Kinetic Analysis of the Binding of Human Matrix Metalloproteinase-2 and -9 to Tissue Inhibitor of Metalloproteinase (TIMP)-1 and TIMP-2*

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The dissociation constants (Kd) of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 for the active and latent forms of matrix metalloproteinase (MMP)-2 and MMP-9 were evaluated using surface plasmon resonance (SPR) and enzyme inhibition studies. SPR analysis shows biphilic kinetics with high (nM) and low (μM) affinity binding sites of TIMP-2 and TIMP-1 for MMP-2 (72- and 62-kDa species) and MMP-9 (92- and 82-kDa species), respectively. In contrast, binding data of TIMP-2 to an MMP-2 45-kDa active form lacking the C-terminal domain and to an MMP-2 C-terminal domain (CTD) fragment displays monophosphatic kinetics with Kd values of 315 and 60 nM, respectively. This suggests that the CTD contains the high affinity binding site, whereas the catalytic domain contains the low affinity site. Also, binding of TIMP-2 to pro-MMP-2 is stronger at both the high and low affinity sites than the corresponding binding of TIMP-2 to the MMP-2 62-kDa form demonstrating the importance of the N-terminal prodomain. In addition, the Kd value of TIMP-1 for the MMP-2 62-kDa species is 28.6 nM at the high affinity site, yet neither the MMP-2 45-kDa species nor the CTD interacts with TIMP-1. Enzyme inhibition studies demonstrate that TIMPs slow binding inhibitors with monophosphatic inhibition kinetics. This suggests that a single binding event results in enzyme inhibition. The kinetic parameters for the onset of inhibition are fast (koff = 10^8 M^-1 s^-1) with slow off rates (kcat = 10^-5 s^-1). The inhibition constants (Ki) are in the 10^-7 to 10^-5 M range and correlate with the values determined by SPR.

The gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are two members of the MMP family, a group of zinc-dependent endopeptidases known to hydrolyze many components of the extracellular matrix (1). Like other MMPs, the gelatinases are produced in a latent form (pro-MMP) requiring activation and are inhibited by TIMPs (1–3). A unique characteristic of the gelatinases is the ability of their zymogens to form tight non-covalent and stable complexes with TIMPs. It has been shown that pro-MMP-2 binds TIMP-2 (4), whereas pro-MMP-9 binds TIMP-1 (5). Although the physiological significance of the proenzyme-inhibitor complex is not completely understood, the complex may play a role in zymogen stabilization and activation (6–8). The interactions of TIMP-2 with pro-MMP-2 and of TIMP-1 with pro-MMP-9 were previously examined by analysis of enzyme activity using truncated enzymes and inhibitors (9–11). These studies demonstrated that the CTD of gelatinases increases the rate of association of the TIMPs for the active enzymes. Studies with activated and C-terminally truncated enzymes demonstrated that the catalytic domain is also involved in TIMP binding (9, 11). However, to date, no quantitative binding analyses of TIMP-1 or TIMP-2 for the latent forms of MMP-2 and MMP-9 have been described. We report herein the first such quantitative binding analysis by surface plasmon resonance (SPR) using highly purified recombinant enzymes and inhibitors (for reviews of SPR see Refs. 12–14). In addition, we report a quantitative analysis of the affinities of TIMP-1 and TIMP-2 for the active forms of either MMP-2 or MMP-9 both in the presence and absence of a substrate. These studies quantitatively define the nature of the unique interactions of MMP-2 and MMP-9 forms with TIMP-1 and TIMP-2.

EXPERIMENTAL PROCEDURES

Buffers—Buffer B (10 mM sodium acetate (pH 4.5)), buffer W (7.8 mM NaH₂PO₄, 8 mM Na₂HPO₄, pH 7.2), 137 mM NaCl, 0.1 mM CaCl₂, 3 mM KCl, 1.5 mM KH₂PO₄, and 0.02% Tween 20, buffer C (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35), buffer HA (25 mM Tris (pH 7.5), 25 mM NaCl, and 0.02% Brij-35), buffer R (50 mM Tris (pH 7.5), 5 mM CaCl₂, 0.01% Brij-35), and phosphate-buffered saline (10 mM NaPO₄, pH 7.2, 150 mM NaCl) were used.

Proteins and Enzymes—Molecular weight marker proteins for SDS-PAGE were purchased from Bio-Rad. Human recombinant stromelysin 1 was the generous gift of Dr. Paul Cannon (Center for Bone and Joint Research, Palo Alto, CA). A recombinant C-terminal fragment of human MMP-2, comprising amino acids 440–660 (15), was the generous gift of Dr. G. I. Goldberg (Washington University, St. Louis, MO).

