Preparation of Active Recombinant TIMP-1 from *Escherichia coli* Inclusion Bodies and Complex Formation with the Recombinant Catalytic Domain of PMNL–Collagenase†

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ABSTRACT: TIMP-1 is a member of the family of tissue inhibitors of metalloproteinases involved in regulating the activity of extracellular matrix degrading metalloproteinases. The TIMP-1 cDNA was obtained by reverse transcription–polymerase chain reaction (RT–PCR) amplification of the corresponding mRNA from human fibroblasts. Cloning and expression of the TIMP-1 cDNA were performed in *Escherichia coli*. In the host vector system chosen, rTIMP-1 is stored intracellularly in its denatured, insoluble form in inclusion bodies. We report a new method for the purification and renaturation of rTIMP-1 from *E. coli* inclusion bodies to an active inhibitor of matrix metalloproteinases (80% yield), presumably containing the correct assignment of the six disulfide bonds. A resin with the covalently bound recombinant catalytic domain of the PMNL–collagenase as the affinity ligand provided an effective means for the separation of correctly folded, active rTIMP-1 from inactive forms with mismatched disulfides. TIMP-1 and TIMP-2, the two most extensively examined members of the family of tissue inhibitors of metalloproteinases, are known to form a complex with the activated forms of most matrix metalloproteinases and the latent forms of the 92-kDa and 72-kDa gelatinases, respectively. In this study, we report on the complex formation of the recombinant catalytic domain of the PMNL–collagenase with TIMP-1, nonglycosylated recombinant TIMP-1, and recombinant TIMP-2. The K_i values for the different inhibitors were determined in a kinetic assay using a fluorogenic substrate peptide. In this assay, rTIMP-2 had a more effective inhibitory capability against the recombinant catalytic domain of the PMNL–collagenase than TIMP-1. As for the PMNL–collagenase, the N-terminal catalytic domain is sufficient for enzyme–inhibitor interaction and binding.

The matrix metalloproteinases (MMPs[1]) are a family of zinc-dependent endopeptidases with proteolytic activities toward several components of the extracellular matrix. As MMPs are the key elements in extracellular matrix degradation, they have been implicated in both the remodeling processes associated with mammalian growth and development and the pathological conditions such as rheumatoid arthritis and cancer (Matrisian, 1992). So far, nine members of the MMP family have been identified. All share an amino-terminal propeptide domain (maintaining latency), a zinc binding catalytic domain, and a C-terminal hemopexin-like domain which, in the case of PMNL–collagenase, is important for substrate specificity (Schnierer et al., 1993; Knäuper et al., 1993). Both of the gelatinases have an additional fibronectin-like domain, which is responsible for the association of denatured collagen (gelatin) (Banay et al., 1991; Collier et al., 1992), and the 92-kDa gelatinase has a unique α_2 (V) collagen-like domain. The catalytic domain of the MMPs is sufficient for proteolytic activity. This is true for matrilysin, whose active form consists only of the catalytic domain (Quantin et al., 1989; Woessner & Taplin, 1988; Crabbe et al., 1992) for recombinant C-terminally-truncated stromelysin (Marcy et al., 1991) and for the recombinant and native catalytic domains of PMNL–collagenase (Schnierer et al., 1993; Knäuper et al., 1993).

The enzymatic activity of the MMPs is inhibited by the family of tissue inhibitors of metalloproteinases (TIMPs) which is specific for these enzymes. Of the TIMPs characterized so far, two have related primary structures and inhibitory properties: TIMP-1, a 30-kDa glycoprotein (Docherty et al., 1985), and TIMP-2, a 23-kDa nonglycosylated protein (Stetler-Stevenson et al., 1989). Both interact with the activated MMPs, forming a 1:1 stoichiometric complex. Site-directed mutagenesis experiments on TIMP-1 revealed that the anchored sequence between Cys3 and Cys13, containing residues His7 and Gln9, is an important primary structural feature governing the interaction of the TIMP-1 molecule with the catalytic domain of matrilysin (O'Shea et al., 1992). Recently, Willenbrock et al. (1993) proposed a model for the 72-kDa gelatinase/TIMP-2 interaction in which both the C-terminal and N-terminal domains of the enzyme are involved. The binding sites on the collagenases are not known, and the actual mechanism of inhibition of MMPs by TIMP is not yet entirely understood.

In this study, we describe the cloning and expression of the TIMP-1 cDNA in *E. coli*. Purification and effective refolding of recombinant TIMP-1 lead to a homogeneous, biologically active inhibitor of metalloproteinases with an 80% yield. Furthermore, we have investigated the complex formation of the recombinant catalytic domain of the PMNL–collagenase

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid); PMNL, polymorphonuclear leukocytes; rcd-PMNL-c, recombinant catalytic domain PMNL–collagenase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; RT–PCR, reverse transcription–polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol.

