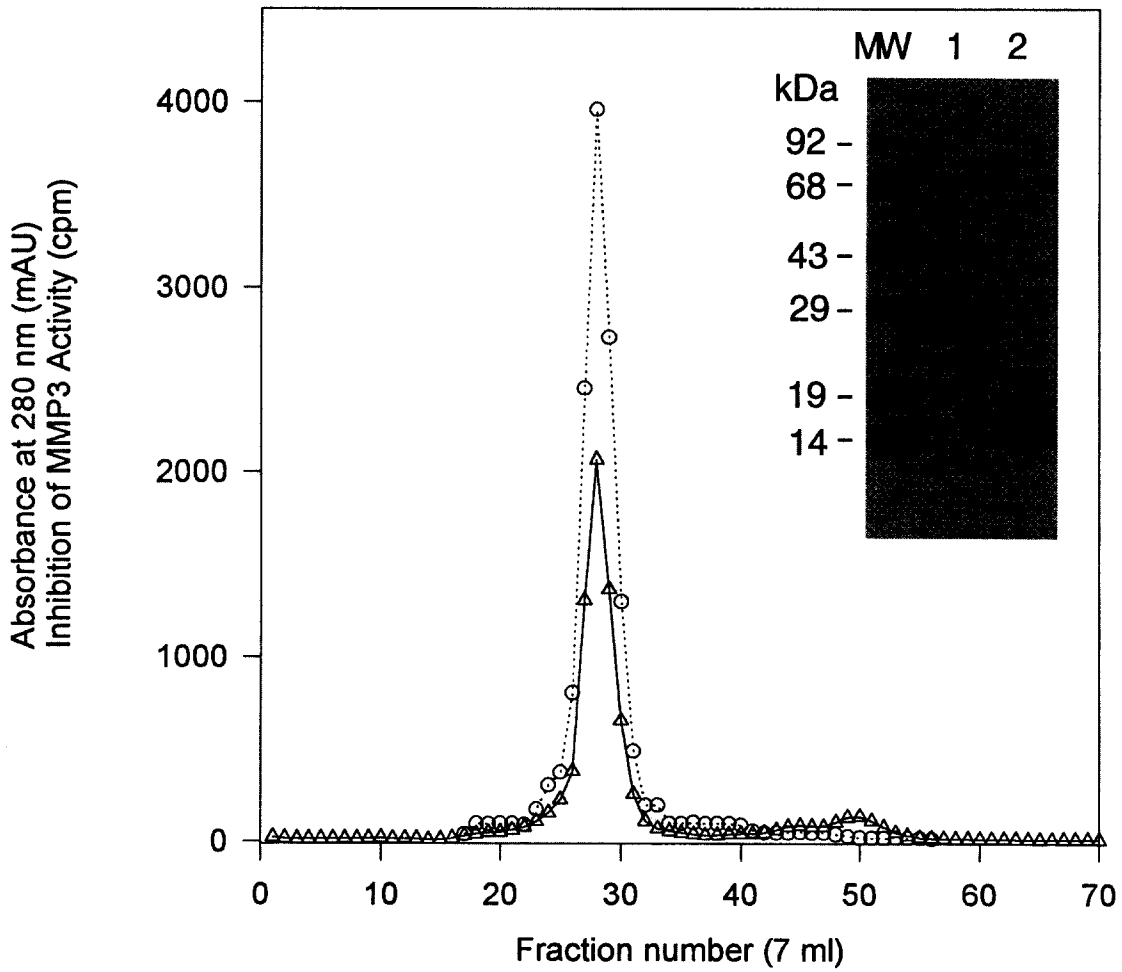

Fig. 1. Purification of folded N-TIMP-1 by cation exchange chromatography with CM-cellulose CM-52. Chromatography was carried out as described in section 2.6. TIMP activity against MMP-3 was assayed as described in Section 2.8. (△—△) Absorbance at 280 nm; (○···○) TIMP activity, which is expressed as [^{3}H]carboxymethylated transferrin (cpm) released by MMP-3 alone-(cpm) released by MMP-3 incubated with N-TIMP-1. The inset shows the results of SDS-PAGE under reducing conditions of recombinant TIMP-1 (lane 1) and N-TIMP-1 (lane 2). The molecular weights of standard molecular weight markers are given on the left.

