Recombinant interstitial collagenase (rMMP-1) forms insoluble inclusion bodies when over-expressed in Escherichia coli. We surveyed conditions for renaturation of purified rMMP-1 in 6 M guanidine hydrochloride (GdnHCl) and found that optimal folding occurred when the denatured protein was diluted at 4 °C in 2 M guanidine HCl, 20% glycerol, 2.5 mM reduced and oxidized glutathione, and 5 mM CaCl2, followed by buffer exchange to remove denaturant and thiols. The circular dichroism spectrum and catalytic constants of the refolded enzyme were similar to those of native MMP-1. The propeptide, which comprises approximately 20% of the mass of pro-rMMP-1, was not required for folding to a functional enzyme. Size exclusion chromatography and spectroscopic measurements at intermediate [GdnHCl] revealed two intermediate folding states. The first, observed at 1 M GdnHCl, had a slightly larger Stokes’ radius than the folded protein. CD and fluorescence analysis showed that it contained ordered tryptophan residues with a higher quantum yield than the fully folded state. The second intermediate, which appeared between 2 and 4 M GdnHCl, exhibited properties consistent with the molten globule, including secondary structure, lack of ordered tryptophan, exposed hydrophobic binding sites, and a Stokes’ radius between that of the folded and unfolded states.

Interstial collagenase (EC 3.4.24.7) is a member of the matrix metalloproteinase family of enzymes which function in remodeling the extracellular matrix. Elevated MMP activity is associated with several physiological and pathological processes, including embryogenesis, arthritis, corneal ulceration, cancer metastasis, and periodontal disease (1, 2). Procollagenase is secreted from a variety of cell lines in culture, including fibroblasts (3) and umbilical vein endothelial cells (4), as a mixture of glycosylated 57-kDa and unglycosylated 52-kDazymogens that are activated by removal of an amino-terminal propeptide to give the corresponding 48- and 42-kDa proteinases. MMP-1 (fibroblast collagenase) and its neutrophil homolog (MMP-8) initiate the breakdown of collagen fibrils by cleaving collagen types I, II, and III at a single location in each α chain to generate three-quarter and one-quarter length fragments, which are then degraded by other proteinases. Sequence and homology analysis coupled with functional comparison of full-length, truncated, and chimeric constructs indicate that MMP-1 consists of three domains: an N-terminal propeptide of approximately 80 residues, a 19-kDa catalytic domain of approximately 160 residues, and a C-terminal domain of 209 residues. The C-terminal domain binds collagen and shares sequence similarity with the plasma protein hemopexin and vitronectin, which also interacts with extracellular matrix proteins (5). The pexin domain is essential for the expression of collagenolytic activity by MMP-1, since the catalytic domain alone, while active against artificial substrates, does not hydrolyze native collagen (6–8). The catalytic and pexin domains are linked by a proline-rich segment of about 17 residues.

The three-dimensional structures of 19-kDa MMP-1 and the homologous domain of MMP-8 have been determined (9–13). These studies reveal a catalytic unit consisting of a five-stranded β-sheet structure and three α-helical segments. Binding sites for catalytic and structural zinc ions as well as three calcium ions in MMP-1 and two in MMP-8 are present. More recently, the crystal structure of full-length porcine MMP-1 was published (13). The collagen-binding pexin domain is composed of antiparallel β-sheets that fold into a motif described as a β-propeller. The pexin domain contains a single calcium binding site situated near the apex of the propeller (13). As expected, the catalytic domain of the pig enzyme is similar to that of the human 19-kDa derivatives.

The precise role of the C-terminal pexin domain in conferring collagenolytic specificity to MMP-1 is not known. One means of exploring this question is through the study of mutant collagenases generated by recombinant DNA methodology. A prerequisite for such a study is to optimize the folding reaction of rMMP-1 and to compare the recombinant and wild-type proteinases. This report summarizes a procedure developed to optimize the yield of renatured recombinant collagenase from inclusion bodies produced by over-expressing the protein from a plasmid in Escherichia coli. The unfolded and refolded states of the proteinase were characterized in terms of catalytic constants, Stokes’ radius, fluorescence, and CD spectroscopic properties. To aid in elucidating the refolding pathway, we measured the dependence of various structural probes on denaturant concentration. The results suggest that unfolding in the presence of denaturant proceeds through two intermediates, one of which exhibits properties consistent with those of
the “molten globule” state detected in the folding pathway of many proteins (14).