Chromatographic Supports—Gelatin-agarose (4% cross-linked), heparin-agarose, Reactive Red 120-agarose, and lectin lentil-Sepharose 4B were purchased from Sigma. A Resource S column and Sephadex-G50 (fine) were purchased from Pharmacia Biotech Inc.

Expression and Purification of Gelatinases and TIMPs—Human pro-MMP-2, pro-MMP-9, and their inhibitors TIMP-1 and TIMP-2 were expressed in a recombinant vaccinia virus mammalian cell expression system, as described earlier (16). Pro-MMP-2 and pro-MMP-9 were purified to homogeneity from the media of infected HeLa cells by gelatin-agarose chromatography, as described previously (16). The protein concentrations of pro-MMP-2 and pro-MMP-9 were determined using their molar extinction coefficients of 122,800 and 114,360 M⁻¹ cm⁻¹, respectively (2). The MMP-2 45-kDa active form was isolated as described (17). The MMP-2 62-kDa species was freshly prepared by incubating pro-MMP-2 with 1 mM p-aminophenylmercuric acid (dissolved in 200 mM Tris) for 30 min at 37 °C. Under these conditions, only the MMP-2 62-kDa species was detected by gelatin zymography. To isolate the MMP-9 82-kDa species, 1 mg of pro-MMP-9 was incubated...
with 20 μg of a recombinant catalytic domain of stromelysin 1 for 2 h at 37 °C. The sample was subjected to gelatin-agarose column chromatography to remove the stromelysin 1, and the fractions containing the 82-kDa activated MMP-9 were detected by gelatin zymography. The protein concentrations of the active species of MMP-2 (45 kDa) and MMP-9 (82 kDa) were determined by Bradford assay analysis or from their molar extinction coefficients (18). The MMP-2 (45 kDa) and MMP-9 (82 kDa) species were distributed in aliquots, flash frozen in liquid nitrogen, and stored at −80 °C. Both enzymes were stable for at least 12 months at −80 °C, as determined by gelatin zymography.

Recombinant human TIMP-2 was purified from media of infected HEK293 cultures, as described (16), with the exception that instead of the CM-Sepharose matrix, the medium containing TIMP-2 was chromatographed on a Resource S column. TIMP-1 was purified by lectin lentil-Sepharose chromatography, as described (17). The TIMP-1-containing fractions were pooled, dialyzed against buffer HA to an ionic equivalent of less than 50 mM NaCl, and loaded onto a heparin-agarose column (5 ml) equilibrated with the same buffer. After the column was washed with HA buffer supplemented with 100 mM NaCl, TIMP-1 was eluted with a linear gradient of NaCl (200–400 mM) in HA buffer. The TIMP-1-containing fractions were pooled and dialyzed against phosphate-buffered saline. The protein concentrations of the recombinant TIMP-1 and TIMP-2 were determined using their molar extinction coefficients of 26,500 and 39,600 M−1 cm−1, respectively (3). Purified TIMP-1 and TIMP-2 were distributed in aliquots, flash frozen in liquid nitrogen, and stored at −80 °C.

SDS-Polyacrylamide Gel Electrophoresis and Zymography—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (19). The proteins were visualized by staining overnight with a 0.2% solution of Colloammine Brilliant R-250 in 45% methanol and 10% acetic acid and destaining in a solution of 20% methanol and 10% acetic acid. Zymography in a 10% SDS-PAGE containing 0.1% gelatin was performed as described (20).

Radioiodination of TIMPs—TIMP-1 and TIMP-2 were iodinated with carrier-free Na125I (100 mCi/ml, American Corp.) using IODO-GEN (Pierce). Briefly, glass vials were coated with 2 μg of IODO-GEN dissolved in 100% chloroform and dried with a stream of dry nitrogen. Carrier-free Na125I (100 mCi/ml, Amersham Corp.) using IODO-GEN was performed as described (20).

Determination of Kinetic and Equilibrium Constants by SPR—TIMP-gelatinase interaction studies were performed using a Fison Iasys instrument (M5T) research grade cells (Fison Iasys) were used for all experiments. The carboxymethyl dextran matrix of the sensor cell was conditioned (115 nm) in buffer HA to an ionic equivalent of less than 50 mM NaCl, and stored at −80 °C. TIMP-2 was covalently coupled to the activated matrix in buffer HA, and the dissociation rate constant. For TIMP-1 pro-MMP-2 and MMP-9 (latent and active species), MMP-2 CTD, TIMP-1, and TIMP-2 were incubated for 2 h at 37 °C. The sample was subjected to gelatin zymography.