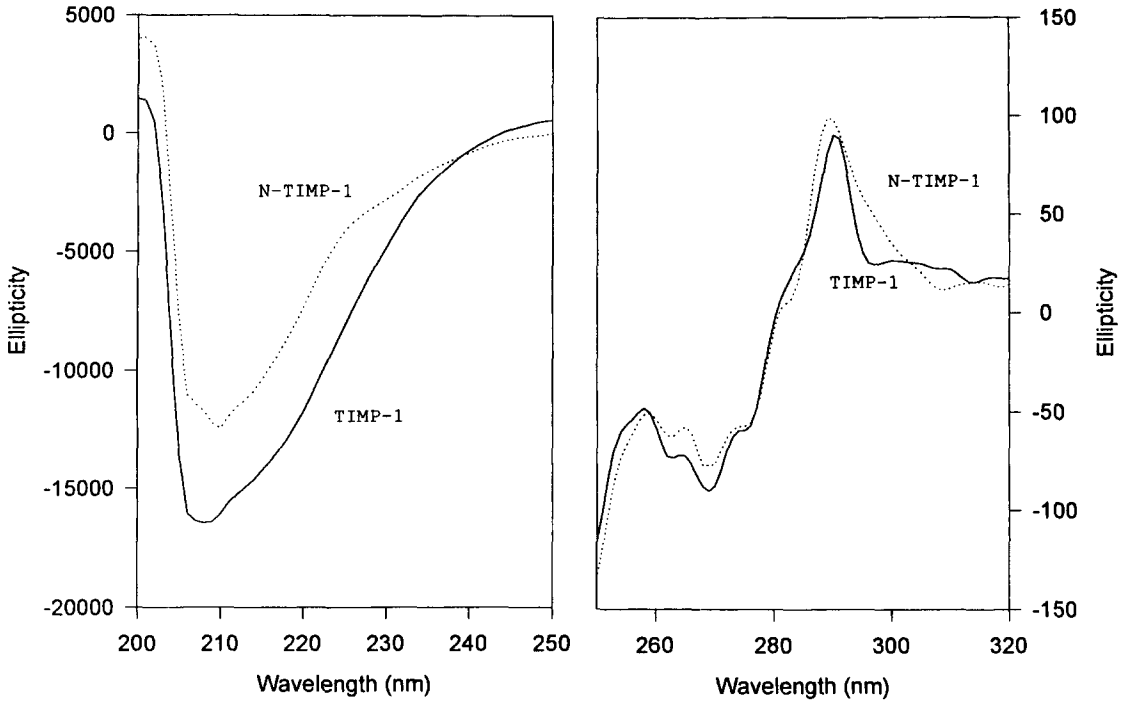

Fig. 2. Near-and far-UV CD spectra of recombinant TIMP-1 and N-TIMP-1. Spectra were collected as described. To facilitate the direct comparison of spectra, ellipticities are expressed as $\theta \times$(number of residues/100) where θ is the mean residue ellipticity in degree cm^2 $dmol^{-1}$.

unfolded N-TIMP-1 was purified by ion exchange chromatography and gel filtration with Sephacryl S-300 in the presence of 0.5 M NaCl. Electrophoresis with agarose gels and SDS-PAGE polyacrylamide gels, respectively, showed that significant amounts of DNA and a mixture of proteins of different sizes were present in the void volume peak, while the second A_{280nm} peak contained a reasonably pure form of unfolded N-TIMP-1 (M_r 14000).

