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Expression and purification of mouse TIMP-1 from *E. coli*

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Tissue inhibitors of metalloproteinases (TIMPs) constitute a family of secreted glycoproteins involved in regulating extracellular matrix degradation in both normal and malignant tissues. We have expressed a cDNA clone of mouse TIMP-1 as a 22-kDa protein with 12 cysteine residues in *E. coli* and purified protein that shows inhibitory activity against collagenase following renaturation by chemical means. The low specific activity and circular dichroism measurements suggest, however, that the renaturation of the mouse recombinant (non-glycosylated) protein is not efficient under the conditions we have used, indicative of either thermodynamic instability or the transition to kinetic intermediates which have very low in vitro refolding rates.

Tissue inhibitor of metalloproteinase; Collagenase; Collagen; Protein refolding

1. INTRODUCTION

Matrix metalloproteinases (MMPs) make up the collagenase gene family (which includes interstitial collagenase, MMP-I; stromelysin, MMP-II, and type IV collagenase, MMP-III); they are effective in the degradation and turnover of extracellular matrices, which are composed of collagens, proteoglycans, and glycoproteins such as fibronectin and laminin [1,2]. Activation of the proenzyme forms of the MMPs secreted by connective tissue cells and inhibition of these enzymes by specific inhibitor proteins (TIMP; or tissue inhibitor of metalloproteinase) are two points at which breakdown of connective-tissue matrix may be controlled.

TIMP-1 [3–8] is a 28.5-kDa glycoprotein that forms a 1:1 complex with activated interstitial collagenase [3–5] and occurs ubiquitously among tissues and species (for review see [2]). As with matrix metalloproteinases, a family of TIMPs exists, the two members most vigorously studied being the 28.5-kDa protein (TIMP-1) [3–8] and the 21-kDa protein (TIMP-2) [9,10]. Khokha et al. [11] recently showed that down-regulation of TIMP-1 mRNA levels via antisense RNA resulted in the conversion of immortal, non-tumorigenic and non-invasive Swiss 3T3 cells into invasive, tumorigenic and metastatic cells. These results corroborated previous observations of an inverse correlation between TIMP levels and the invasive potential of both mouse and human tumor cells [12,13] and extended them by revealing for the first time an unexpected role for TIMP as a tumor suppressor.

Although it has been found that the N-terminal domain of TIMP is involved in metalloproteinase inhibitory activity [14], the exact mechanism of TIMP binding and inhibition of MMPs is unknown. To understand how TIMP interacts with the MMPs we have sought to produce TIMP in quantities large enough to study. We chose a host-vector system in which TIMP is made in *E. coli* as an intracellular inclusion body. Since TIMP in this intracellular structure is found as a complex of inactive, aggregated protein, the inactive species must be purified and renatured in vitro. Here, we report the synthesis in *E. coli* of inactive TIMP-1 (a non-glycosylated 22-kDa protein), its purification from inclusion bodies, and its refolding to a form presumably containing 12 disulfide bonds [15].

Abbreviations: IPTG, isopropylthiogalactoside; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; collagenolysis inhibition.

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2. MATERIALS AND METHODS

2.1. *Construction of TIMP-1 expression vector*

In order to express mouse TIMP-1 (henceforth written as mTIMP-1) in *E. coli* we employed pET-3c, a plasmid vector that allows cloning of target DNAs at sites where they will be minimally transcribed by RNA polymerase but selectively and actively transcribed by T7 RNA polymerase in BL21 (DE3) [16]. We fused a mTIMP-1 cDNA (with its own leader sequence eliminated) between the *Nde*I and *Bam*HI sites of pET-3c. This cDNA, derived from mouse embryo fibroblasts [17], was placed under the control of the T7 promoter (ϕ10) of the vector (Fig. 1). The resultant TIMP-1 expression vector, pET-3cT, was transformed into *E. coli* BL21pLysS [18] and selected for ampicillin resistance.

