The N-Terminus of Collagenase MMP-8 Determines Superactivity and Inhibition: A Relation of Structure and Function Analyzed by Biomolecular Interaction Analysis†

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ABSTRACT: Tissue inhibitors of metalloproteinases (TIMPs) are the physiological, specific inhibitors of matrix metalloproteinases (MMPs) forming tight, noncovalent complexes. Therefore they control the proteolytic activity of MMPs toward the extracellular matrix. To analyze the inhibition of the "activated" and "superactivated" variants of human neutrophil collagenase (MMP-8) by TIMP-2, we determined complex dissociation constants using biomolecular interaction analysis (BIA). As it is known that the association rate constants can exceed the limits of the BIA instruments, the biomolecular interaction analysis was used to examine the equlibrium situation. The dissociation constants were determined by fitting the parameters of the mathematical term for the binding of collagenase onto the TIMP-coupled sensor chip surface to the saturation curve derived from individual sensorgrams. The resulting values are in the nanomolar range and correlate with the results of fluorescence kinetics. These data reveal that TIMP-2 (the recombinant inhibitory domain of human TIMP-2 and bovine TIMP-2 isolated from seminal plama) is a better inhibitor of the activated neutrophil collagenase than of the superactivated variant (the recombinant catalytic domain of human MMP-8). It has been demonstrated by X-ray analysis that the N-terminal heptapeptide only of superactivated MMP-8 is attached by a salt bridge and hydrophobic interaction to the C-terminal helix. Because these interactions have to be disrupted in the complex formation with TIMP we assume that the activated variant enables higher flexibility and a tighter induced fit in the complex formation. Therefore superactivation of MMP-8 correlates with weaker inhibition by TIMP-2.

Human matrix metalloproteinases are a family of zinc- and calcium-dependent endopeptidases that exhibit proteolytic activity toward most of the constituents of the extracellular matrix. Therefore they are highly involved in physiological processes such as embryonic development, angiogenesis, tissue remodeling, wound healing and pathological processes such as rheumatoid arthritis, periodontosis, tumor formation, metastasis, multiple sclerosis, liver fibrosis, cystic fibrosis, and aneurysms (for a review, see ref *4*).

Because of the substrate specificity and domain structure the family of matrix metalloproteinases can be divided into matrilysin, collagenases, stromelysins, metalloelastase, gelatinases, and membrane-type MMPs.[1] Collagenases such as fibroblast collagenase, neutrophil collagenase, and collagenase-3 exhibit the unique ability to cleave triple-helical collagen (*21*, *32*, *43*). They are secreted as inactive multidomain proenzymes, with the active-site zinc blocked by an unpaired cysteine within the strongly conserved PRCGVPD sequence motif of the propeptide (*58*). Upon activation this ∼80 residue N-terminal peptide is removed in a stepwise process (*29*, *45*). The zinc- and calcium-binding catalytic domain of about 170 residues is followed by the hemopexin-like domain of about 210 residues, which is important for substrate specificity (*30*, *57*).

The neutrophil collagenase (MMP-8) is synthesized during the early phases of cellular differentiation, stored as a highly glycosylated protein in the specific granules of neutrophils, and secreted as proenzyme after stimulation by inflammatory mediators (*20*, *29*, *40*, *62*). In vitro, activation cleavage initiated by proteinases, mercurials, or oxidants leads to active species having either Phe79, Met80, or Leu81 as the N-terminal residue and differing significantly in activity (*5*, *18*, *29*, *31*, *39*, *61*). The species upon stromelysin-1 activation is the Phe79 form, and because it is approximately 3.5-fold more active than the two other species, this phenomenon has been called superactivation (*31*, *53*). Likewise fibroblast collagenase can be superactivated by stromelysin-1 with an increase of proteolytic activity up to 12-fold (*24*, *41*, *61*).

