

Fluorescence Quenching Studies of Matrix Metalloproteinases (MMPs): Evidence for Structural Rearrangement of the proMMP-2/TIMP-2 Complex upon Mercurial Activation

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Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which are secreted from cells as zymogens and can be activated by treatment with organomercurial reagents or limited proteolysis. The proenzyme forms of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are found in complex with tissue inhibitor of metalloproteinases (designated proMMP-2/TIMP-2 and proMMP-9/TIMP-1, respectively). The proposed mechanism of activation by mercurial compounds involves the induction of a conformational change in the zymogen which leads to propeptide auto-processing. To investigate the possibility of conformational differences in MMPs, solute quenching of MMP intrinsic fluorescence was used to probe the relative exposure of tryptophan residues in latent and mercurial-activated MMPs. Our data demonstrate that fluorescence quenching of the proMMP-2/TIMP-2 complex by either acrylamide or iodide is significantly increased following mercurial activation. In contrast, no significant change in tryptophan accessibility accompanies mercurial treatment of either proMMP-2 or TIMP-2 alone, or mercurial-activated MMP-2 mixed with TIMP-2. To determine whether the enhanced fluorescence quenching was unique to the activated proMMP-2/TIMP-2 complex, similar experiments were performed using MMP-1, MMP-3, and MMP-9/TIMP-1 complex. In all cases, both latent and mercurial-treated MMPs exhibited similar fluorescence quenching profiles, suggesting that there are no significant conformational differences between the zymogen and activated forms of MMP-1, -2, -3, or -9/TIMP-1. The en-

hanced fluorescence quenching observed with mercurial-treated proMMP-2/TIMP-2 is indicative of increased exposure of a previously buried tryptophan residue(s), providing evidence for a structural rearrangement of the activated complex. These data, together with our previous biochemical observation that mercurial treatment of proMMP-2/TIMP-2 exposes the MMP-2 active site without propeptide processing (Y. Itoh *et al.* (1995) *Biochem. J.* 308, 645–651), suggest that the activated proMMP-2 in the complex may represent a transitional conformational intermediate in MMP activation. © 1996 Academic Press, Inc.

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Matrix metalloproteinases (MMPs)² are a family of zinc-dependent proteinases which function in the degradation of collagen and other extracellular matrix macromolecules [reviewed in (1)]. Included in the MMP family are interstitial collagenase (MMP-1), gelatinase A (MMP-2), gelatinase B (MMP-9), and stromelysin-1 (MMP-3). ProMMP-2 is purified in tight complex with tissue inhibitor of metalloproteinases (TIMP)-2 (designated proMMP-2/TIMP-2), while proMMP-9 copurifies in a stable stoichiometric complex with TIMP-1 (designated proMMP-9/TIMP-1) [for a review, see (2)]. Structural interrelationships are evident in the domain organization of the MMPs. All members of the family except matrilysin (MMP-7) contain three homologous structural domains including the NH₂-terminal propeptide

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² Abbreviations used: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

domain, the metal binding catalytic domain, and the COOH-terminal domain (1, 3). The NH₂-terminal domain contains an highly conserved sequence PRCG-[V/N]PD, the cysteine residue of which is proposed to coordinate with the zinc atom in the active site metal binding domain, thereby maintaining latency of the zymogen (4–7). The COOH-terminal domain contains an hemopexin-like region which is involved in regulation of substrate specificity for some members of the MMP family (8). Both MMP-2 and MMP-9 contain a domain with similarity to the type II domain of fibronectin, whereas MMP-9 has an additional collagen-like insertion (9, 10). ProMMP-2 and proMMP-9 form complexes with TIMP-2 and TIMP-1, respectively, through an interaction involving the C-terminal domains of both the gelatinases and the TIMPs (11–15).

The extracellular activity of MMPs is regulated in part by secretion as inactive proenzymes. MMP zymogens can be converted to active enzymes *in vitro* by treatment with chaotropic and organomercurial reagents or by limited proteolytic cleavage (4, 16, 17). Activation is thought to proceed via disruption of the interaction between the unpaired cysteine residue in the amino-terminal propeptide and the active site zinc atom (4, 5). It has been proposed that an initial step in proMMP activation by nonproteolytic agents involves the induction of a conformational change in the zymogen which then results in autocatalytic cleavage of the proenzyme and bimolecular processing to the fully active mature enzyme (5, 16–19). To investigate further the possibility of conformational alterations in MMPs upon activation, we examined the accessibility of tryptophan residues using fluorescence quenching agents.