Fractions containing N-TIMP-1 from the Sephacryl S-300 separation were subjected to the dialysis procedure described in section 2 to generate native protein. When this was carried out using protein concentrations >100 μg/ml, a large proportion of the protein precipitated during dialysis; precipitation was greatly reduced by reducing the protein concentration to 50 μg/ml. Folded N-TIMP-1 was subsequently concentrated and purified by chromatography with CM-52 (Fig. 1) to give a single peak in which protein concentration and activity coincided. This material gave a single band of 14 kDa on SDS-PAGE whereas the CHO cell expressed recombinant full-length TIMP-1 was estimated to be 28.5 kDa (Fig. 1). Both proteins were positive on Western blotting with anti-TIMP-1 antiserum (data not shown).

The structural state of the in vitro folded N-TIMP-1 was investigated by CD and NMR spectroscopy. The near-and far-UV CD spectra of N-TIMP-1 are similar, but distinct from those of the full-length TIMP-1 obtained by mammalian expression, both proteins having positive complex spectra in the near-UV range with a maximum around 290 nm and typical negative ellipticities in the far-UV range with minima at 208 nm (Fig. 2).

Recombinant TIMP-1 and N-TIMP-1 showed similar high stabilities to thermal denaturation. After heating solutions of either protein (10 μg/ml) at 95°C for 15 min full activity was restored (>95%) on cooling to room temperature. In contrast, when the unfolding of N-TIMP-1 was monitored by near-UV CD ellipticity thermal unfolding was observed between 45 and 60°C. In this case, unfolding was accompanied by precipitation and was irreversible; neither the near-nor far-UV spectrum was restored on cooling to 30°C after heating to 60°C. This appears likely to reflect the fact that a far higher concentration (400 μg/ml) was used in the CD study which enhances the aggregation of unfolded or partially folded forms of the protein. At pH 2.0 the spectrum is reduced in magnitude but not eliminated indicating a higher level of stability to acid.

NMR spectra determined for ^{15}N-substituted N-TIMP-1 indicate that the protein folded from inclusion bodies is homogeneous in conformation. A homogeneous preparation is expected to have one backbone amide NMR peak for each non-proline residue. At least 92% of the 119 possible backbone amide peaks are reproducibly resolved in the 2D spectra correlating amide protons with the attached ^{15}N nuclei of [^{15}N]N-TIMP-1 by heteronuclear multiple quantum coherence (HMQC) (Fig. 3) and heteronuclear single quantum coherence (HSQC) spectra (data not shown). Furthermore, essentially all of the expected side chain peaks from Asn, Gln and Arg residues (the Arg peaks are not plotted in Fig. 3) can be counted, provided presaturation of the water resonance is avoided. The spectral dispersion, linewidths, and patterns of NOEs among backbone amide and α protons (not shown) suggest that N-TIMP-1 is correctly folded and well suited for high resolution structural studies.

In agreement with these results, titration of MMP-3 (45 nM) with increasing concentrations of N-TIMP-1 (Fig. 4) showed that full inhibition was acheived at a 1.08:1 molar ratio of inhibitor to enzyme, indicating that all molecules in the N-TIMP-1 preparation are active. Equilibrium inhibition constants for the recombinant full-length TIMP-1 and recombinant N-TIMP-1 for MMP-1, MMP-2 and the full-length MMP-3 (45 kDa) and the catalytic domain of MMP-3 (23.5