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(rcd-PMNL-c) with native TIMP-1, recombinant TIMP-1 (rTIMP-1), and recombinant TIMP-2 (rTIMP-2). Kinetic studies reveal that rTIMP-2 is a more potent inhibitor for rcd-PMNL-c than TIMP-1.

MATERIALS AND METHODS

Materials

Recombinant TIMP-2 (rTIMP-2) was a gift from Dr. Yves A. DeClerck (Children's Hospital of Los Angeles) and Dr. Keith Langley (Amgen Center, Thousand Oaks, CA). Oligonucleotides were synthesized on a DNA synthesizer from LKB Pharmacia and purified using Nensorb Prep columns (NEN Research Products, Wilmington, DE). The synthetic substrates DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH and DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 were from Bachem. Reverse transcriptase, Bacto tryptone, and yeast extract were obtained from Gibco BRL. Taq DNA polymerase was purchased from Perkin-Elmer Cetus. Divicell ONB-carbonate was obtained from Eurochrom, and the chromatography materials HiLoad 16/60 Superdex 75 and Sephacryl S-200 were purchased from Pharmacia.

Methods

RT–PCR and Cloning of TIMP-1 cDNA. Human fibroblast RNA was purified according to the method of Chirgwin et al. (1979) and reverse-transcribed with BRL reverse transcriptase. After reverse transcription, PCR was performed and the TIMP-1 cDNA was selectively amplified using two oligonucleotide primers: "timpstart" 5′-C AGC CAT ATG TGC ACC TGT GTC CCA CC-3′ and "timpend", 5′-C GGG GGA TCC TCA GGC TAT CTG GGA CC-3′. Timpstart incorporates sequences for a unique *Nde*I site and an initiating methionine. Timpend introduces sequences for a stop codon and a unique *Bam*HI site. For amplification of the TIMP-1 cDNA, 28 cycles of PCR (94 °C, 75 s; 57 °C, 75 s; 71 °C, 90 s) were carried out using Taq DNA polymerase. The amplified 574-bp fragment was treated with Klenow DNA polymerase, digested with *Nde*I and *Bam*HI, and ligated into the *Nde*I and *Bam*HI sites of the T7 expression vector pET 11a (Studier et al., 1990).

Sequencing of TIMP-1 cDNA. Sequencing of the TIMP-1 cDNA in the plasmid pET-11a-TIMP-1 was performed according to the dideoxy termination method (Sanger et al., 1977) and showed the complete and correct coding sequence of TIMP-1.

Expression of rTIMP-1. For expression of rTIMP-1, the *E. coli* strain HMS 174 [DE3] was transformed with pET-11a-TIMP-1 to allow T7 RNA polymerase mediated transcription of the cloned TIMP-1 cDNA. A culture of HMS 174 [DE3] [pET-11a-TIMP-1] was grown overnight at 37 °C in medium containing 10 g of Bacto tryptone, 5 g of yeast extract, and 10 g of NaCl per liter and 100 μg/mL ampicillin. Two liters of the same medium were inoculated with 20 mL of the overnight culture at 37 °C. When a cell density corresponding to an OD_{578} of 0.6 was reached, IPTG was added to a final concentration of 0.4 mM, and the incubation was continued for another 4 h at 37 °C to maximize the production of rTIMP-1.

Recovery of rTIMP-1 from Inclusion Bodies. The cells were pelleted at 5000*g* for 10 min at 4 °C, resuspended in 100 mL of lysis buffer (100 mM Tris-HCl (pH 7.5), 20 mM EDTA, and 2 mg/mL lysozyme), and incubated for 15 min at room temperature. Next, 2.9 g of NaCl and 1 mL of Triton X-100 were added, and the mixture was stored on ice for another 10 min. This lysate was centrifuged for 10 min at 10000*g*, and the resulting pellet was suspended in 50 mM Tris-HCl (pH 7.5) and 6 M urea for washing. After centrifugation of this suspension at 10000*g* for 10 min, solubilization of the resulting pellet was achieved in a buffer containing 50 mM Tris-HCl (pH 8.5), 8 M urea, 1 mM EDTA, and 200 mM β-mercaptoethanol. This mixture was stirred for 2 h at room temperature, and insoluble material was removed by centrifugation at 12000*g* for 25 min. Acetic acid was added dropwise to the supernatant until a pH of 6.2 was reached. This solution (crude extract) was immediately frozen and stored at −20 °C until further purification.