EXPERIMENTAL PROCEDURES

Materials

The recombinant human fibroblast collagenase expression system was supplied by Dr. G. McGeegan, Glaxo Laboratories, Research Triangle Park, NC. Native procollagenase was purified by the method of Kakalits et al. (15) from growth medium of phorbol ester-stimulated human umbilical vein endothelial cells. Procollagenase was activated by incubation with 1.5 mM p-aminophenylmercuric acetate at 25 °C for 2 h (16).

The MMP substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-o-Arg-NH₂ was synthesized by Stack and Gray (17). Guanidine HCl (ultrapure) was from U. S. Biochemical Corp.; ANS and type I collagen from calf skin were from Sigma; Brij 35 was obtained as a purified product from Pierce Chemical Co.

Methods

Purification of rMMP-1—The purification procedure was modified from that of Dr. G. McGeegan. E. coli JM103 harboring the expression vector were grown in LB broth with 50 μg/ml ampicillin to an absorbance of 0.8 at 550 nm. Expression of recombinant enzyme was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside with continued growth for 90 min. Bacteria were harvested by centrifugation and treated with lysozyme (0.25 mg/ml) in lysis buffer (200 mM NaCl, 50 mM Tris, 5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM o-phenanthroline, pH 7.6) for 30 min. The suspension was frozen in liquid nitrogen, thawed (four freeze-thaw cycles), and then sonicated on ice with a Branson sonifier at 70 watts. Incubation bodies were pelleted by centrifugation and washed three times with wash buffer (50 mM Tris, 100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM o-phenanthroline, 0.5% Triton X-100, pH 7.6). The washed pellet was resuspended in denaturing buffer (20 mM Tris, 6 M GdnHCl, 5 mM reduced diithiothreitol, pH 7.6) by homogenization with a Dounce homogenizer. After centrifugation, the supernatant was loaded on a gel filtration column (Bio-Gel A-0.5m, 2.5 × 88 cm) and eluted with 20 mM Tris, pH 7.6, 3 × 6 M GdnHCl, 5 mM reduced diithiothreitol at a flow rate of 12 ml/h. MMP-1 was recovered in the major protein peak and showed a single band of 42 kDa by SDS-polyacrylamide gel electrophoresis.

Folding Procedure—Optimal refolding was achieved when rMMP-1 in 6 M GdnHCl was diluted into folding buffer (50 mM Tris, 5 mM CaCl₂, 200 mM NaCl, 50 μM ZnSO₄, 0.05% Brij 35, pH 7.6) containing 2.5 mM GSH, 2.5 mM GSH, 20% glycerol, and a final GdnHCl concentration of 2.3 M. The prefolding reaction was allowed to proceed at 4 °C for at least 1 h. GdnHCl, glutathione, and glycerol were subsequently removed either by a gel filtration spin column or by dialysis against folding buffer without Zn²⁺. For the spin-column procedure, Sephadex G-50 was equilibrated in folding buffer without Zn²⁺. The gel was poured into a 38 ml volume followed by centrifugation for 2 min at 2000 rpm in a Beckman centrifuge (TJ-6) to remove buffer. Prefolded rMMP-1 (100 μl) was loaded onto the spin column which was then centrifuged for 2 min at 3000 rpm to elute the protein. The recovery of protein could be increased by adding an additional 100 μl of buffer and recentrifuging for 1 min at 3000 rpm.

Protein Assays—To allow protein estimation in the presence of interfering substances, a filter paper dye-binding assay was used with bovine serum albumin as standard (18).

Kinetic Assays—Enzyme activity with DnpS as substrate was measured as described by Stack and Gray (17). Collagenolytic activity was measured in solution at 25 °C as described by Welgus et al. (19) using a Bio-Rad 620 video densitometer (19). Kinetic constants were estimated using the program Enzfitter (Biosoft, Cambridge, UK).

Fluorescence—Fluorescence spectra were recorded at 25 °C with an SLM-Aminco SPF-500C spectrophotometer interfaced to an IBM personal computer. Software supplied by the manufacturer was used for data collection and manipulation. Appropriate buffer blanks were subtracted from each spectrum. All fluorescence spectra are expressed in arbitrary units and are uncorrected for instrumental response. Stokes’ Radii—SEC-HPLC was carried out with a Bio-Sil Sec 125–5 size exclusion column connected to a Spectra Physics SP 8700XR HPLC system equipped with a Kratos Spectroflow 75 UV/Vis detector (set at 220 nm) and a SP 4290 integrator. The column was calibrated with blue dextran 2000, bovine serum albumin, ovalbumin, human erythrocyte carbonic anhydrase, pancreatic ribonuclease, horse heart cytochrome c, and pyridoxine phosphate. Stokes’ radii were estimated from the calibration curve as described by Fish et al. (22).