Both the catalytic domain of MMP-8 with the N-terminal Phe79 and the N-terminal Met80 have been expressed in *Escherichia coli* (*52*, *57*) and crystallized (*6*, *53*) (Figure 1). Determination of the X-ray structures has revealed that the N-terminal Phe79 ammonium group makes a salt link with the side chain carboxylate group of Asp232. In contrast to the Met80 variant with its shortened N-terminus, this results in hydrophobic interactions of the N-terminal peptide with the C-terminal helix and therefore in a more ordered

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[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; BIA, biomolecular interaction analysis; RU, response unit; DMSO, dimethyl sulfoxide; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; SDS, sodium docyl sulfate; Tris-HCl, Tris-(hydroxymethyl)aminomethane hydrochloride.

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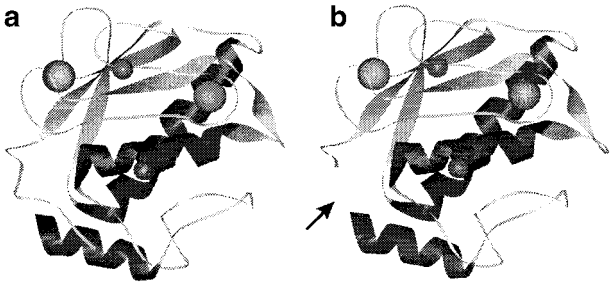

FIGURE 1: Structure of the catalytic domain of MMP-8 (*6*, *53*): (a) cdMMP-8^{Phe79} and (b) cdMMP-8^{Met80}. The arrow indicates that the N-terminal less ordered amino acids are not localized in the X-ray structure analysis.

N-terminus and probably in stabilization of the catalytic site via strong hydrogen bonds made by the adjacent Asp233 with the "Met-turn" that forms the base of the active-site residues (*53*).

The physiological antagonists of this proteinase family are the tissue inhibitors of metalloproteinases (TIMPs) comprising at least four proteins with molecular weights ranging from about 21 kDa up to 28.5 kDa for TIMP-1 that is highly glycosilated (*9*, *19*, *58*, *61*). They consist of 184−195 amino acids which include 12 highly conserved cysteine residues involved in the formation of six disulfide bonded loops (*66*). MMP inhibitory activity of TIMP-1 and TIMP-2 was located to the three N-terminal loops (*11*, *42*, *49*, *65*, *67*), while the C-terminal loops include additional binding sites for the gelatinases outside their catalytic domain (*22*, *65*). Because of these two major domains there are several binding modes comprising binary complexes such as progelatinase B/TIMP-1 (*64*), gelatinase A/TIMP-2 (*15*), or MT1-MMP/TIMP-2 (*56*), ternary complexes such as progelatinase A/TIMP-2/MMP-8 (*35*) or gelatinase B/TIMP-1/low molecular weight stromelysin-1 (*3*6), and quaternary complexes such as progelatinase B/lipocalin/TIMP-1/gelatinase B (*37*). Besides their inhibitory activity TIMP-1 and TIMP-2 exhibit also cell growth-promoting activity for a wide range of cells, for example erythroid cells (*14*, *16*, *60*), keratinocytes (*3*), or fibroblasts (*46*) probably mediated by TIMP receptors (*1*, *3*, *23*). Moreover TIMP-2 bound to a receptor on breast cancer cells enables the simultaneous binding of active gelatinase A (*10*). Because of these various interactions concerning the balance between TIMPs and MMPs or the cellular activity, it is of increasing interest to analyze complex formation at a molecular level.

One of the recent methods to analyze these biomolecular interactions is the BIA systems combining surface plasmon resonance detection, a suitable sensor chip chemistry, and an integrated flow system (*12*, *26*). The biomolecular interaction between an analyte and a ligand coupled to the sensor chip surface can be detected, because surface plasmon resonance measures changes in refractive index close to the sensor surface proportional to the surface concentration (g/m^2) of the analyte.

EXPERIMENTAL PROCEDURES

Equipment and Reagents. BIAcore2000^{TM} system, sensor chip CM5, NHS, EDC, and 1 M ethanolamine-HCl, pH 8.5, are from Biacore AB, Germany. The spectrofluorescence photometer is a Perkin-Elmer LS 50 B. RNA isolation and plasmid purification kits have been purchased from Qiagen. Oligonucleotides are from Genosys. Tth-polymerase and restriction endonucleases have been obtained from New England Biolabs. The ultrafiltration equipment is from Amicon, and the chromatographic material is from Pharmacia. The fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and the control Mca-Pro-Leu have been purchased from Bachem. All other chemicals have been obtained from Fluka or Merck.