Purification and Refolding of rTIMP-1. A 15-mL aliquot of the frozen crude extract was thawed and loaded onto a Sephacryl S-200 column (1000 mL) previously equilibrated with equilibration buffer containing 20 mM Tris-HCl (pH 6.9), 4 M urea, 10 mM DTE, 200 mM NaCl, 1 mM EDTA, and 0.005% Triton X-100. Equilibration and elution were performed at a flow rate of 7.5 mL/h. Fractions were assayed for rTIMP-1 by Western immunoblotting of SDS–polyacrylamide gels, as previously described (Bläser et al., 1991), and those containing rTIMP-1 were pooled and dialyzed against reduction buffer (100 mM Tris-HCl (pH 7.5), 150 mM DTE, and 4 M urea). Protein content was determined in the dialysate by the Bradford method (Bradford, 1976). Oxidation buffer (100 mM Tris-HCl (pH 7.5), 80 mM oxidized glutathione, and 4 M urea) was added to this dialysate, so that a final rTIMP-1 concentration of 20 μg/mL was reached. To reduce the concentration of the denaturing and reducing reagents and to initiate refolding, this mixture was diluted at 4 °C with dilution buffer (100 mM Tris-HCl (pH 8.7), 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Triton X-100) to a final concentration 0.3 M urea. This dilution step was performed by adding 6 mL/h dilution buffer while gently stirring, thus allowing reshuffling of the mismatched disulfide bonds. This reaction mixture was then concentrated 15-fold in an Amicon ultrafiltration device equipped with a YM 5 membrane. After concentration, the buffer was exchanged against TIMP–buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, and 0.005% Triton X-100) by dialysis. The inhibitory activity of rTIMP-1 was analyzed by a modified method of Masui et al. (1977), and the concentration of rTIMP-1 was measured by TIMP-1 ELISA.

rcd-PMNL-c Affinity Resin Purification of rTIMP-1. rTIMP-1 was purified by using rcd-PMNL-c linked to bead cellulose. The affinity resin was prepared by coupling 6 mg of rcd-PMNL-c to 5 mL of Divicell ONB-carbonate (8.2 mmol of active groups/mL). A column (7 × 1 cm i.d.) was filled with this material and equilibrated with a buffer containing 20 mM Tris-HCl and 50 mM NaCl, pH 7.5. rTIMP-1 in equilibration buffer was loaded onto the column, and nonadsorbed proteins were removed by washing with the same buffer at a flow rate of 2 mL/h. rTIMP-1 was eluted with equilibration buffer containing 0.2% SDS.

Recombinant Catalytic Domain of PMNL–Collagenase (rcd-PMNL-c). As reported, rcd-PMNL-c was obtained after activation of the recombinant C-terminally-truncated form of PMNL–collagenase with $HgCl_2$. Purification of rcd-PMNL-c to homogeneity was achieved by hydroxamate affinity chromatography (Schnierer et al., 1993), and activity was determined by proteolytic degradation of the synthetic octapeptide (Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH) as described by Masui et al. (1977).

Isolation and Purification of Native TIMP-1 from Human PMN Leukocytes. Free PMNL–progelatinase and the com-

plex of progelatinase TIMP-1 were purified simultaneously from buffy coat as recently published for the isolation of PMNL–progelatinase (Tschesche et al., 1992). The free enzyme and the complex coeluted from the gelatin Sepharose column (Sopata et al., 1982) at a DMSO concentration of 1% in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3. Free TIMP-1 was obtained from the complex by SDS gel filtration (Wilhelm et al., 1989).

Complex Formation and Purification of rcd-PMNL-c/rTIMP-2. A HiLoad Superdex gel permeation chromatography column was equilibrated in a buffer containing 20 mM Tris, 5 mM $CaCl_2$, 0,5 mM $ZnCl_2$, and 200 mM NaCl (pH 7.5). A sample of 400 μL of rcd-PMNL-c in equilibration buffer was prepared (0.35 mg/mL), loaded onto the column, and chromatographed at a flow rate of 0.08 mL/min. After this run, a 400-μL sample of rTIMP-2 in the same buffer (0.44 mg/mL) was loaded onto the column and chromatographed under the same conditions. Formation of the complex of rcd-PMNL-c and rTIMP-2 was achieved by mixing the samples described above and incubating them for 3 h at 37 °C. The subsequent mixture was loaded onto the column and chromatographed under the same conditions used for the free enzyme and the free inhibitor.

rcd-PMNL-c/TIMP-1 Sandwich ELISA. Microtitration plates were coated with 100 μL of polyclonal rabbit anti-TIMP-1-IgG at a concentration of 2 mg/L in coating buffer (50 mM sodium carbonate, pH 9.6) at 4 °C overnight. After washing twice with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Tween 20), samples were diluted in BSA/TBST (1% bovine serum albumin in TBST) and incubated overnight at 4 °C. After four washes with TBST, 100 μL of the conjugate solution (polyclonal anti-collagenase-IgG, 1:2000 in BSA/TBST) (Tijssen et al., 1984) was subjected to the wells and incubated for another 2 h at room temperature. After six washes with TBST, the substrate reaction was performed by incubation with ABTS solution (1 g/L in 0.1 M citric acid, adjusted to pH 4.2 with Na_2HPO_4, 0.5 g/L Tween 20, and 1.3 mM H_2O_2). The absorption values were determined at 405 nm using a Dynatech Microreader MR 4000 (Denkendorf, FRG).