Intrinsic fluorescence emission of tryptophan residues in a protein provides a sensitive probe of structural properties of the protein microenvironment [for reviews, see (20, 21)]. Quenching of protein intrinsic fluorescence can be used to probe the relative exposure of fluorescent residues in proteins under a specified set of conditions. Two commonly used quenching agents are acrylamide and iodide, both of which function through collision with the fluorophore, such that a change in quenching properties is reflective of a change in accessibility to the quenching agent. Acrylamide is a polar nonionic quencher which can penetrate the protein surface, whereas iodide is a large polar anion with access to only surface Trp residues (21, 22). In this study, we have utilized both acrylamide and iodide as quenchers to probe conformational alterations in MMPs which may accompany zymogen activation and report that fluorescence quenching of the proMMP-2/TIMP-2 complex is significantly increased following mercurial activation, whereas both latent and mercurial-treated MMP-1, MMP-2, MMP-3, MMP-9/TIMP-1, and TIMP-2 exhibit similar fluorescence quench profiles. These data provide biophysical evidence for a significant structural rearrangement of the proMMP-2/

TIMP-2 complex upon activation by a mercurial compound.

EXPERIMENTAL

MMP purification and activation. ProMMP-2/TIMP-2 complex and proMMP-2 free of TIMP-2 were purified from the conditioned medium of human uterine cervical fibroblasts by gelatin-Sepharose affinity chromatography and gel permeation chromatography on Sephacryl S-200 as previously described (23). The purified proMMP-2/TIMP-2 was free of uncomplexed proMMP-2 or TIMP-2 as confirmed by the loss of MMP inhibitory activity of TIMP-2 and the lack of gelatinolytic activity of MMP-2 upon treatment of the complex with 1 mM 4-aminophenylmercuric acetate. TIMP-2 was purified by dissociation of the proMMP-2/TIMP-2 complex with 8 M urea and 20 mM EDTA. The resulting proMMP-2 and TIMP-2 were separated by gel permeation chromatography on Sephacryl S-200 equilibrated with 50 mM Tris-HCl, pH 7.5, containing 8 M urea, 10 mM EDTA, 1 M NaCl, and 0.02% NaN₃. TIMP-2-containing fractions were concentrated, dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.4 M NaCl, 10 mM CaCl₂, 0.02% NaN₃ (TNC buffer) and rechromatographed on Sephacryl S-200 equilibrated with TNC buffer. The concentration of TIMP-2 was calculated by titration with a known amount of MMP-3. ProMMP-1 and proMMP-3 were purified from the conditioned medium of human rheumatoid synovial fibroblasts as described previously (18, 24, 25). ProMMP-9/TIMP-1 was purified from the conditioned medium of U937 monocytic leukemia cells (26).

Latent MMPs were activated by incubation with 1 mM HgCl₂ at 37°C for 1 h followed by chromatography on prepacked Sephadex G-25 gel filtration columns (PD10, Pharmacia, Piscataway, NJ) to remove HgCl₂. TIMP-2 was also treated with HgCl₂ as described above. ProMMPs and TIMP-2 (not treated with mercurial) were also applied to PD10 columns under identical conditions. In addition, proMMPs and TIMP-2 were denatured by incubation for 18 h at room temperature in 6.7 M guanidine-HCl (21) followed by chromatography on PD10 equilibrated with 6.7 M guanidine-HCl.

Fluorescence measurements. Steady state fluorescence measurements were performed using a Shimadzu RF-540 spectrofluorometer with temperature maintained constant at 25°C using a refrigerated circulating water bath. An excitation wavelength of 295 nm was used to minimize interference from tyrosine groups, emission was monitored in the range of 300–400 nm (using 5 nm excitation and emission slit widths), and spectra were corrected for buffer blank. Proteins were diluted from a stock solution to a final concentration of 100 nM in 20 mM Hepes, pH 7.4, containing acrylamide (0–0.5 M) or iodide (0–0.2 M) in a total volume of 500 μl. In iodide quenching experiments, constant ionic strength was maintained using NaCl. In addition, the KI stock solution contained 0.1 M Na₂S₂O₃ to prevent formation of I₃⁻ which absorbs at 290 nm. Excitation of fluorescence at a wavelength of 295 nm also prevents absorbance of exciting light by iodide (20). Iodide concentrations of less than 0.3 M were used to prevent protein denaturation. In acrylamide quenching experiments, fluorescence intensities were corrected for the attenuation of the exciting light intensity due to absorption by acrylamide (21). With both iodide and acrylamide, no large changes in the wavelength of maximal fluorescence were observed at any quencher concentration, indicating the absence of protein denaturation (27). The initial absorbance of protein solutions was less than 0.1 at the excitation wavelength to avoid the inner filter effect (4). Experimental error in fluorescence measurements was estimated to be less than 5%.