2.2. *Recovery of recombinant mTIMP-1 from inclusion bodies*

E. coli BL21 cells transformed with pET-3cT plasmid were grown at 30°C overnight in shaker flasks in medium containing 10 g of tryptone, 5 g of NaCl, 19 mM NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4,

4 g of glucose, 1 mM $MgSO_4$ per liter (M9.ZB medium) and 75 μg/ml carbenicillin. The culture was induced with 0.4 mM IPTG for 4 h after it reached an OD_{600} of 0.5. The cells were centrifuged at 4,400 × g for 15 min at 4°C, resuspended in 80 ml of lysis buffer (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.3 M NaCl) – per 500 ml of original culture volume – and sonicated (15–20 s pulses at 40% maximum intensity) using a Sonic Dismembrator (Model 300) before being frozen at −70°C for 30 min. After partially thawing the bacterial cell lysate at 37°C, 100 μl of 100 mM phenylmethane-sulphonyl fluoride was added and the mixture allowed to sit on ice for 20 min. Four ml of 10% NP-40 (in lysis buffer) were then added, and the mixture allowed to sit on ice for another 30 min. After brief sonication, the solution was centrifuged at 18,000 × g for 20 min and the pellet resuspended in 20 ml of 10 mM Tris-HCl (pH 8.0)/1 M NaCl. The solution was centrifuged at 20,000 × g for 15 min and the pellet resuspended in 5 ml of solubilization buffer (8 M urea, 50 mM Tris-HCl, pH 7.5, and 0.1 M β-mercaptoethanol). After incubation overnight at 4°C, the insoluble material was removed by centrifugation at 31,000 × g for 20 min.

2.3. *Purification and renaturation of mTIMP-1*

The supernatant from the 31,000 × g spin was applied to a Bio-Rex 70 column equilibrated with 8 M urea, 50 mM Tris-HCl, pH 7.5, and 50 mM β-mercaptoethanol. Samples (120 mg protein) were loaded into a 2.6 × 4.2 cm column and washed extensively. The column was eluted with 0.25 M NaCl in this same buffer. Fractions (2 ml) were collected and assayed for protein content by the Bradford method. Fractions containing protein were then analyzed by electrophoresis on an SDS-20% polyacrylamide gel using the Pharmacia Phast System [19]. Fractions containing TIMP-1 (based on the mobility of proteins in 20% acrylamide 'Phast' gels) were pooled.

A modified procedure of Kohno et al. [20] was used to refold mTIMP-1. β-Mercaptoethanol was added to the mTIMP-1 enriched protein solution to a concentration of 140 mM in 6 M urea, 50 mM Tris-HCl, pH 7.5. The protein was then diluted to 200 μg/ml with the same buffer and cystamine was added to 292 mM. This solution was gently rocked in 50-ml Falcon culture tubes overnight at 4°C to form mixed disulfide derivatives of TIMP. The next day urea was gradually diluted with 50 mM Tris-HCl, pH 9.0 (at about 50 ml/30 min) to 0.3 M at 4°C to initiate refolding. The protein solution (10 μg/ml) was then exposed to air without stirring at 4°C for 24 h to allow reshuffling of mismatched disulfide bonds. Following the overnight incubation, the protein solution was concentrated by ultrafiltration on an Amicon YM10 membrane to about 15% of its volume and then concentrated to its final volume (20-fold overall) in Centricon-10 devices which had been previously treated with 5% Triton X-100. The buffer solution was then exchanged by dialysis to 50 mM Tris-HCl, pH 7.5 (4 × 4 liters). The protein was then examined by SDS-PAGE on the Phast system. Further purification was by reverse phase HPLC; a linear gradient of 0.05% TFA in water to 0.05% TFA in acetonitrile was used to elute the column.

2.4. *Analysis of refolded recombinant mTIMP-1 for activity*

To determine the inhibitory activity of mTIMP-1, rat tail collagen (type I) which was ^3H-labeled by the method of Birkedal-Hansen and Dano [21] was incubated overnight at room temperature with *p*-aminophenylmercuric acetate-activated human collagenase in the presence or absence of mTIMP-1. The extent of cleavage of the α_1 and α_2 subunits of collagen I to the α_1A and α_2A fragments was analyzed by SDS-PAGE of the reaction mixtures using the Phast system, followed by autoradiography and densitometry of the autoradiogram. Percent inhibition of collagen digestion by collagenase was calculated by the following formula:

% inhibition = (% collagen degradation by standard collagenase) − (% degradation in the presence of inhibitor)/(% collagen degradation by standard collagenase)

3. RESULTS

Examination of the bacterial lysates by SDS-polyacrylamide gel electrophoresis for the presence of protein with an apparent molecular weight of about 22 kDa (indicative of mTIMP-1) revealed that *E. coli* BL21 cells transformed with pET-3cT plasmid (Fig. 1) expressed mTIMP-1 protein when induced with IPTG (Fig. 2; Inset: lane 2). Based on several gel scans with an LkB Ultrascan Laser densitometer (data not shown), this *E. coli* strain appeared to produce recombinant mTIMP-1 at about 10–15% of total cell protein.