Kinetic Experiments. rcd-PMNL-c was assayed using the synthetic fluorogenic substrate DNP-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (Stack & Gray, 1989). Experiments were performed at 37 °C in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, and 5 mM $CaCl_2$. Hydrolysis of the substrate was followed continuously by the increase in fluorescence, which was measured with a Hitachi F-4010 fluorescence spectrophotometer at λ_{ex} = 280 nm and λ_{em} = 345 nm. rcd-PMNL-c and TIMPs were preincubated for 10 min at 37 °C, and reactions were started by addition of the substrate. The initial linear rates of peptide hydrolysis by rcd-PMNL-c were determined in the presence of increasing amounts of TIMPs at substrate concentrations of 5, 10, and 15 μM. The resulting data were used to construct Dixon plots, from which K_i values were calculated.

RESULTS

E. coli Expression of rTIMP-1. E. coli HMS 174 [DE3] cells contain a genomic copy of the T7 RNA polymerase gene under the control of the *lac* promotor. Addition of IPTG to a culture of HMS 174 [DE3] transformed with the constructed pET-11a-TIMP-1 expression plasmid induces the production of T7 RNA polymerase and the expression of rTIMP-1. The expression of rTIMP-1 under the control of T7 RNA polymerase was efficient. On the basis of several gel scans with an LKB Ultrascan laser densitometer, HMS 174 [DE3] [pET-11a-TIMP-1] appeared to produce rTIMP-1 at about 15% total cell protein.

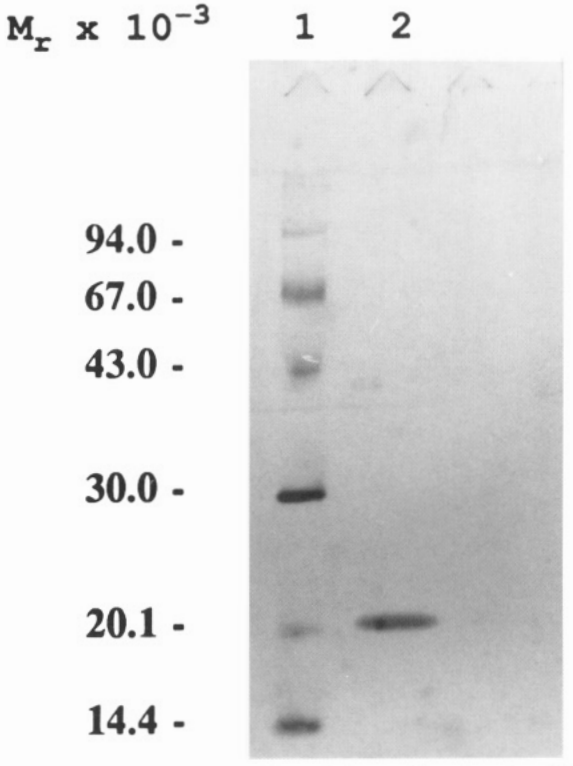

FIGURE 1: SDS–polyacrylamide gel electrophoresis of purified, refolded rTIMP-1 after affinity chromatography on a resin with covalently bound rcd-PMNL-c: lane 1, molecular weight markers; lane 2, rTIMP-1 (0.3 μg). The homogeneity of active rTIMP-1 is documented after silver staining.

Purification and Refolding of rTIMP-1. rTIMP-1 is generated in *E. coli* in inclusion bodies as an insoluble, denatured, nonglycosylated protein with an apparent molecular weight of 20 000. The inclusion body fraction was solubilized in 8 M urea. Gel permeation chromatography of the solubilized inclusion bodies effectively eliminated contaminating *E. coli* proteins and resulted in 85% enrichment of rTIMP-1. Refolding of the purified rTIMP-1 to the biologically active inhibitor was achieved by slowly diluting the denaturing and reducing reagents from the protein mixture. Dilution was carried out in the presence of reductants and oxidants over 60 h. It was essential for the success of the refolding procedure that during the long-term dilution process the amount of oxidized glutathione was slowly increased. Additional essential parameters for effective refolding of rTIMP-1 are a protein concentration of 20 μg/mL and a concentration of the reductant DTE of 150 mM before dilution with the buffer containing 80 mM oxidized glutathione is started. Under these conditions, the recombinant protein remained soluble in denaturant-free buffer. Purification of the correctly folded rTIMP-1 was finally achieved by the highly effective affinity chromatography with rcd-PMNL-c covalently bound to agarose. Elution of rTIMP-1 from this affinity resin was accomplished in a buffer containing SDS. Purification to homogeneity was revealed by silver staining of an SDS–PAGE (Figure 1), and the identity of rTIMP-1 was shown by Western immunoblotting with a specific anti-TIMP-1 antiserum (Figure 2). The refolding yield was 80%, and the inhibitory activity of refolded rTIMP-1 was determined by inhibition of the proteolytic degradation of a synthetic octapeptide by PMNL–collagenase and rcd-PMNL-c.