Inhibitory Domain of Human TIMP-2. Total RNA was isolated from human synoviocytes SW 982 (ATTC: HTB-93) by Qiagen RNeasy RNA preparation kit and reverse-transcribed with BRL SuperScript reverse transcriptase according to standard procedures (*55*). The TIMP-2 cDNA was amplified by standard PCR with a sense primer introducing a *Nde*I recognition site and the start codon (5'-CATATGTGCAGCTGCTCCCCGG-3') and an antisense primer comprising a stop codon and a *Sal*I recognition site (5'-GTCGACTTATGGGTCCTCGATGTCG-3'). The cDNA was integrated into the pCRII vector (Invitrogen) and subsequently used as a template for a second PCR reaction generating the idTIMP-2 cDNA by using the sense primer and an antisense primer which introduces the stop codon and the *Sal*I recognition site behind the codon of Cys^{126} (5'-GTCGACTTAGCACTCGCAGCCC-3'). After digestion with *Nde*I and *Sal*I the idTIMP-2 cDNA was ligated into the *Nde*I and *Sal*I sites of the expression vector pET12b (Novagen) and used for transformation of the *E. coli* strain BL21[DE3]. Highly expressed idTIMP-2 was recovered from inclusion bodies by standard procedures (*55*), purified by Sephacryl S-200 gel filtration chromatography, and refolded with a glutathione/dithiothreitol redox system as described before (*7*, *28*). The homogeneous product was concentrated by ultrafiltration up to 250 μg/mL in native buffer (5 mM Tris/HCl, pH 7.0, 100 mM NaCl, 5 mM $CaCl_2$) and finally dialyzed against coupling buffer (10 mM sodium acetate pH 4.0, 100 mM NaCl).

Bovine TIMP-2. Bovine TIMP-2 was prepared stepwise from bovine seminal plasma by using Sephadex G-50 size exclusion chromatography, DEAE-Sephadex A25 ion exchange chromatography, and heparin affinity chromatography as described before (*8*). The solution of bTIMP-2 was finally dialyzed against coupling buffer and adjusted to 250 μg/mL, too.

Catalytic Domain of Neutrophil Collagenase. The N-terminal Met80 catalytic domain of MMP-8 (cdMMP-8^{Met80}) was expressed, refolded, and purified as described before (*52*, *57*). Briefly, the cDNA coding Met80-Gly242 was amplified by PCR from the plasmid pSVB30 bearing the cDNA for the short form of procollagenase (*57*). The sense primer 5'-TGGTCATATGTTAACCCCAGGAAACCCC-3' incorporates sequences for a *Nde*I site and the start codon, while the antisense primer 5'-GTTGGATCCTCATCCATAGATGGCCTGAAT-3' introduces sequences for the stop codon and a *Bam*HI site. After digestion of the PCR product with *Nde*I and *Bam*HI, this fragment was cloned into the *Nde*I and *Bam*HI sites of the T7 expression vector pET11a (Novagen) and used for transformation of the *E. coli* strain BL21[DE3]. The highly expressed cdMMP-8^{Met80} was renatured by dialyzing the inclusion bodies, solubilized in 6 M urea and 0.1 M β-mercaptoethanol, against a buffer containing 0.1 M NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, and 20 mM Tris/HCl, pH 7.5, and purified by hydroxamate affinity

chromatography.

The N-terminal Phe79 catalytic domain of MMP-8 (cdMMP-8^{Phe79}) was obtained by expression and renaturation of the short form of procollagenase (*57*) followed by activation with 10 nM stromelysin-1. The enzyme was purified as described for cdMMP-8^{Met80} and stored in sample buffer containing 5 mM Tris/HCl, pH 7.0, 5 mM $CaCl_2$, and 0.1 M NaCl.