Partial purification by cationic exchange on Bio-Rex 70 of the urea-solubilized inclusion bodies (Fig. 2) resulted in >90% enrichment of mTIMP-1 protein (Fig. 2; Inset: lane 5). Refolding of this partially purified protein by gradual dilution of the urea (to 0.3 M) allowed more than 40% of the protein to remain soluble in denaturant-free buffer after extensive dialysis. SDS-polyacrylamide gel electrophoresis of this refolded material under reducing conditions showed predominantly one protein band (Fig. 2; Inset: lane 6) indicative of no significant degradation of mTIMP-1 during refolding. A more compact protein structure may account for the anomalous migration (slightly higher mobility) of refolded TIMP-1. Similar banding patterns of mTIMP-1 under both reducing and non-reducing conditions (data not shown) indicated a primarily monomeric protein species.

Final purification of mTIMP-1 was accomplished using reverse-phase HPLC (Fig. 3). The HPLC-purified mTIMP-1 protein (Fig. 3; Inset: lane 3) migrated on SDS-PAGE similarly to human TIMP-1, henceforth written as hTIMP-1 (Fig. 3; Inset: lane 2). This HPLC-purified mTIMP-1 was more than 95% pure as judged by gel scanning (data not shown).

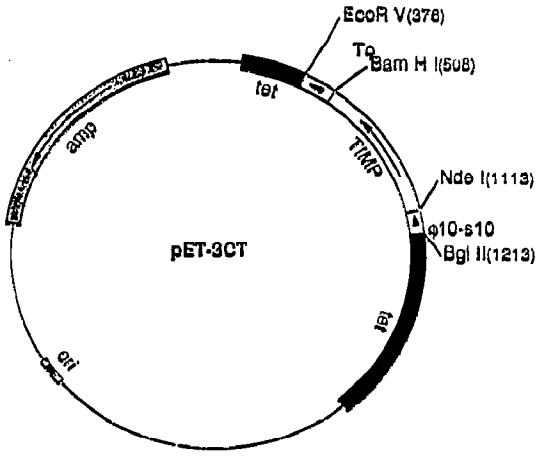

Fig. 1. Structure of the pET-3cT expression vector. The T7 DNA segment of this vector contains the φ10 promoter plus the s10 translation initiation region for the gene 10 protein and the Tφ transcription termination signal. mTIMP-1 is joined directly to the gene 10 initiation codon at the *Nde*I site.