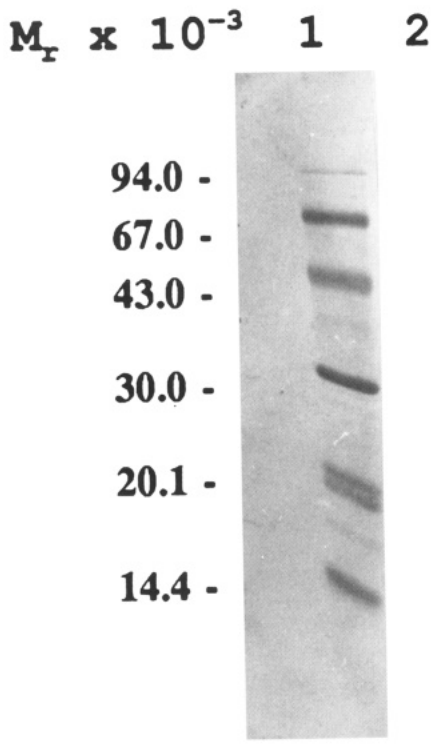

FIGURE 2: Western blot of the purified active rTIMP-1 with specific anti-TIMP-1 IgG after SDS gel electrophoresis: lane 1, molecular weight markers; lane 2, rTIMP-1 (0.6 μg).

rcd-PMNL-c/rTIMP-2 Complex. The catalytic domains of PMNL–collagenase (rcd-PMNL-c) and rTIMP-2 were chromatographed, one after the other, on a HiLoad Superdex column, and the retention times for both were determined. A mixture of rcd-PMNL-c and excessive amounts of rTIMP-2 was preincubated and analyzed by gel permeation chromatography on the same column under the same conditions used for the free enzyme and the free inhibitor. The elution profile of the chromatogram indicated complete separation of two components: one component was eluted with a retention time identical to that of rTIMP-2, and the other component was a new, larger molecular species which traversed the column more rapidly (Figure 3). The two fractions were analyzed by SDS–PAGE and identified as rTIMP-2 and the complex of rcd-PMNL-c and rTIMP-2, which was dissociated in the presence of SDS (Figure 4). The complex no longer had any proteolytic activity toward synthetic collagenase substrates. After 4 months' storage at 4 °C, no degradation of either component of the complex could be observed in SDS–PAGE after silver staining.

PMNL–Collagenase/TIMP-1 ELISA. Further proof that the catalytic domain of PMNL–collagenase is sufficient for complex formation with TIMPs was gained by the use of a newly developed collagenase/TIMP-1 complex sandwich ELISA (Figure 5). During this procedure, a polyclonal anti-TIMP-1 antibody was used to capture the complex and an enzyme-linked polyclonal anti-collagenase antibody for detection. There was no ELISA reaction when the components were subjected individually to the test system. Preincubation of TIMP-1 and rTIMP-1 with rcd-PMNL-c resulted in a clear reaction, indicating a stable complex between rcd-PMNL-c/TIMP-1 and rcd-PMNL-c/rTIMP-1.

Thus, TIMP-1, rTIMP-1, and rTIMP-2 were able to complex with rcd-PMNL-c. Activity of the catalytic domain against synthetic peptide substrates was, in all cases, completely abolished by each of the inhibitors.