Homogeneity of the protein preparations was analyzed by HPLC and SDS−PAGE, and the N-terminal sequences were verified by automated Edman-degration. Protein concentrations were determined by UV spectroscopy at 280 nm (*50*) with the molar extinction coefficients ϵ(cdMMP-8^{Phe79}) = 28 420 M^{-1} cm^{-1}, ϵ(cdMMP-8^{Met80}) = 28 420 M^{-1} cm^{-1}, ϵ(idTIMP-2) = 14 815 M^{-1} cm^{-1}, and ϵ(bTIMP-2) = 33 180 M^{-1} cm^{-1}. Moreover TIMP concentrations were determined by active-site titration (*44*) against the collagenase, the concentration of which had been quantified by amino acid analysis.

Biomolecular Interaction Analysis. Immobilization of the recombinant inhibitory domain of human TIMP-2 (idTIMP-2) and bovine TIMP-2 (bTIMP-2) by amine coupling was performed according to standard procedures at a flow rate of 5 μL/min (*51*). After activation of the sensor chip CM5 by a 7 min pulse of 0.05 M NHS/0.2 M EDC, the coupling was achieved by a 10 min pulse of ligand solution (about 250 μg/mL idTIMP-2 or bTIMP-2 in 10 mM sodium acetate, pH 4.0, and 100 mM NaCl). The excess active groups on the dextran matrix were blocked with 1 M ethanolamine-HCl, pH 8.5. Another flow cell was activated/deactivated without any ligand to obtain a control surface for unspecific binding. Finally the sensor chip was equilibrated with running buffer (5 mM Tris/HCl, pH 7.0, 5 mM $CaCl_2$, and 10 mM NaCl).

The binding assays were performed at 25 °C with running buffer, using analyte concentrations ranging from 0.12 to 1.96 μM cdMMP-8^{Phe79} and from 0.04 to 2.45 μM cdMMP-8^{Met80}. After a 4 min pulse of analyte the flow cells were washed with running buffer until constant response level. To regenerate the ligand for the next interaction, we achieved dissociation of the ligand analyte complexes by using a 1 min pulse of 100 mM 1,10-phenanthroline. Finally the flow cells were re-equilibrated with running buffer. The homogeneous 1:1 interaction of immobilized ligand (L) and analyte (A) on the sensor chip surface may be described by the dissociation constant K_i.

$$K_i = \frac{[A][L]}{[AL]}$$

The dissociation constant can be determined in a three-step procedure: (I) Plot of RU as a function of time for each analyte concentration (sensorgram): $RU = f(t)$. (II) Plot of the RU increase (ΔRU) versus the total analyte concentration (free and ligand-bound analyte) to obtain the saturation function: $\Delta RU = f([A_{tot}])$. (III) Simultaneous fitting of the three parameters ΔRU_{max} (maximal possible RU increase), $[L_{tot}]$ (concentration of free and analyte-bound ligand), and K_i to the saturation function (II) to obtain the K_i value by the following equation:

$$\Delta RU = \frac{\Delta RU_{max}}{[L_{tot}]}\left(\frac{[K_i] + [L_{tot}] + [A_{tot}]}{2} - \sqrt{-[L_{tot}][A_{tot}] + \left(\frac{[K_i] + [L_{tot}] + [A_{tot}]}{2}\right)^2}\right)$$

This equation results from a stepwise substitution, starting with the dissociation constant (f subscript = free, b subscript = bound to ligand or analyte):

$$K_i = \frac{[L_f][A_f]}{[AL]}$$

$$K_i = \frac{([L_{tot}] - [A_b])([A_{tot}] - [A_b])}{[A_b]}$$

$$[A_b] = \frac{\Delta RU[L_{tot}]}{\Delta RU_{max}}$$

Fluorometric Assay. In the fluorometric assay the collagenase variants cdMMP-8^{Phe79} and cdMMP-8^{Met80} catalyze the cleavage of the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 at the Gly-Leu bond (*34*). Turnover (25 °C, 50 mM Tris/HCl, 100 mM NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$, pH 7.5) was followed by the increase of fluorescence measured with a Perkin-Elmer fluorescence spectrophotometer LS 50 B (λ_{ex} = 328 nm, λ_{em} = 393 nm) calibrated with Mca-Pro-Leu (*34*). Turnover never exceeded more than 5% of substrate hydrolysis. At substrate concentrations of 1.0 μM and TIMP concentrations in the range of 10−100 nM reactions were started by addition of 1.0 nM enzyme. The increase of fluorescence was monitored until steady-state velocity was attained (v_s). The progress curves were analyzed by the GraFit program (Erithacus Software) using the integrated rate equation $P = v_s t + (v_o - v_s)(1 - e^{-kt})/k$ (*65*) in which P is the product concentration, v_o is the initial velocity, and k is the apparent first-order rate constant for the establishment of equilibrium between TIMP and MMP. Linear regression of a plot of $v_o/(v_s - 1)$ against the TIMP concentration provides $1/K_i$ (i.e., the reciprocal dissociation constant in the presence of substrate) (*54*).