Fluorescence data were analyzed using the Stern-Volmer equation for steady state collisional quenching:

$$F_0/F = 1 + K_{sv}[Q],$$

where F_0 is the fluorescence intensity in the absence of quencher, F is

fluorescence intensity in the presence of quencher, K_{SV} is the Stern–Volmer constant for collisional quenching, and Q is the quencher concentration (20, 21). Whereas plots of F_0/F are linear for a population of homogeneously emitting fluorophores, downward curvature is observed in proteins containing multiple heterogeneous fluorophores. To analyze heterogeneously emitting systems, the modified Stern–Volmer equation was used:

$$F_0/(F_0 - F) = 1/[Q]f_a K_Q + 1/f_a$$

where f_a is the fraction of Trp residues accessible to quenching agent with a quench constant K_Q (22). The values f_a and K_Q are determined from plots of $F_0/(F_0 - F)$ vs $1/[Q]$.

RESULTS AND DISCUSSION

It has been proposed that activation of proMMPs by nonproteolytic agents is initiated by a structural perturbation of the zymogen which results in processing to the mature enzyme (5, 16). To analyze this proposed conformational change, the accessibility of Trp residues to solvent was examined by quenching intrinsic fluorescence of MMPs with acrylamide and iodide. MMPs contain multiple Trp residues dispersed throughout the protein primary structure, although the propeptides of MMPs-1, -2, -3, and -9 lack Trp residues. If a Trp residue(s) is in the vicinity of a portion of the protein structure undergoing a conformational change, alterations in the properties of intrinsic protein fluorescence are

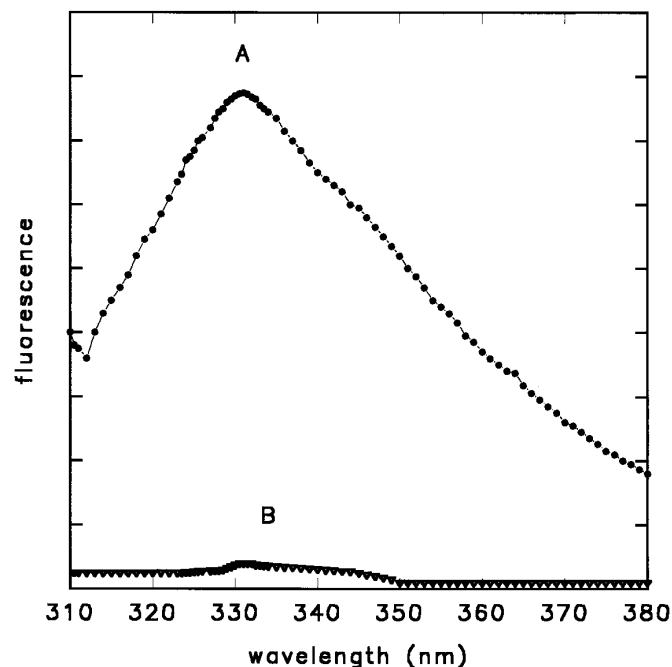


FIG. 1. Fluorescence emission spectrum of proMMP-2. ProMMP-2 was diluted to a final concentration of 100 nM in 20 mM HEPES, pH 7.4, and fluorescence emission was monitored at 25°C using an excitation wavelength of 295 nm. (A) proMMP-2, (B) 20 mM HEPES, pH 7.4.