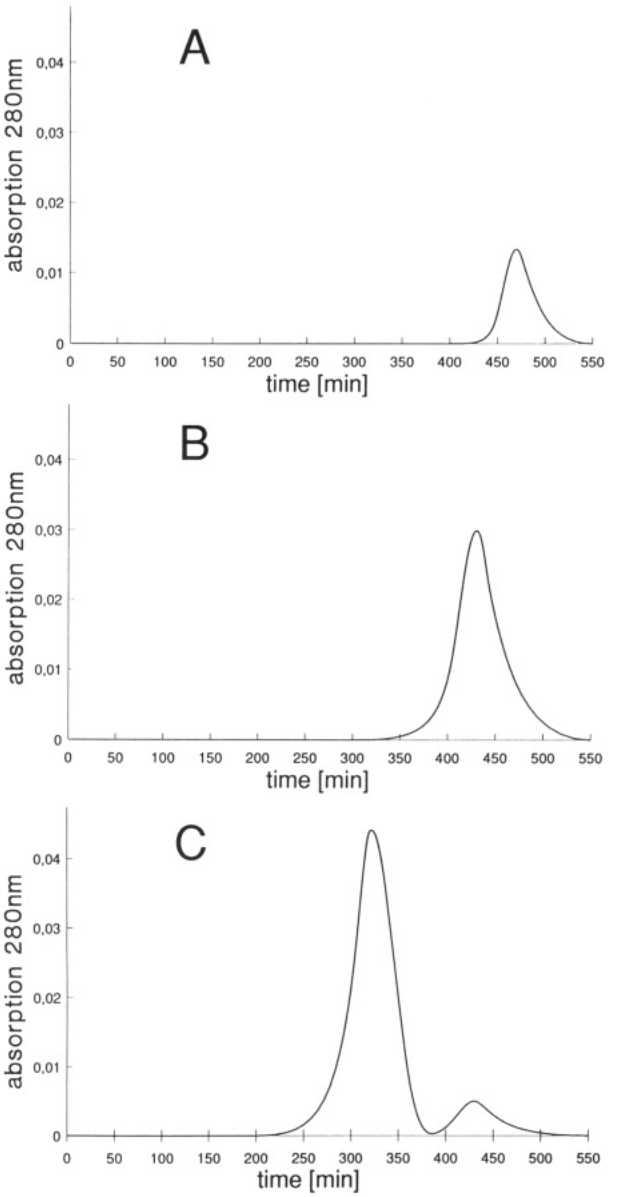

FIGURE 3: Gel permeation chromatography of the enzyme, the inhibitor, and their complex, demonstrating the formation and purification of the rcd-PMNL-c/rTIMP-2 complex. Pure rcd-PMNL-c (A), pure rTIMP-2 (B), or a mixture of the two with excessive amounts of rTIMP-2 (C) were chromatographed on HiLoad Superdex (for details, see Methods). The mixture showed a peak at a retention time distinct from that for either rcd-PMNL-c or rTIMP-2, corresponding to the enzyme–inhibitor complex.

Inhibition Kinetics. Analysis of the inhibition of rcd-PMNL-c by TIMP-1, rTIMP-1, and rTIMP-2 was performed in a continuous fluorometric assay with a quenched fluorescent peptide substrate. The addition of increasing amounts of the inhibitors to a constant amount of rcd-PMNL-c resulted in a decrease in the initial linear rates of substrate hydrolysis. These experiments were carried out at three different substrate concentrations, and K_i values were determined from Dixon plots (Figure 6). In this assay, TIMP-1 with a K_i of 2.8×10^{-9} M and rTIMP-1 with a K_i of 18×10^{-9} M proved to be weaker inhibitors for rcd-PMNL-c than rTIMP-2, which has a K_i value of 5.5×10^{-10} M. As both TIMPs are potent inhibitors of rcd-PMNL-c, these kinetic studies give further evidence that the C-terminal hemopexin-like domain of the PMNL–collagenase is not essential for inhibitor binding by this enzyme.

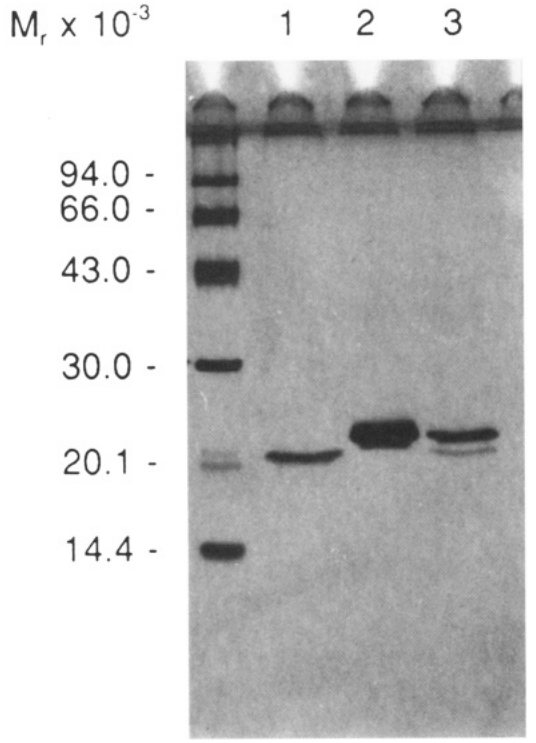

FIGURE 4: SDS–polyacrylamide gel electrophoresis of the fractions corresponding to the peaks of the chromatograms in Figure 3. Proteins were visualized by silver staining: lane 1, rcd-PMNL-c (0.4 μg); lane 2, rTIMP-2 (0.25 μg); lane 3, complex of rcd-PMNL-c and rTIMP-2 (0.2 μg of the complex) which is dissociated in the presence of SDS. That the amount of rTIMP-2 seems to exceed the amount of rcd-PMNL-c is due to the fact that rTIMP-2 is stained much better than rcd-PMNL-c.

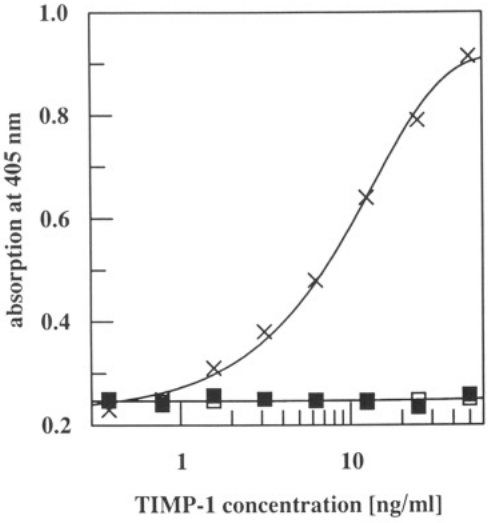

FIGURE 5: TIMP-1 (■), rcd-PMNL-c (□), and the rcd-PMNL-c/TIMP-1 complex (×) were subjected to sandwich ELISA in equal concentrations (concentration of TIMP-1 given in the graph). All samples were serially diluted in TBST.