Gelatin-Agarose Chromatography of TIMP-Gelatinase Complexes—Pro-MMP-9 and pro-MMP-2 (200 pmol) were combined with TIMP-1 and TIMP-2 (600 pmol), respectively, in buffer C (final volume of 0.1 ml) and incubated for 40 min at 25 °C. These mixtures were then applied to a gelatin-agarose column (0.1 ml) and equilibrated with buffer C, and high affinity binding sites, respectively. The binding constants for each analyte protein were determined in duplicate using at least five different concentrations of analyte (8.7–624 nM), in a final volume of 200 μl, where the response increased as a function of analyte concentration. For TIMP-1 pro-MMP-9 and MMP-9 (82 kDa) were both titrated from 10 to 200 nM; pro-MMP-2 and MMP-2 (62 kDa) were titrated from 20 to 250 and from 20 to 200 nM, respectively; MMP-2 (45 kDa) and the MMP2 CTD were titrated from 5 to 40 and from 50 to 1250 nM, respectively. For TIMP-2 pro-MMP-9 and MMP-9 (82 kDa) were titrated from 100 to 1000 nM; pro-MMP-2 and MMP-2 (62 kDa) were titrated from 50 to 500 and from 15 to 330 nM, respectively. Furthermore, each analyte protein (200 nm) was subjected to analysis using a derivatized sensor cell to determine the amount of nonspecific binding to the carboxymethyl dextran matrix. In each case, less than a 7-arc second decrease was observed. The binding curves were analyzed using the nonlinear data fitting program “Iasys Faisfit” using both monophasic and biphasic models to obtain the first-order association rate constant and the dissociation rate constant.

Binding of TIMPs to Gelatinases in Solution—125I-TIMP-1 or 125I-TIMP-2 were incubated at 1:1 or 3:1 molar ratios with the latent and active forms of MMP-2 and MMP-9 for 30 min at 25 °C in buffer C. Binding reactions were carried out in 500 μl (final volume) where the concentration of the gelatinases was 25 nM or in 40 μl (final volume) where the concentration of enzymes was 450 nM. After binding, a 50-μl aliquot of gelatin-agaroase matrix (50:50 slurry in buffer C) was added to each sample followed by incubation for 30 min at 25 °C. Each sample was centrifuged, washed three times with 400 μl of buffer C, and the resulting supernatant discarded. The radioactivity in the pellets was measured and a Pictet Siaflia gammarid clone for 3 ml of PMP-9 (0.5 ng) Lane 2, 125I-TIMP-9 (82 kDa) (0.15 ng). Lane 3, pro-MMP-9 (0.8 ng). Lane 4, MMP-2 (62 kDa) (0.2 ng). Lane 5, MMP-2 (45 kDa) (0.1 ng). Lane 6, MMP-2 CTD (100 ng). Lane 7, TIMP-1 (100 ng). Lane 8, TIMP-2 (115 ng).
the flow-through fraction was collected. The column was washed with 0.4 ml of buffer C and 0.1-ml fractions were collected. Twenty-five microliters of each fraction were analyzed by SDS-PAGE. Quantitation of the TIMPs and gelatinases in the complexes was determined by densitometric scanning of Coomassie Blue-stained gels, as described above, using known amounts of standard proteins.

Fluorometric Activity Assay for MMP-2 and MMP-9—The active forms of MMP-2 and MMP-9 were assayed for activity using the fluorescence quenching substrate MOAcPLGLA2pr(Dnp)-AR-NH2 (Peptide Institute, Inc., Japan, and first described by Knight et al. (21)). The peptide substrate was dissolved in 100% Me2SO. Each assay was carried out at 25 °C in 2 ml (final volume) of 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM CaCl2, 0.01% Brij-35, and Me2SO (1% v/v), containing 1 mM catalytic site of MMP-2 and MMP-9 species were allowed to bind to TIMP-1 and examined by SPR as described under “Experimental Procedures.” A, pro-MMP-9 (92 kDa), 40 nM, B, MMP-9 (82 kDa), 40 nM, C, pro-MMP-2 (72 kDa), 250 nM, D, MMP-2 (62 kDa), 65 nM, E, MMP-2 (45 kDa), 440 nM, F, MMP-2 CTD, 625 nM. The asterisk indicates the end of the association phase.