TABLE I
MMP Tryptophan Fluorescence in the Presence of Hydrophilic Quenchers^a

Protein	Form	Percentage decrease in fluorescence	
		Acrylamide	Iodide
MMP-1	pro	31.2	17.8
	active	34.0	15.7
	Gu-HCl	61.2	32.9
MMP-2	pro	28.4	19.4
	active	25.9	19.8
	Gu-HCl	44.8	34.0
MMP-3	pro	34.5	13.1
	active	34.1	13.4
	Gu-HCl	55.5	26.6
MMP-2/TIMP-2	pro	38.0	21.3
	active	63.9	42.9
	Gu-HCl	57.8	43.8
MMP-9/TIMP-1	pro	29.4	17.4
	active	26.6	14.0
	Gu-HCl	42.9	21.5

^a Latent (pro), active, or guanidine–hydrochloride (Gu–HCl)-treated MMPs were prepared as described under Experimental. Fluorescence was determined using an excitation wavelength of 295 nm and monitoring emission in the range of 300–400 nm. Percentage decrease in fluorescence is reported at the highest concentration of quenching agent added (0.2 M iodide, 0.5 M acrylamide).

expected, including a change in the wavelength of maximum emission and/or altered susceptibility to quenching agents. Therefore, monitoring intrinsic fluorescence of MMPs and quantitation of the relative abilities of acrylamide and iodide to quench Trp fluorescence in either the proenzyme or active form should enable detection of conformational alterations of the enzyme upon activation. A representative fluorescence emission spectrum is shown in Fig. 1.

A progressive decrease in Trp fluorescence was observed upon addition of quenching agent to MMPs-1, -2, -3, MMP-2/TIMP-2 and MMP-9/TIMP-1. At the highest concentration of quenching agent added (0.2 M iodide, 0.5 M acrylamide), fluorescence quenching ranged from 13.1–43.8% with iodide, whereas acrylamide resulted in a greater overall decrease in protein fluorescence, with quenching ranging from 25.9 to 63.9% (Table I). Data for MMPs unfolded by treatment with 6.7 M guanidine–HCl are included for comparison. These data indicate that MMP fluorescence is more efficiently quenched by acrylamide than iodide, suggesting that most, if not all Trp residues are not localized on the protein surface. This is supported in part by the conserved location of most Trp residues in the MMP family. Alternatively, the presence of amino acids with negatively charged side chains in the vicinity of the Trp residue(s) would repel iodide and effectively decrease the quenching efficiency.