DISCUSSION

Proteins of eukaryotic organisms expressed in *E. coli* are often generated as inactive, insoluble aggregates known as inclusion bodies and therefore require *in vitro* refolding. The purification and refolding of rTIMP-1 from *E. coli* inclusion bodies to a biologically active protein is somewhat difficult as TIMP-1 is a highly disulfide-bonded protein containing 12 cysteines, all of which are apparently engaged in intrachain disulfide linkages (Stricklin et al., 1983). Former results of

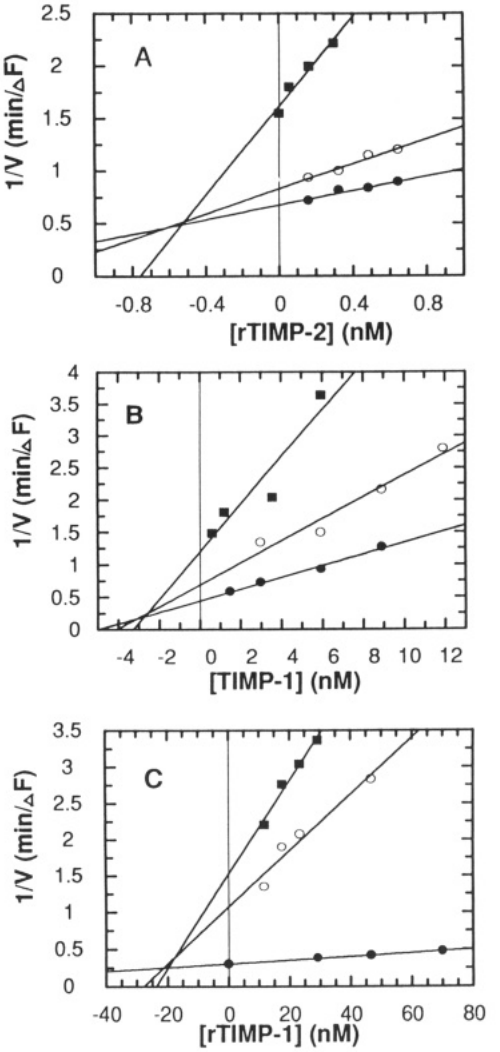

FIGURE 6: Dixon plots for the inhibition of rcd-PMNL-c by rTIMP-2 (A), TIMP-1 (B), and rTIMP-1 (C). Initial rates of peptide hydrolysis expressed in arbitrary units were determined by the fluorescence assay under the conditions described in the Methods section. The peptide concentrations were as follows: (■) 5 μM; (○) 10 μM; (●) 15 μM.

Kohno et al. (1990) on the refolding of rTIMP, also recovered in denatured form from *E. coli* inclusion bodies, could not be reproduced in our laboratory. This may be due to the fact that the rTIMP-1 described by Kohno et al. has a 23 amino acid N-terminal extension. It may be possible that this N-terminal extension, signaling a secretory destination, is lost upon expression, but this is not stated in the report by Kohno et al. (1990).

In vitro refolding of the denatured rTIMP-1 to a biologically active inhibitor was achieved by slowly reducing the concentration of the denaturing and reducing reagents upon shifting the relation of oxidants and reductants. Formation of insoluble aggregates during the refolding procedure was avoided by very low rTIMP-1 concentrations (20 μg/mL). The overall yield of purified, active rTIMP-1 obtained by this method is about 10 mg/L of *E. coli* cell suspension. Very low protein concentrations during refolding are extremely important. Doubling the rTIMP-1 concentration to 40 μg/mL causes the formation of insoluble aggregates and drastically reduces the recovery of active rTIMP-1. rcd-PMNL-c covalently bound to agarose provided an effective means for the rapid purification of biologically active rTIMP-1. With this affinity resin, correctly folded rTIMP-1 could be separated from

Table I: Comparison of Approximate K_i Values of MMP-8, the MMP-8 M_r 40 000 Fragment, and rcd-PMNL-c with rTIMP-2, TIMP-1, and rTIMP-1[a]

enzyme	K_i (M)		
	rTIMP-2	TIMP-1	rTIMP-1
MMP-8	6.0×10^{-10}	4.7×10^{-9}	
MMP-8 M_r 40 000 fragment	1.5×10^{-10}	4.0×10^{-9}	
rcd-PMNL-c	5.5×10^{-10}	2.8×10^{-9}	18×10^{-9}

[a] The data for inhibition of MMP-8 and the MMP-8 M_r 40 000 fragment by the TIMPs were obtained from Knäuper et al. (1993).

intermediate, inactive conformations with mismatched disulfides.