Circular Dichroism—CD spectra were measured at ambient temperature (−23°C) with a Jasco J-710 spectropolarimeter interfaced to a Compaq 486 computer. The instrument was calibrated with d-camphor sulfonic acid using a molar ellipticity of 7800 degrees cm²/dmol at 290.5 nm (23). All solutions were centrifuged at 12,000 rpm before measurement. CD spectra represent the average of three runs and have buffer blanks subtracted. Instrumental calibration was confirmed using hen lysozyme and myoglobin as control proteins; the spectra obtained were essentially identical to published spectra (24).

RESULTS

Effect of Refolding Conditions on Yield of Active Enzyme

Preliminary experiments showed that substantial amounts of protein precipitated when the denaturant was removed from purified rMMP-1 by dialysis. To minimize protein precipitation and thereby increase the yield of active rMMP-1, we investigated several different refolding procedures. We modeled our approach after that of Brems (25) who showed that precipitation of recombinant bovine growth hormone can be minimized by “prefolding” the protein at an intermediate concentration of denaturant followed by removal of residual denaturant. The data of Fig. 1 show that recovery of rMMP-1 activity was highest and protein precipitation was lowest when folding proceeded from an intermediate GdnHCl concentration (2.3–3.0 M) prior to complete removal of denaturant. Below 2.3 M GdnHCl, decreased recovery of enzyme activity and soluble protein correlated with increased precipitated protein.

We next investigated the effect of the redox potential of the folding medium on the yield of active enzyme. MMP-1 contains a pair of cysteine residues in the COOH-terminal collagen binding domain that have been reported to form a disulfide bond essential for collagenolytic activity (8, 26). To catalyze disulfide shuffling and promote intramolecular disulfide pairing rather than intermolecular pairing during refolding, rMMP-1 was incubated in the presence of different ratios of oxidized to reduced thiol reagent (27). The data of Fig. 2 demonstrate that the recovery of enzyme activity was highest when renaturation proceeded in the presence of 2.5 mM GSSG and 2.5 mM GSH. The glutathione redox buffer system was more effec-

2 G. McGeegan, personal communication.
The effects of glycerol, a protein stabilizing agent, and elevated temperature, which enhances hydrophobic interactions, were also assessed. Glycerol in the presence of GSH/GSSG increased the yield of active refolded protein and decreased the proportion of precipitated protein from 59 to 6.4%. The extent of precipitation was also found to be temperature-dependent. Protein precipitation increased from about 6% at 4°C (as indicated above) to 32% at 25°C and 47% at 35°C.

MMP-1 binds Ca\(^{2+}\) which contributes to its stability (28–31). To determine if Ca\(^{2+}\) plays a role in the folding reaction, rMMP-1 was incubated in different concentrations of Ca\(^{2+}\) ranging up to 10 mM. The different Ca\(^{2+}\) levels were maintained at all folding steps until the enzyme activity assay. The samples were then assayed in 11 mM Ca\(^{2+}\). No catalytically active protein was obtained when refolding took place in the absence of added Ca\(^{2+}\). The yield of active enzyme increased with increasing Ca\(^{2+}\) to 5 mM and remained constant to the highest concentration tested (9.5 mM).

### Characterization of Refolded rMMP-1

#### Intrinsic Fluorescence

The intrinsic fluorescence of a protein is a sensitive indicator of the local environment of its tryptophanyl residues (32). MMP-1 has three tryptophans in the catalytic domain and four in the pexin domain. The fluorescence emission spectra of unfolded and refolded MMP-1 are shown in Fig. 3. The emission maximum of the refolded protein was at 332.5 nm while that of the denatured enzyme was at 356 nm. In addition to the blue shift in emission maximum that occurred on folding, there was an increase in quantum yield of the folded protein. Both results are consistent with a folding reaction in which most if not all of the tryptophanyl residues are incorporated into an environment that is inaccessible to solvent.

#### Circular Dichroism

The far UV CD spectra of refolded rMMP-1 and native proMMP-1 were compared as an indicator of the secondary structure of the two species. Figs. 4, A and B, show that the far UV CD spectra of refolded rMMP-1 and native proMMP-1 are similar, suggesting that the recombinant protein exists in a conformational state with significant secondary structure which is similar but not identical to that of the native protein. Since the proenzyme contains an additional 80 residues of polypeptide, it is not surprising that the two proteins do not give identical spectra. As expected, the CD spectrum of the denatured protein differed from that of the folded protein.