RESULTS

Protein Preparation. The recombinant catalytic domain of human neutrophil collagenase (cdMMP-8^{Phe79} and cdMMP-8^{Met80}) expressed in *E. coli* was purified by hydroxamate affinity chromatography as described before. As the hydroxamate ligand in this final chromatographic step binds specifically to the active site of the collagenase, elution of the MMP variants under nondenaturing conditions provides a homogeneious preparation of active enzyme. Concentrations were determined photometrically and by amino acid analysis with a deviation of under 3%. These MMP preparations were used to quantify the concentration of the TIMPs by active-site titration (*44*). The "functional" concentrations of TIMPs were at least 95% of the "photometrical" concentrations and were used to calculate the dissociation constants.

TIMP/MMP Interaction. For efficient aminocoupling of a ligand on a CM5 sensor chip surface, it is necessary to preconcentrate the ligand in the surface matrix. Because in the CM series the sensor chip surface carries a net negative

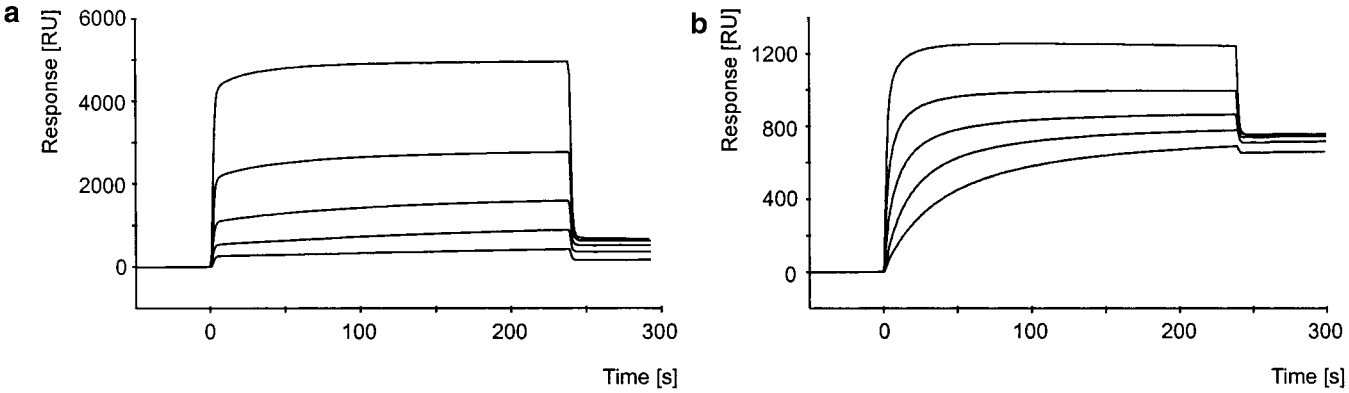

FIGURE 2: Sensorgrams for the binding of (a) cdMMP-8^{Phe79} and (b) cdMMP-8^{Met80} to idTIMP-2. The concentrations of MMPs were (a) 0.12, 0.25, 0.49, 0.98, and 1.96 μM and (b) 0.15, 0.31, 0.61, 1.23, and 2.45 μM.