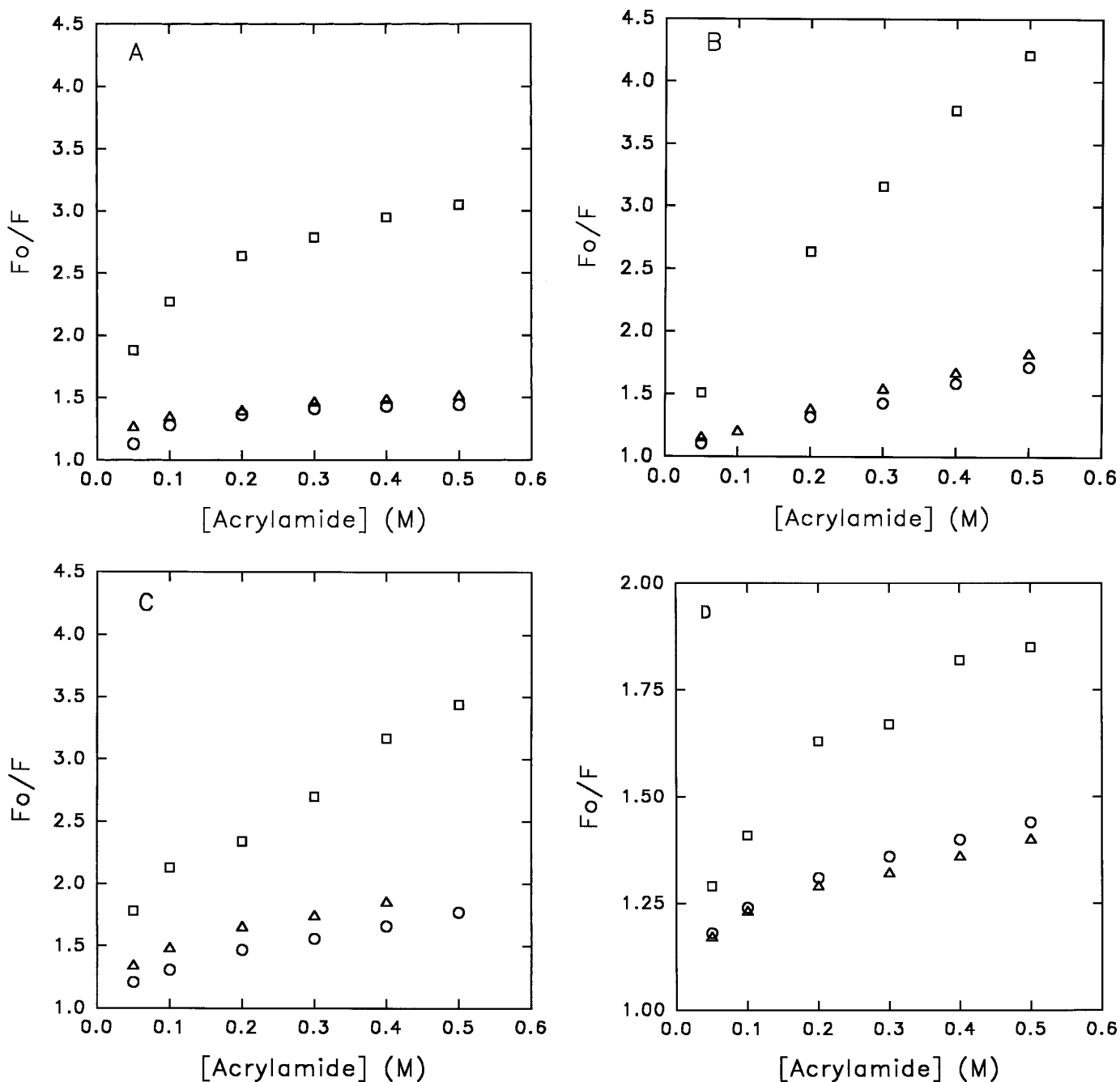


FIG. 2. Quenching of MMP-1, -2, -3, and -9/TIMP-1 intrinsic fluorescence by acrylamide. Increasing concentrations of acrylamide were added to proMMPs (○), HgCl₂-activated MMPs (△), or guanidine-HCl-treated proMMPs (□) and fluorescence emission was monitored as described under Experimental. Data are plotted according to the Stern-Volmer equation. (A) MMP-1, (B) MMP-2, (C) MMP-3, (D) MMP-9/TIMP-1.

Data obtained for acrylamide quenching of the proenzyme, active, and guanidine-treated forms of MMPs-1, -2, and -3 were analyzed by the Stern-Volmer equation and are shown in Figs. 2A-2C, respectively. In several cases, downward curvature of the Stern-Volmer plots is observed, indicative of greater than one

population of Trp residues with unequal accessibility to quencher. Comparison of the proenzyme (circles) and activated (triangles) forms of MMPs-1, -2, and -3 indicates that little change in Trp accessibility to either acrylamide (Fig. 2) or iodide (data not shown) accompanies activation. In contrast, guanidine unfolding re-