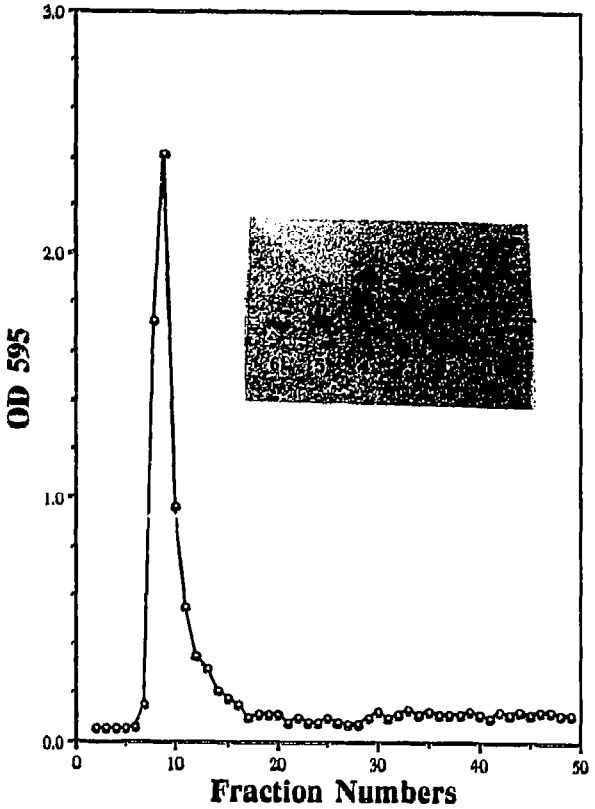

Fig. 2. Cationic-exchange chromatography of recombinant mTIMP-1. Solubilized inclusion body protein was applied to a 2.6 × 4.2 cm Bio-Rex 70 column and eluted with 0.25 M NaCl. Fractions of 2 ml were collected and assayed for protein. Inset: SDS-polyacrylamide gel electrophoresis of mTIMP-1 on a Phast 20% gel. (Lane 1) Uninduced cell lysate of *E. coli* B21(DE3) cells transformed with pET-3cT; (lane 2) cell lysate of transformed cells induced with 0.4 mM IPTG; (lane 3) solubilized inclusion body protein from induced cells; (lane 4) flow-through of solubilized inclusion body protein applied to the cationic exchanger; (lane 5) denatured mTIMP-1 protein eluted from the urea-equilibrated Bio-Rex 70 column with 0.25 M NaCl; (lane 6) soluble (denaturant-free) recombinant mTIMP-1 refolded by dilution. Arrow indicates the position of mTIMP-1.

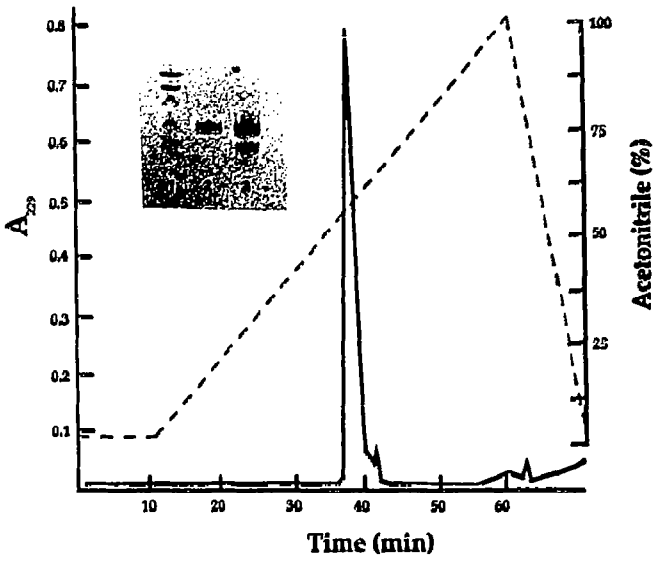

Fig. 3. HPLC of refolded recombinant mTIMP-1. Approximately 0.7 mg of refolded TIMP-1 was applied to a 10 mm × 15 cm semi-prep column of C_{18}-ultrasphere (Beckman) equilibrated in 0.05% TFA. A linear gradient of acetonitrile containing 0.05% trifluoroacetic acid was used to elute the protein. The flow rate was maintained at 1 ml/min, and 2 ml fractions were collected. Absorbance was recorded at 229 nm and peak areas evaluated by a Beckman 427 integrator. Inset: SDS-PAGE of refolded recombinant mouse TIMP-1 after final purification by HPLC. (Lane 1) Low molecular weight markers; (lane 2) hTIMP-1; (lane 3) mTIMP-1 which was purified by HPLC.

Assays to determine the ability of the refolded mTIMP-1 protein to inhibit the degradation of collagen by interstitial collagenase were performed in order to assess the efficiency of renaturation. As indicated in Fig. 4, HPLC-purified, refolded mTIMP-1 possessed inhibitory activity. Although inhibitory activity by mTIMP-1 was not observed with undiluted collagenase (Fig. 4, lane 3), in contrast to hTIMP-1 (Fig. 4, lane 2), inhibition was apparent (Fig. 4, lane 6) when the collagenase was diluted by one half (Fig. 4, lane 4), i.e. under conditions such that the collagen subunits were only partially degraded. On closer examination (Fig. 5), mTIMP-1 displayed a much lower specific activity (for 50% inhibition, a ratio of TIMP-1 to collagenase of about 190/1 was needed) than hTIMP-1 (a ratio of TIMP-1 to collagenase of about 1.4/1 for 50% inhibition).