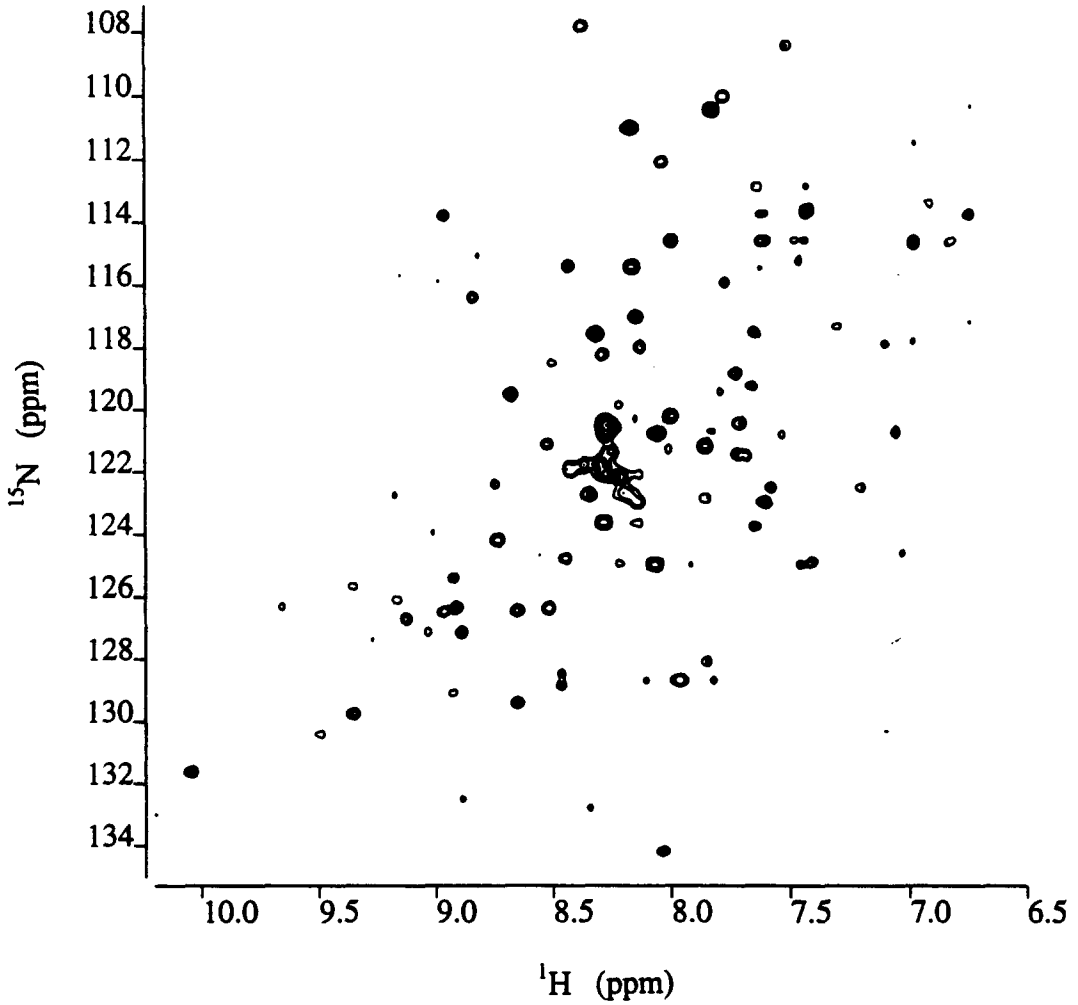

Fig. 3. HMQC spectrum of ^{15}N-enriched N-TIMP-1 at 500 MHz. Protein (0.6 mM) was dissolved in 92% H2O/8% D_2O containing 25 mM deuterated sodium acetate, pH 6.0 and 20 mM $CaCl_2$. Water suppression was achieved using 1-1 excitation and 1-1 refocusing pulses instead of presaturation, as described by Sklenar and Bax [39].

kDa) were determined using quenched fluorescent peptide substrates as described. The results, summarized in Table 1, show that K_i values of N-TIMP-1 for MMP-1, MMP-2 and full-length and truncated forms of MMP-3 are in the nanomolar range but they are 6–8 times higher than those of full length TIMP-1.