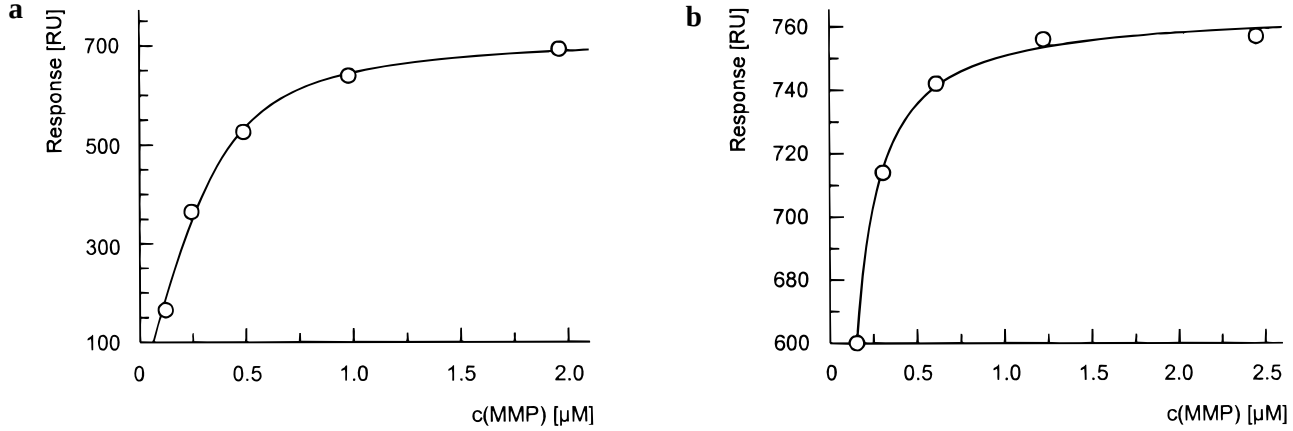

FIGURE 3: Saturation curves for the complexation of idTIMP-2 with (a) cdMMP-8^{Phe79} and (b) cdMMP-8^{Met80}.

charge resulting from unactivated carboxyl groups, preconcentration of ligand has been tested by binding of positively charged ligand in binding buffer (10 mM sodium acetate, pH 4.0, 100 mM NaCl). Although TIMPs and collagenases have been tested as ligands at concentrations up to 250 μg/mL in binding buffer, only electrostatic adsorption of TIMPs to the unactivated sensor chip leads to binding rates of several hundred response units. Therefore the collagenases are used as analytes and the TIMP ligands aminocoupled to the CM5 sensor chip according to standard procedure. Simultaneously a third flow cell has been activated/deactivated without ligand as a reference cell for unspecific binding.

When performing a series of complex association and dissociation cycles on a single sensor chip surface, it is important to avoid any baseline shift by incomplete complex dissociation. Therefore several reagents have been tested to dissociate the TIMP/collagenase complexes. Neither running buffer nor running buffer at pH 9.0 with 3 M NaCl and with 2% (v/v) Triton X-100 is able to dissociate the complexes. Even intense purging with solutions of 10 mM sodium acetate, pH 5.0, 100 mM glycine, pH 3.0, 10% (v/v) DMSO, 4 M urea, and 1% (w/v) SDS do not lead to complex dissociation of more than five percent measured in resonance units (data not shown). However a solution of 100 mM 1,10-phenanthroline allows one to restore the baseline completely even after more than 20 complex association and dissociation cycles.

After overlaying the individual sensorgrams of each concentration series (Figure 2), we plotted the final resonance unit levels against the respective concentration of analyte (Figure 3). These saturation curves are used to fit simultaneously the three parameters ΔRU_{max} (maximal possible RU increase), $[L_{tot}]$ (concentration of free and analyte-bound ligand), and K_i using $\Delta RU = f([A_{tot}])$. The dissociation constants range from 9.7 to 81.4 nM with a mean standard deviation of 2.8 nM (Table 1).

Table 1: Complex Dissociation Constants[a]

TIMP	collagenase	
	superactivated (nM)	activated
idTIMP-2	81.4 ± 4.8	18.8 ± 3.0
	(40.7 ± 2.8)	(13.7 ± 1.0)
bTIMP-2	37.3 ± 2.4	9.7 ± 0.8
	(19.7 ± 1.0)	(11.1 ± 0.6)

[a] K_i values are ± sd. K_i values in parentheses are from flourescence kinetics.