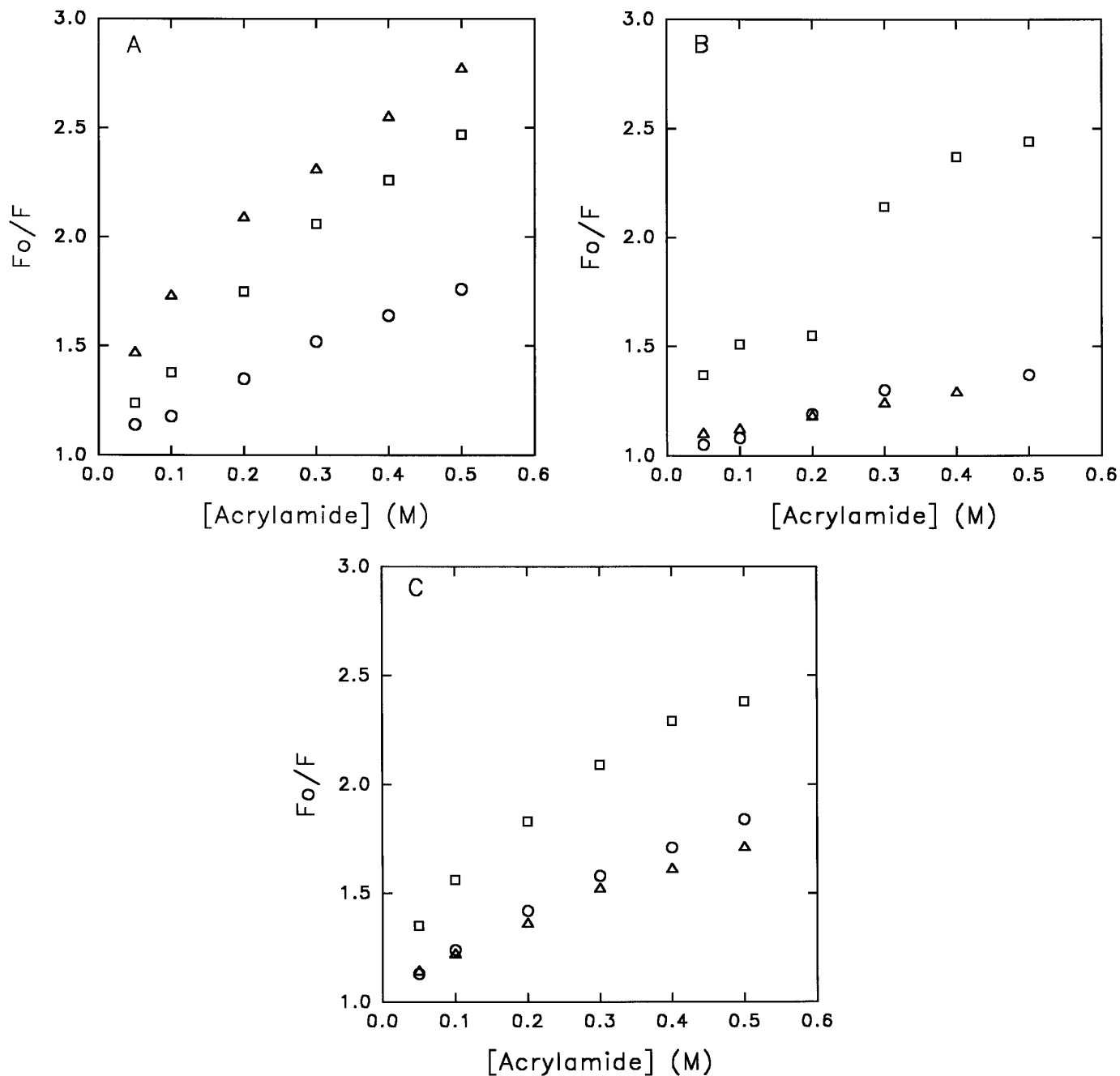


FIG. 3. Analysis of MMP-2/TIMP-2 quenching by acrylamide. Increasing concentrations of acrylamide were added to native (○), HgCl₂-treated (△), or guanidinium-HCl-treated (□) proteins and fluorescence emission was monitored as described under Experimental. (A) proMMP-2/TIMP-2 complex, (B) TIMP-2, (C) proMMP-2 + TIMP-2 (treated as described above and mixed prior to fluorescence determination). Data are plotted according to the Stern-Volmer equation.

sults in increased solute quenching of Trp fluorescence (squares). These data suggest that the active conformations of MMPs-1, -2, and -3 are not significantly different than the proenzyme forms. This is in agreement with X-ray crystallographic data which indicate little overall conformational change in the catalytic domain of proMMP-3 as a consequence of activation (29). How-

ever we cannot rule out the possibility that a conformational change does occur which does not alter the solvent accessibility of any MMP Trp residues or, alternatively, that compensatory changes in Trp environments occur.

In contrast to results obtained with free MMPs, the proMMP-2/TIMP-2 complex exhibits significant en-

TABLE II
Fluorescence Parameters of Acrylamide Quenching^a

Protein	Pro- or native		HgCl ₂ -treated		GuHCl-treated	
	f_a	K_Q (M ⁻¹)	f_a	K_Q (M ⁻¹)	f_a	K_Q (M ⁻¹)
MMP-2/TIMP-2	0.50	5.9	0.69	17.1	0.74	7.1
MMP-2	0.63	3.3	0.53	5.9	0.89	12.7
TIMP-2	0.60	1.6	0.33	4.7	0.60	15.3
MMP-2 + TIMP-2	0.68	4.0	0.51 ^b	6.4 ^b	0.65	12.7

^a Latent (pro), HgCl₂-treated, or guanidine-hydrochloride-treated (GuHCl) MMPs were prepared as described under Experimental. Fluorescence was determined using an excitation wavelength of 295 nm and monitoring emission in the range of 300–400 nm. Fractional accessibilities (f_a) and quench constants (K_Q) were calculated using the modified Stern–Volmer equation as described under Experimental.