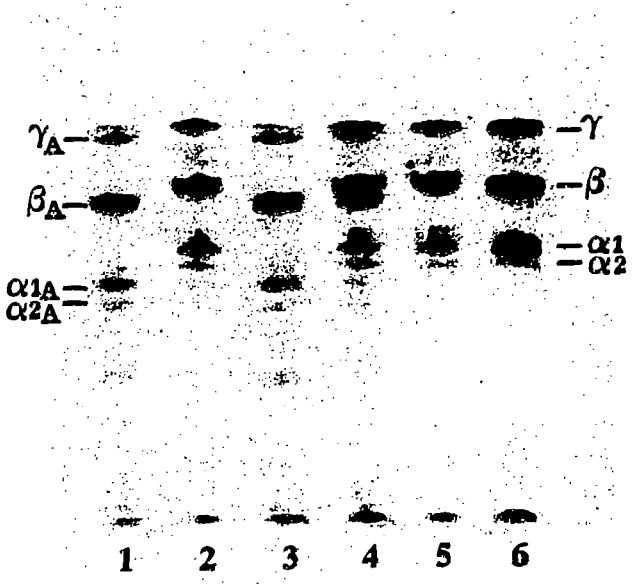

Fig. 4. Analysis by SDS-PAGE of the inhibitory activity of recombinant mouse TIMP-1. One microliter aliquots of HPLC-purified inhibitor were incubated overnight with 0.50 μl of APMA-activated human collagenase in the presence of 5 μl of labeled collagen (15,000 cpm, 2.5 μg) and 5 mM $CaCl_2$ to determine the extent of collagen degradation. Total reaction volumes: 10 μl. (Lane 1) Collagen plus undiluted collagenase; (lane 2) collagen, undiluted collagenase plus hTIMP-1; (lane 3) collagen, undiluted collagenase plus mTIMP-1; (lane 4) collagen plus 1:1 diluted collagenase; (lane 5) collagen, 1:1 diluted collagenase plus hTIMP-1; (lane 6) collagen, 1:1 diluted collagenase plus mTIMP- 1.

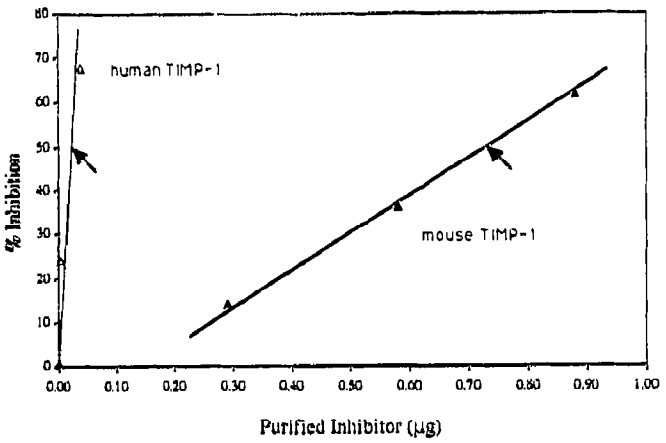

Fig. 5. Assessment of the specific activity of purified mTIMP-1. Increasing amounts of hTIMP-1 or mTIMP-1 were incubated overnight with either 1.3×10^{-3} nmol (in the case of hTIMP-1) or 1.7×10^{-4} nmol (in the case of mTIMP-1) of human collagenase, which was rate-limiting here, and then assayed for collagenolysis inhibition. The tailed arrows indicate the amount of TIMP-1 protein needed to achieve 50% inhibition of collagen degradation by collagenase.

4. DISCUSSION

The overall yield of active mTIMP-1 obtained by our method represents about 0.5%. Far ultraviolet circular dichroic spectra of refolded, HPLC-purified mTIMP-1 at different pHs [19] revealed that at the physiological pH range (pH 6.5–7.5) a pH-induced conformational change occurred (data not shown) in the recombinant protein as reflected by a large increase in random coil secondary structure. Since native, glycosylated mTIMP-1 is normally stable at physiological pH, the conformational change observed here suggests that although some of the protein can refold properly, most of it is in an inactive, relatively unstable, state.

The fact that recombinant hTIMP-1 is apparently more efficiently refolded [20] than recombinant mTIMP-1 (mTIMP-1 is about 30% non-homologous at the amino acid level with respect to hTIMP-1 [21]) might mean that it contains an amino acid sequence that allows more thermodynamically favorable or stable conformations to occur. Alternatively, the low specific activity of recombinant mTIMP-1 may reflect the accumulation of intermediate conformations with mismatched disulfides that temporally stabilize structures that are not at the global minimum energy level and whose rates of rearrangement to both the correct number and types of disulfide bonds normally found associated with the final native product are extremely low. The conformers accumulating here can be viewed as structures akin to kinetic folding mutants of TIMP-1.

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