4. Discussion

The aim of the present project was to develop a system for the expression and mutational analysis of the N-terminal domain of TIMP-1 which provides sufficient protein for proper physical and structural characterization. Other groups have reported mammalian expression systems for full length and C-terminally truncated forms of TIMP-1 and TIMP-2 [15–17] The expression of full-length TIMP-1 in *E. coli* as inclusion bodies together with procedures for folding and isolation of active protein have been described by others [23–26]. Although the production of active protein in high yield has been described, Cocuzzi et al. [23] reported that their recombinant protein preparations have a high proportion of non-native molecules. This may be also the case with the expression system described by Kleine and co-workers [25] since their *E. coli* expressed recombinant TIMP-1 has a 7-fold lower affinity for neutrophil collagenase (MMP-8) as compared with the natural protein. Although the properties of truncated forms of TIMP-1 expressed in mammalian cells have been described by other investigators [15], a bacterial expression system for N-TIMP-1 was not developed previously.

The procedure described here utilizes the high level expression provided by pET vectors in conjunction with a separation and folding protocol. The method reproducibly provides high yields of native protein (about 20 mg/l) and is more facile in generating a more concentrated solution of folded protein than in methods previously described for full length TIMP-1. An important aspect of the procedure is the partial purification of the protein extracted from inclusion bodies in the denatured state prior to folding, a step that removes anionic materials such as DNA which may associate strongly with the unfolded basic TIMP-1 molecule and stabilize the unfolded state under folding conditions.

CD spectroscopy shows that purified N-TIMP-1 has a spectrum that is similar to but distinct from that of full-length TIMP-1 expressed in a eukaryotic system (Fig. 2). N-TIMP-1 unfolds reversibly when heated to 95°C at low concentrations, but denatures and aggregates at lower temperatures when unfolding was monitored by CD spectroscopy at much higher concentrations. This contrasts with the reversible

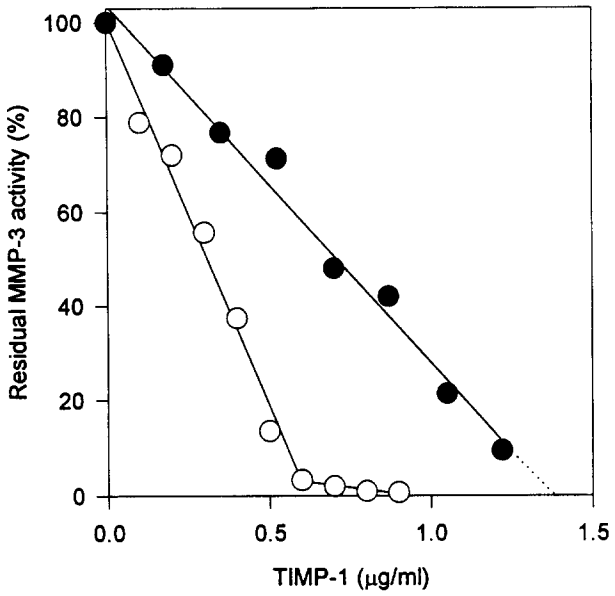

Fig. 4. Titration of MMP-3 with recombinant TIMP-1 and N-TIMP-1. MMP-3 (45 kDa) was incubated with equal volumes of solutions of TIMP-1 solution at different concentrations at 37°C for 30 min. and the residual MMP-3 activity was measured using *S*-[^{3}H]carboxymethylated transferrin. The final concentration of MMP-3 in the assay was 45 nM. (●) Full-length CHO K1 expressed TIMP-1; (○) *E. coli* expressed N-TIMP-1.

nature of the chemical denaturation of mammalian-expressed glycosylated and unglycosylated forms of N-TIMP-1 by guanidine HCl described by Williams and co-workers [17] and indicates that stability measurements of N-TIMP-1 variants are best determined by reversible chemical denaturation rather than by thermal unfolding.

Bacterial expression systems provide an economical means of generating isotopically substituted protein for multinuclear NMR studies, by growth on minimal medium containing appropriate isotopically labeled precursors as the sole source of protein nitrogen and carbon. NMR studies of ^{15}N-enriched N-TIMP-1 generated in this fashion has provided strong evidence that the in vitro folded protein is homogeneous in conformation, as well as being a promising subject for the determination of its solution structure by multinuclear NMR.