In the fluorometric assay the turnover of fluorogenic substrate by the collagenase variants was monitored in the presence of TIMPs until steady-state velocity was attained. Fitting of the integrated rate equation and linear regression of the quotient of the initial and steady-state velocitiy against the TIMP concentration results in the determination of apparent K_i values (i.e., complex dissociation constants in the presence of substrate). They range from 11.1 to 40. 7 nM with a mean standard deviation of 1.4 nM (Table 1).

DISCUSSION

The complex formation between matrix metalloproteinases and their inhibitors, the four known members of the TIMP

family, is a major aspect of physiological regulation of MMP activity in a lot of processes concerning the remodeling of the extracellular matrix. Moreover a pathological imbalance between MMPs and TIMPs is involved in a variety of diseases such as rheumatoid arthritis, periodontitis, liver fibrosis, chronic inflammations, tumor formation, and metastasis (*4*). Therefore there is a basic interest in analyzing these interactions of enzyme and inhibitor at a molecular level.

A recent method to examine the complex formation and dissociation of biomolecules is the biomolecular interaction analysis (BIA) that is used in this study (*51*). Interestingly there seems to be no free choice of ligand and analyte because only the TIMP molecules can be preconcentrated sufficiently on the CM 5 sensor chip surface under standard conditions at pH 4.0. One reason might be that the catalytic domain of MMP-8 is a very acidic protein with a calculated isoelectric point of only 4.6. In addition this molecule comprises only one lysine residue in contrast to 12 and 15 lysine redidues in idTIMP-2 and bTIMP-2 which are involved in aminocoupling. While cdMMP-8 seems to be a poor candidate for standard aminocoupling, the TIMP molecules were used as ligands leading to high coupling rates. Because the BIAcore2000 equipment enables programming and automated performing of a series of complex association and dissociation cycles on four parallel flow cells, it is important to dissociate the TIMP/MMP complexes quantitatively and to regenerate an analyte-free sensor chip surface with a constant response level after each cycle. Although a variety of potential complex denaturing conditions such as acidic and basic buffers, high ionic strength, detergents, and urea have been tested, only a solution of 100 mM 1,10-phenanthroline leads to complete dissociation of the TIMP/MMP complexes. This correlates with the very recent X-ray analysis of the crystallized bTIMP-2/cdMT1-MMP complex, demonstrating that the complex formation is achieved by direct interaction of the active-site zinc ion with the N-termial amino group of TIMP (*13*). As association rate constants of 2.1×10^7 and 3.8×10^7 M^{-1} s^{-1} have been reported for TIMP-2 complexed with gelatinase A (*47*, *65*), the BIA system with its diffusion-controlled limit of about 10^6 M^{-1} s^{-1} cannot be used to determine the association kinetics. In addition, there is no other way to analyze the kinetics of the TIMP-2/MMP-8 interaction because there is no complex dissociation under physiological conditions. The rapid decay of resonance units (and therefore a part of the increase in the beginning, too) is not caused by complex dissociation but by the change of buffer, especially the higher content of NaCl in the sample buffer. Such a change in buffer composition will also lead to a change in refractive index and thus to a shift in signal level (*27*). The final constant response level, however, indicates that there is no detectable complex dissiociation under physiological conditions (Figure 2).

Therefore the BIA measurement is analyzed by a different approach based on the saturation effect of the ligand-coated sensor chip surface by the analyte. Because the catalytic domain of MMP-8 comprises no C-terminal binding site for TIMPs (unlike, e.g., gelatinases), the mathematical term of the increase of response is a relative simple function of the total concentration of MMP-8. By computer-aided iterative fitting of this equation to the saturation curve of the TIMP-coated sensor chip surface, the dissociation constants can be determined.