The CD spectra were analyzed in terms of secondary structure using the fitting program LINCOMB of Perzel et al. (33, 34); the results are summarized in Table I. Briefly, LINCOMB assumes that the measured CD spectrum can be represented by a linear combination of five reference CD spectra obtained from the CD spectra of 25 proteins of known secondary structure. Reference or "template" spectra represent contributions to the far UV CD spectrum of a protein by \(\alpha\)-helix, \(\beta\)-turn, and/or parallel \(\beta\)-sheet, aromatic and disulfide, unordered and/or \(\gamma\)-turn and antiparallel \(\beta\)-sheet. By this method of analysis, both rMMP-1 and native proMMP-1 gave comparable

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**Fig. 2.** Effect of thiol reagents on the recovery of activity during refolding of rMMP-1. rMMP-1 was diluted to 4.6 \(\mu\)M in 2.3 M GdnHCl containing different ratios of oxidized and reduced thiol reagent. GSH = GSSG, 2.5 mM of each reagent; DTTox = DTTred, 2.5 mM each of oxidized and reduced DTT; GSSG, 5 mM GSSG; GSH, 5 mM GSH; DTTox, 5 mM oxidized DTT; 5 GSH:1 GSSG, 5 mM GSH + 1 mM GSSG; 1 GSH:5 GSSG, 1 mM GSH + 5 mM GSSG. Buffers were purged with argon to eliminate oxygen. Enzyme activity was assayed with DnpS after GdnHCl and thiol reagent were removed by a spin column procedure.

**Fig. 3.** Fluorescence emission spectra of folded and unfolded rMMP-1. Folded rMMP-1 was diluted to 0.2 \(\mu\)M in folding buffer without GdnHCl (---) or 6 M GdnHCl (----). After incubating the sample at 25°C for 24 h, fluorescence emission spectra were recorded from 310 to 450 nm. Excitation wavelength was 280 nm.

**Fig. 4.** Far UV CD of folded and unfolded rMMP-1 and proMMP-1. Panel A, folded rMMP-1 (1.9 \(\mu\)M) was incubated without GdnHCl (---) or with 6 M GdnHCl (----) in folding buffer at 25°C for 24 h. CD spectra were then recorded after centrifugation. Panel B, the CD spectrum of proMMP-1 (2.4 \(\mu\)M) was measured in folding buffer. In both panels, the dashed line represents the best fit of the experimental data using the program LINCOMB (Refs. 33 and 34; see text and Table I).
The experimental data of Fig. 4 were analyzed using the LINCOMB program of Perczel et al. (33, 34). This program fits the experimental spectrum to a linear combination of five standard spectra representing the four secondary structural elements below plus contributions from aromatic and disulfide chromophores. The percentage of each secondary structural type was calculated after subtracting the contribution of aromatic and disulfide chromophores.

| Structure              | rMMP-1 | proMMP-1 |
|-----------------------|--------|----------|
| α-Helix               | 29.9   | 30.5     |
| Antiparallel β-sheet  | 12.2   | 20.8     |
| β-Turn/parallel sheet| 30.3   | 7.9      |
| γ-Turn/unordered      | 32.4   | 37.7     |
| Other*                | 16.1   | 28.1     |

*Contributions to the measured CD spectra from aromatic groups and disulfide bonds.

amounts of α-helix (approximately 30%) and β-structure (30–40%). However, the contribution of disulfide and aromatic chromophores to the CD spectrum of the proenzyme appears to be larger than in the recombinant enzyme missing the propeptide (28 versus 16%, respectively).

Kinetic Constants—Values of \(K_m\) and \(k_{cat}\) of rMMP-1 and MMP-1 from HUVEC were determined with both collagen and a synthetic peptide substrate (Table II). For comparison, published kinetic constants for fibroblast MMP-1 (19, 35) are also listed in Table II. The \(K_m\) of rMMP-1 for collagen was identical to that of fibroblast and HUVEC MMP-1. The \(k_{cat}\) values were similar for the enzymes from the three sources. The small differences may be explained by the previously reported finding that \(k_{cat}\) of collagenase depends on the activation procedure (36, 37). For DnpS, the catalytic efficiency of rMMP-1 was also comparable to that of native MMP-1 (35).