Other investigators have reported that N-TIMP-1 appears to have a lower inhibitory activity than full-length TIMP-1 for some metalloproteinases, based on measurements of association rate constants [3,15,16,40], but a direct comparison of K_i values for the two inhibitors with the same MMPs under the same conditions has not been reported. Our results, using recombinant forms of the two proteins show that N-TIMP-1 is lower in affinity for MMP-1, MMP-2 and MMP-3 by factors of 6–8. Although Baragi et al. [41] reported that C-terminal domain-truncated MMP-3 has a reduced affinity for TIMP-1 compared with full-length MMP-3, our data shows that the K_i values of TIMP-1 (and N-TIMP-1) for MMP-3 and MMP-3(ΔC) are closely similar. The lack of involvement of the C-terminal domain of MMP-3 in the interaction with TIMP-1 has also been indicated by the similarity of the rate constants for the association of TIMP-1 with the two forms of MMP-3 [40]. However, our study indicates that the K_i values for N-TIMP-1 for both MMP-3 and MMP-3(ΔC) are 6–8 fold higher than the corresponding parameters for TIMP-1. This suggests that the C-terminal domain of TIMP-1 makes some contribution to the interaction with the catalytic domain of MMP-3. It should be noted, however, that when the K_i values are used to calculate Gibbs free energy changes (ΔG) associated with the TIMP-MMP interactions ($\Delta G = -RT\ln K_i$) the difference between the two-domain full-length TIMP-1 and the single-domain N-TIMP-1 for binding to these three proteases is only 8–9% of the total free energy associated with the TIMP-1-MMP interaction (about 1 out of 13 kcal/mol). Therefore, contacts between these metalloproteinases and the C-terminal domain of TIMP-1 in these cases play only a minor role in stabilizing the complex.

Acknowledgements: This work was supported by grants AR40994 and AR39198 from the National Institutes of Health, USA.

References

[1] Woessner, J.F. Jr. (1991) FASEB J. 5, 2145–2154.
[2] Docherty, A.J.P., O'Connell, J., Crabbe, T., Angal, S. and Murphy, G. (1992) Trends Biotechnol. 10, 200–207.
[3] Murphy, G. and Willenbrock, F. (1994) Methods Enzymol. 248, 496–510.
[4] Hayakawa, T., Yamashita, K., Tanzawa, K., Uchijima, E. and Iwata, K. (1992) FEBS Lett. 298, 29–32.
[5] Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. (1994) J. Cell. Sci. 107, 1373–1379.
[6] Yang, T.I. and Hawkes, A.P. (1992) Proc. Natl. Acad. Sci. USA 89, 10676–10680.
[7] Docherty, A.J.P., Lyons, A., Smith, B.J., Wright, E.M., Stephens, P.E., Harris, T.J.R., Murphy, G. and Reynolds, J.J. (1985) Nature 318, 66–69.
[8] Boone, T.C., Johnson, H.J., DeClerck, Y.A. and Langley, K.E. (1990) Proc. Natl. Acad. Sci. USA 87, 2800–2804.
[9] Pavloff, N., Staskus, P.W., Kishnani, N.S. and Hawkes, S.P. (1992) J. Biol. Chem. 267, 17321–17326.
[10] Silbiger, S.M., Jacobsen, V.L., Cupples, R.L. and Koski, R.A. (1994) Gene 141, 293–297.
[11] Apte, S.S., Mattei, M-G. and Olsen, B.R. (1994) Genomics 19, 86–90.
[12] Williamson, R.A., Marston, F.A.O., Angel, S., Koklitis, P., Panico, M., Morris, H.R., Carne, A.F., Smith, B.J., Harris, T.J.R. and Freedman, R.B. (1990) Biochem. J. 268, 267–274.
[13] Carmichael, D.F., Sommer, A., Thompson, R.C. anderson, D.C., Smith, C.G., Welgus, H.G. and Stricklin, G.P. (1986) Proc. Natl. Acad. Sci. USA 83, 2407–2411.
[14] Apte, S.S., Olsen, B.R. and Murphy, G. (1995) J. Biol. Chem. 270, 14313–14318.
[15] Murphy, G., Houbrechts, A., Cockett, M.I., Williamson, R.A., O'Shea, M. and Docherty, A.J.P. (1991) Biochemistry 30, 8097–8102.
[16] Willenbrock, F., Crabbe, T., Slocombe, P.M., Sutton, C.W., Docherty, A.J.P., Cockett, M.I., O'Shea, M., Brocklehurst, K, Phillips, I.R. and Murphy, G. (1993) Biochemistry 32, 4330–4337.
[17] Williamson, R.A., Bartels, H., Murphy, G. and Freedman, R.B. (1994) Protein Eng. 7, 1035–1040.
[18] Cocuzzi, E.T., Walther, S.E. and Denhardt, D.T. (1994) Inflammation 18, 35–43.
[19] Gomez, D.E., Lindsay, C.K., Cottam, D.W., Nason, A.M. and Thorgeirsson, U.P. (1994) Biochem. Biophys. Res. Commun. 203, 237–243.
[20] Marston, A.O. (1986) Biochem. J. 240, 1–12.
[21] Stricklin, G.P. (1987) Collagen Relat. Res. 6, 219–228.
[22] Tolley, S.P., Davies, G.J., O'Shea, M., Cockett, M.I., Docherty, A.J.P. and Murphy, G. (1993) Proteins: Struct. Funct. Genet. 17, 435–437.
[23] Cocuzzi, E.T., Walther, S.E., Rajan, S. and Denhardt, D.T. (1992) FEBS Lett. 307, 375–378.
[24] Kohno, T., Carmichael, D.F., Sommer, A. and Thompson, R.C. (1990) Methods Enzymol. 185, 187–195.