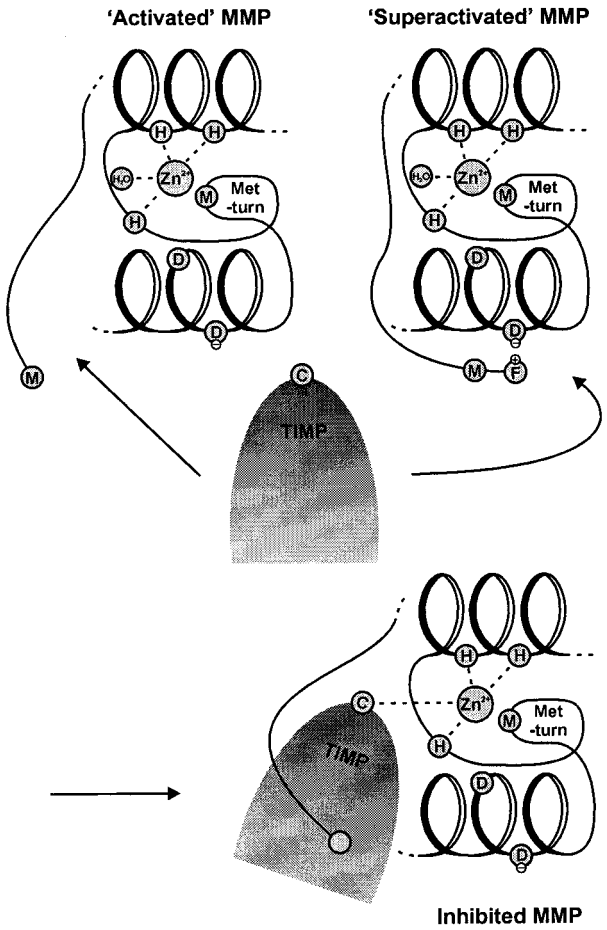

FIGURE 4: Schematic model of the different induced fit in the inhibition of the activated and superactivated collagenase by TIMP.

Both the residuals from the fitting of the BIA data and the initial velocities from the fluorometric assay were independent of the TIMP concentration, supporting the assumption of a bimolecular process (*65*). The results of both methods clearly correlate, especially regarding the internal relation of the constants. One reason for the small differences between BIA and fluorometry could be the nonspecific amine immobilization of the ligand resulting in reduced binding affinities (*38*). Moreover relatively high K_i values in the micromolar range have been reported measuring the complex dissociation of TIMP and C-terminal truncated gelatinase by BIA (*48*). Because the complex formation on the sensor chips is performed with immobilized ligands, at high reactant concentrations relative to K_i values, and in the absence of substrate, the BIA dissociation constants are only relative values and not directly comparable with the fluorometric dissociation constants that are apparent K_i values. The fact that none of the K_i values is in the subnanomolar range is in accordance with previous studies of complexes of truncated MMPs having higher dissociation constants than complexes of full-length molecules due to the lack of C-terminal interactions (*2*, *25*, *33*, *47*, *48*, *65*).

Both methods reveal that the mean dissociation constants of the superactivated variant are 4.1 times (BIA) and 2.4 times (fluorometry) higher than the constants of the activated variant (Table 1). These significantly higher dissociation constants of the complexes with the superactivated MMP-8

demonstrate that the activated form of the collagenase with N-terminal Met80 is better inhibited by TIMP-2 than the superactivated form with the N-terminal Phe79. The attached N-terminal residues with the ammonium group of Phe79 forming a salt link with the side chain carboxylate of Asp232 stabilize the globular protein structure as shown by X-ray analysis [ref *53*, Figure 1]. This putative more rigid structure of the Phe79-stabilized catalytic domain of human neutrophil collagenase seems to be the reason not only for the higher activity of this variant but also for the more difficult induced fit of the complex formation of enzyme and inhibitor. This interpretation is substantiated by analysis of the X-ray structure of the complex of TIMP-1 and the catalytic domain of stromelysin-1, which comprises the homologous residues Phe83 and Asp237 that do not form a salt bridge due to the binding of TIMP-1 (*17*). The flexibility of the N-terminal peptide seems to be a precondition of the binding of TIMPs as demonstrated also by the very recent X-ray analysis of the cdMT1-MMP/bTIMP-2 complex (13). Therefore we assume that binding of the N-terminal residues and the salt link formation of the superactivated collagenase (Phe79 variant) have to break up when TIMP-2 binds to this variant (Figure 4). Correspondingly, binding to the activated collagenase (Met80 variant) is stereochemically facilitated by the absence of this interfering N-terminal peptide and salt bridge. We conclude that there is a functional correlation of enzyme structure, superactivation, and weaker inhibition by TIMP.

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