Characterization of Folding Intermediates

Stokes’ Radius—Size exclusion chromatography has been a valuable method for detecting potential folding intermediates that are stabilized by low concentrations of denaturant (14, 33–41). Typically, such intermediates exhibit Stokes’ radii that are larger than that of the fully folded protein but smaller than the denatured state. We used SEC-HPLC to assess the possibility that such states might exist for rMMP-1 at low GdnHCl concentrations.

Above 4 M GdnHCl, all of the protein was fully unfolded and eluted at 6 min. The retention times, partition coefficients, and Stokes’ radii of these species are summarized in Table III.

ANS Binding to 1.8 M GdnHCl—ANS binds to hydrophobic regions of proteins in an association reaction characterized by increased ANS quantum yield and a blue shift in its emission maximum (41). Fig. 6 shows that the emission maximum of ANS in 1.8 M GdnHCl shifts from 530 to 475 nm and the quantum yield increases when rMMP-1 is added. No change in fluorescence properties of ANS was noted with rMMP-1 in 0 or 6 M GdnHCl (data not shown). These results are consistent with the hypothesis that in 1.8 M GdnHCl, rMMP-1 is in a state with hydrophobic binding sites that are accessible to ANS. These binding sites are not present in the folded or unfolded states.

Spectroscopic Analysis of Intermediates—To gain further insight into the physical nature of the intermediates detected by SEC-HPLC, we analyzed the dependence of several structural probes on denaturant concentration. These probes include fluorescence quantum yield, fluorescence emission maximum, near UV CD (sensitive to tryptophanyl environment and packing) for UV CD (an indicator of backbone structure), and catalytic activity. Fig. 7 shows CD spectra obtained in the near UV...
region between 280 and 310 nm at GdnHCl concentrations between 0 and 4 M. The inflection appearing between 290 and 300 nm in the CD spectrum of the refolded protein is typical of tryptophanyl residues constrained in an asymmetric region of the protein. This inflection in the near UV CD spectrum persisted at 2 M GdnHCl. However, it disappeared at 3 M GdnHCl, indicating that any tertiary structure involving these residues was destroyed at this denaturant concentration. Changes in emission maximum and quantum yield were also noted at these concentrations of denaturant; these will be discussed below.

Table III

| State   | GdnHCl (M) | Retention | Kd (Å) | Rs (Å) |
|---------|------------|-----------|--------|--------|
| N       | 0          | 8.1       | 0.64   | 22.7   |
| I₁      | 1-2        | 7.6       | 0.50   | 26.9   |
| I₂      | 2-4        | 6.7       | 0.28   | 32.9   |
| U       | >4         | 6.0       | 0.16   | 36.3   |

Fig. 7. Near UV CD of folded rMMP-1 in different concentrations of GdnHCl. The CD spectrum of folded rMMP-1 (5.5 M) was recorded from 310 to 250 nm. Solid GdnHCl was then added to the sample to give 0.8, 1.0, 2.0, 3.0, and 4.0 M denaturant. The increase in volume caused by the added GdnHCl was calculated using its partial specific volume of 0.75 μl/mg. The protein concentration of each sample was determined by dye-binding assay (18) after the spectrum was recorded.

Fig. 8 summarises the effect of GdnHCl concentration on enzyme activity, secondary structure, and tertiary structure as monitored by the tryptophanyl residues. The parameters displayed are ellipticity at 222 and 290 nm, fluorescence emission maximum, quantum yield, and enzyme activity. Inspection of Fig. 8A shows that catalytic activity disappeared by 1.5 M GdnHCl. In the range of [GdnHCl] between 0 and 1 M, there was a slight biphasic shift in emission maximum from 332 to about 340 nm, suggesting an increase in solvent accessibility of one or more tryptophanyl residues. Accompanying this shift...
was an increase in emission intensity and in tryptophanyl asymmetry as assessed from the near UV CD data (Fig. 8B).

The species displaying these characteristics probably corresponds to I1 (elution time of 7.6 min) in SEC-HPLC. Between 1.2 and 2 M GdnHCl, the emission intensity declined and remained relatively constant at a level of about 65% of that of the refolded enzyme. Above 2 M GdnHCl, the emission maximum increased to about 352 nm and remained at this wavelength as the CD shoulder at 290 nm disappeared, indicating disruption of tertiary structure. Secondary structure, as indicated by the relative constant ellipticity at 222 nm, was retained. This probably corresponds to the SEC-HPLC state I2 at -6.7 min. With a further increase in denaturant to 4 M GdnHCl, secondary structure was lost, the emission maximum increased to 356 nm (typical of fully exposed tryptophan), and emission intensity dropped to about 15% of that of the refolded protein. This corresponds to the unfolded state observed in the molecular sieve experiments at -6 min.