[25] Kleine, T., Bartsch, S., Bläser, J., Schnierer, S., Triebel, S., Balentin, M., Gote, T. and Tschesche, H. (1993) Biochemistry 32, 14125–14131.
[26] Negro, A., Onisto, M., Masiero, L. and Garbisa, S. (1995) FEBS Lett. 360, 52–56.
[27] Ogata, Y., Itoh, Y. and Nagase, H. (1995) J. Biol. Chem. 270, 18506–19511.
[28] Suzuki, K., Enghild, J.J., Morodomi, T., Salvesen, G. and Nagase, H. (1990) Biochemistry 29, 10261–10270.
[29] Okada, Y., Morodomi, T., Enghild, J.J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G. and Nagase, H. (1990) Eur. J. Biochem. 194, 721–730.
[30] Ito, A. and Nagase, H. (1988) Arch. Biochem. Biophys. 267, 211–216.
[31] Sarkar, G. and Sommer, S.S. (1990) BioTechniques 8, 404–407.
[32] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[33] Grobler, J.A., Wang, M., Pike, A.C.W. and Brew, K. (1994) J. Biol. Chem. 269, 5106–5114.
[34] Cockett, M.I., Bebbington, C.R. and Yarrington, G.T. (1990) BioTechnology 8, 662–667.
[35] Nagase, H. (1995) Methods Enzymol. 248, 449–470.
[36] Nagase, H., Fields, C.G. and Fields, G.B. (1994) J. Biol. Chem. 269, 20952–20957.
[37] Knight, C.G., Willenbrock, F. and Murphy, G. (1992) FEBS Lett. 296, 263–266.
[38] Morrison, J.F and Walsh, C.T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301.
[39] Sklenar, V. and Bax, A. (1987) J. Magn. Reson. 4, 469–479.
[40] Nguyen, Q., Willenbrock, F., Cockett, M.I., O'Shea, M., Docherty, A.J.P. and Murphy, G. (1994) Biochemistry 33, 2089–2095.
[41] Baragi, V.M., Fliszar, C.J., Conroy, M.C., Ye, Q-Z., Shipley, J.M. and Welgus, H.G. (1994) J. Biol. Chem. 269, 12692–12697.