The locations of the specific binding sites of the TIMPs on the MMPs are not known to date. The activated forms of the MMPs are inhibited by both TIMPs, forming 1:1 stoichiometric complexes (Cawston et al., 1981; Chin et al., 1985; Goldberg et al., 1989; Umenishi et al., 1991; Wilhelm et al., 1989). In addition, the latent proforms of the 72-kDa and 92-kDa gelatinases form complexes with TIMP-2 and TIMP-1, respectively (Goldberg et al., 1989; Stetler-Stevenson et al., 1989). The fact that these latent enzyme–inhibitor complexes can still be activated *in vitro* and inhibited afterward by further addition of TIMPs indicates the existence of two TIMP binding sites on the gelatinases. Howard and Banda (1991) first proposed a TIMP-2 binding site within the C-terminal domain of the 72-kDa gelatinase after investigating the binding of TIMP-2 to autoproteolytically generated fragments of this enzyme. Genetically engineered deletion mutants of the 72-kDa gelatinase, lacking the C-terminal domain, had a decreased affinity for both TIMPs, which provides further evidence for the existence of a TIMP binding site on this domain which is distinct from the TIMP-2-specific binding site on the proenzyme (Murphy et al., 1992a). Very little data is available on the interaction of the collagenases with TIMPs. Clark and Cawston (1989) reported that the catalytic domain of fibroblast collagenase is not inhibited by TIMP-1, suggesting that the C-terminal domain of this enzyme is involved in inhibitor binding.

In this study, we investigated the interaction of rcd-PMNL-c with TIMPs, attempting to better characterize the location of the binding site of the TIMPs on the PMNL–collagenase. rcd-PMNL-c formed a stable complex with rTIMP-2, which could be purified by gel permeation chromatography. The complex of rcd-PMNL-c with TIMP-1 and rTIMP-1 could be established by sandwich ELISA. Kinetic studies with the enzyme domain and the TIMPs in a fluorometric assay revealed that both TIMPs are potent inhibitors of rcd-PMNL-c, with K_i values comparable to those of the glycosylated native enzyme (Table I). From these findings it can be concluded that, in the case of the PMNL–collagenase, the C-terminal domain is not involved or at least is not essential for the enzyme–inhibitor interaction, which is consistent with recent results from our laboratory (Knäuper et al., 1993) where we reported that the glycosylated M_r 40 000 active fragment of PMNL–collagenase is inhibited by TIMPs.

However, the C-terminal, hemopexin-like domain of the PMNL–collagenase might have some influence on the interaction of this enzyme with TIMPs. De Clerck et al. (1991) reported on an SDS-stable complex of fibroblast collagenase and TIMP-2 visualized by SDS–PAGE. In former work from our laboratory, it was shown that the complex of native PMNL–collagenase and TIMP-1 is also SDS-stable (Bläser et al., 1991). In contrast to the full-length enzymes complexed with TIMPs, the purified rcd-PMNL-c/rTIMP-2 complex was dissociated in the presence of SDS (Figure 4). The complexes of rcd-PMNL-c with TIMP-1 and rTIMP-1 also were not stable in the presence of SDS (data not shown).

Howard et al. (1991) reported that the autocatalytically generated catalytic domain of the 72-kDa gelatinase is not as well-inhibited by TIMP-1 as by TIMP-2. The same is true for the glycoslyated active M_r 40 000 fragment of PMNL–collagenase (Knäuper et al., 1993) and for the nonglycosylated M_r 20 000 rcd-PMNL-c produced in *E. coli*. Why TIMP-2 is the more potent inhibitor of the catalytic domains of these enzymes is not understood.

Currently, no detailed structural information on any member of the MMP family or TIMP family exists. In the case of the MMPs this is mainly due to the instability of the enzymes, which undergo autoproteolytic degradation upon storage. The complexes of rcd-PMNL-c/rTIMP-2 and rcd-PMNL-c/rTIMP-1 no longer showed any proteolytic activity, and the purified complex of rcd-PMNL-c/rTIMP-2 was stable upon storage at 4 °C for several months. Therefore, these complexes may be suitable candidates for X-ray crystallographic experiments. The cloning and expression of recombinant TIMPs and rcd-PMNL-c now enable us to carry out such experiments as sufficient amounts of protein become available.

Results from single-site mutations of rTIMP-1 from eukaryotic cells revealed that the region between Cys3 and Cys13 bears important structural elements for inhibitory activity (O'Shea et al., 1992), and our results show that the catalytic domain of the PMNL–collagenase is sufficient for inhibition and complex formation by TIMP, but only detailed data on these complexes from X-ray structures can elucidate the mechanism of the enzyme–inhibitor interaction on the molecular level. A three-dimensional picture of the enzyme–inhibitor complex will display the structural features of TIMPs essential for inhibition and will thus facilitate the computer-assisted design of potent, selective, low molecular weight inhibitors. Such inhibitors could lead to new therapeutic strategies in the treatment of diseases as diverse as rheumatoid arthritis and cancer.

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