^b These values were obtained with the complex between HgCl₂-treated proMMP-2 and TIMP-2.

hancement in fluorescence quenching by both acrylamide (Fig. 3A) and iodide (data not shown) upon activation, indicating an activation-induced change in the accessibility of Trp residue(s) to quenching agent. This enhanced quenching is reflected by the increased fractional accessibility (f_a) of Trp in the HgCl₂-treated complex, similar to that observed with guanidine-treated proMMP-2/TIMP-2 (Table II), and suggests that a previously buried Trp residue(s) becomes available to solvent. A relatively small increase in the quench constant (K_Q) is observed with guanidine-treated proMMP-2/TIMP-2, suggesting that although more Trp residues are available to acrylamide in the guanidine-treated protein, it is more difficult to quench their fluorescence (22). In contrast, the K_Q of HgCl₂-treated MMP-2/TIMP-2 approaches that of free Trp in solution (17.5 M⁻¹; Ref. 22), demonstrating that all accessible Trp residues are at the protein surface. ProMMP-2 contains 4 Trp in the catalytic domain, 4 in the fibronectin-like domain, and 6 in the C-terminal domain with no Trp residues located in the propeptide, whereas TIMP-2 contains 4 Trp. The large K_Q is not attributable simply to quenching of Trp residues in TIMP-2 (Fig. 3B, Table II). Furthermore, mixing of free MMP-2 and TIMP-2 before or after mercurial treatment did not alter Trp quench curves (Fig. 3C, Table II). Together these data suggest that a conformational rearrangement in MMP-2/TIMP-2 accompanies mercurial treatment of the complex. This phenomenon appears to be unique to the proMMP-2/TIMP-2 complex, as quenching experiments with the MMP-9/TIMP-1 complex showed no differences in fluorescence parameters between latent and mercurial-activated complex (Fig. 2D).

In summary, using fluorescence quenching analysis as a conformational probe, no significant change in Trp environment is detectable following zymogen activation of proMMPs-1, -2, -3 and proMMP-9/TIMP-1. However, activation of proMMP-2/TIMP-2 does result in increased accessibility of previously buried Trp residue(s), providing biophysical evidence for a structural

rearrangement of the activated complex. This observation is in agreement with our recent biochemical studies which also predict a considerable structural rearrangement of the proMMP-2/TIMP-2 complex following activation by a mercurial compound, 4-aminophenylmercuric acetate (23). In this report, we demonstrated that mercurial treatment of proMMP-2/TIMP-2 induces a conformational perturbation which triggers disruption of the Cys⁷⁵-Zn²⁺ interaction in proMMP-2. The exposed active site then rapidly interacts with and is inhibited by the NH₂-terminal domain of TIMP-2 *prior to* propeptide autoproducting, resulting in the formation of an “activated proMMP-2/TIMP-2” complex. This structural rearrangement is supported by the observation that the proMMP-2/TIMP-2 complex can inhibit other active MMPs, while the activated complex is no longer inhibitory. Our fluorescence quenching studies provide additional evidence for a mercurial-induced structural rearrangement of the proMMP-2/TIMP-2 complex. The observed increase in fluorescence quenching may result from solvent exposure of previously shielded Trp residue(s) in the COOH-terminal domain of proMMP-2 or TIMP-2 in the activated complex. If formation of the proMMP-2/TIMP-2 complex shielded Trp from solvent, a decreased fractional accessibility of Trp in the complex formed between exogenously added proMMP-2 and TIMP-2, relative to that of proMMP-2 alone or TIMP-2 alone, would be predicted. However, no large changes in fractional accessibility were observed (Table II). These data suggest that the fluorescence changes associated with mercurial treatment of the proMMP-2/TIMP-2 complex likely reflect a novel structure in the complex between the “activated proMMP-2” (containing the propeptide) and TIMP-2. Such a structure is not formed in the case of the proMMP-9/TIMP-1 complex, as the propeptide of proMMP-9 in this complex is removed upon mercurial activation (28). The conformation of proMMP-2 which is trapped in the activated complex may be representative of a transitional structure induced in proMMP-2

by mercurial treatment prior to propeptide processing. In this scenario, propeptide processing to active MMP-2 is followed by a return to a tertiary structure similar to that of proMMP-2. It is interesting to speculate that mercurial treatment of other MMPs may initiate a similar transition to an unstable structural intermediate and a return to the proMMP conformation accompanying propeptide processing. However, definitive analysis of conformational changes associated with activation of proMMP-2/TIMP-2 and other MMPs must await detailed X-ray crystal structure analysis.

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