**DISCUSSION**

It is well known that heterologous proteins when overexpressed in bacteria often aggregate to form inclusion bodies (42). While isolation of the inclusion bodies serves as a convenient enrichment step, it remains necessary to solubilize the recombinant protein, a process requiring strong denaturants. To generate the fully functional protein, one must then remove the denaturant to allow refolding. That folding often proceeds with poor yield has been attributed to competing pathways of folding and self-aggregation, both of which result in removing surface hydrophobic groups from contact with solvent (25). The first part of the current study was designed to optimize recovery of active protein and to compare the refolded and native enzymes. In common with studies with other recombinant proteins, we found that a critical step was to initiate folding from an intermediate concentration of denaturant, which presumably solubilizes a conformation with exposed hydrophobic groups so that internalization of these groups can occur without protein aggregation. For rMMP-1, a redox buffer to catalyze disulfide shuffling was important; in addition, the GSSG/GSH couple, which may transiently form a charged mixed disulfide with enhanced solubility, was more effective than DTT (43). Furthermore, agents that stabilize the folded conformation such as glycerol and calcium ions significantly affected the yield of refolded protein. Recently, Zhi et al. (44) reported that glycerol promotes renaturation of citrate synthase. Interestingly, the propeptide, which comprises roughly 20% of the mass of the proenzyme, was not necessary for folding to an active enzyme. This situation is in contrast to the proteinase subtilisin, which requires the propeptide to direct folding along a productive pathway (45, 46), but resembles that of cathepsin D, which refolds without its propeptide (47).

The folded recombinant protein exhibited characteristics expected of a native protein. It was catalytically active, contained significant secondary and tertiary structure as judged from CD analysis, was more compact than the unfolded protein, and the emission properties of its tryptophanyl residues suggest that they are inaccessible to solvent. Due to limited amounts of the native protein, we were unable to carry out extensive physical comparisons with the recombinant protein, but kinetic studies showed that the two preparations were virtually indistinguishable with both synthetic and natural substrates. Catalytic competence with collagen as substrate is a particularly stringent test of "nativeness" since both the active site domain and collagen binding domains must be properly folded for activity.

Analysis of spectroscopic and hydrodynamic properties of rMMP-1 as a function of denaturant concentration revealed an interesting unfolding pathway. Unfolding of rMMP-1 apparently occurs in three stages: N -> l1 -> l2 -> U, where N and U represent the folded and unfolded states. The first intermediate is characterized by an increase in emission intensity and is accompanied by a small blue shift in emission maximum. This species, while slightly less compact than the folded protein, has at least one tryptophanyl residue in a less polar environment. The increase in quantum yield suggests a conformational change which removes a charged residue from the vicinity of a tryptophanyl emission. In an effect recently reported in a study of the folding of barnase (48). At somewhat higher GdnHCl (2 M), the emission intensity decreased and the maximum shifted to the red. As judged from the near and far UV CD spectra, tertiary structure is disrupted at this stage to give I2, which appears to fit the definition of the molten globule state (14). This species contains exposure of hydrophobic groups as indicated by the ANS binding experiments and also retains secondary structure as indicated by the CD spectra. The last stage of denaturation occurs between 2 and 4 M denaturant, and is characterized by a sharp decrease in fluorescence intensity, a gradual red shift in emission maximum, and loss of ellipticity at 220 nm.

These results complement and extend those of Lowry et al. (30) who investigated the effect of calcium and zinc on the refolding of 19-kDa collagenase catalytic domain. These workers found that in the absence of metal ions, the catalytic domain was totally unfolded at 1 M GdnHCl as judged by tryptophan emission. Addition of calcium and zinc stabilized the 19-kDa form of the enzyme such that unfolding occurred between 1 and 2 M GdnHCl. Full-length collagenase without calcium and zinc unfolded over a broad range of GdnHCl (1-4 M).

Loss of enzyme activity preceded the loss of tertiary and secondary structure, an indication that catalytic activity of rMMP-1 is more sensitive to denaturants than is gross conformational integrity. The active site of the enzyme is apparently more susceptible to denaturation than the molecule as a whole. A similar phenomenon has been observed in the unfolding reaction of several enzymes including creatine kinase (49), lactate dehydrogenase (50), and d-glycerolphosphate dehydrogenase (51, 52).

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Characterization of Folded, Intermediate, and Unfolded States of Recombinant Human Interstitial Collagenase
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