RESULTS

Analysis of Purified Gelatinases and TIMPs—Determination of the binding constants for gelatinase-TIMP complexes requires pure enzymes and inhibitors. To this end, human recombinant latent and active MMP-2 and MMP-9 and TIMP-1 and TIMP-2 were purified to homogeneity. To address the role of the MMP-2 CTD and the catalytic site of MMP-2, we purified a C-terminally truncated MMP-2 45-kDa form (17) and obtained a purified recombinant CTD fragment (15). Fig. 1, A and B, demonstrates the purity and lack of any contaminating proteins in the samples used for kinetic analyses. Furthermore, active enzymes were not detected in the latent enzymes and vice versa (Fig. 1, A and B). Incubation of the enzymes for 120 min at 25 °C prior to electrophoresis confirmed their stability, an essential requirement for the SPR analysis.

SPR Analysis of Gelatinase-TIMP Interactions—The kinetic and equilibrium constants for gelatinase-TIMP interactions were determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument. Since both TIMP-1 and TIMP-2 are acid stable (3), the inhibitors were chemically linked to the carboxymethyl dextran matrix on a sensor cell as described under “Experimental Procedures.” Dilute solutions of latent or active MMP-2 or MMP-9 and TIMP-1 and TIMP-2 were mixed with 0.4 pmol (0.2 nM) of enzyme. The recovery of enzyme activity was determined by SPR (12–14) using a Fison Iasys™ instrument.
observed when the enzymes or domains thereof were allowed to bind to a sensor cell derivatized in the absence of TIMPs (data not shown).

**SPR Analysis of Gelatinase-TIMP Interactions Reveal Low and High Affinity Sites**—The association rate constant ($k_a$), dissociation rate constant ($k_d$), and equilibrium constant ($K_d$) of MMP-2 and MMP-9 forms for TIMP-1 and TIMP-2 were calculated from the data obtained from the Fison Iasys™ analyses (Table I and Table II). To determine if the data fit the monophasic or biphasic models for nonlinear curve fitting, the following criteria were followed. First, random residuals for the nonlinear curve fitting of both the association and dissociation phases were required. Second, replotting of the ln of the association phase versus time and of the ln of the dissociation phase versus time was required to fit the theoretical plot provided by the software program Iasys Fasfit™. Third, the root mean square deviation following nonlinear curve fitting for each model was required to be less than 1%. For the monophasic model, the root mean square deviation was consistently greater than 5% for the association and dissociation rate constant determinations. However, analysis of the data fit the biphasic model since it showed that the root mean square deviation value ranged between 0.001 and 0.38% for the first-order association rate constant and dissociation rate constant values. As shown in Tables I and II, these analyses indicated the existence of high and low affinity binding sites. The $K_d$ values of TIMP-1 for the MMP-9 latent and active species were 35 and 23.9 nM for the high affinity site and 7.4 and 3.1 μM for the low affinity site, respectively. Interestingly, the $k_a$ and $k_d$ values of TIMP-1 for the latent and active MMP-9 forms and the MMP-2 (62 kDa) active species were similar for both the high and low affinity sites (Table I). With TIMP-2, the $K_d$ values for the latent and active MMP-2 species and the active MMP-9 were 5.2, 23.1, and 57.9 nM for the high affinity site and 0.19, 2.7, 12.7 μM for the low affinity site, respectively (Table II). Binding of the active MMP-2 (45 kDa) species and the CTD to TIMP-2 only fit the monophasic model, where the root mean square deviation value was consistently less than 0.092% for the first-order association rate constant and dissociation rate constant values. This indicates a single binding site with $K_d$ values of 315 and 61.6 nM, respectively (Table II). The lower $K_d$ value for the TIMP-2-MMP-2 CTD complex suggests that the high affinity binding site resides within this domain.

**Analysis of Gelatinase-TIMP Interactions in Solution**—Previous studies suggested a 1:1 stoichiometry of gelatinase-TIMP complexes (9, 10, 24). Due to the nature of the SPR analysis that requires immobilization of TIMPs, we examined the binding of gelatinases to the inhibitors in solution. To this end, unlabeled or radioiodinated TIMPs were allowed to bind to gelatinases, and the resultant complexes were analyzed by gelatin-agarose precipitation and gelatin-agarose chromatography. Binding of TIMP-1 and TIMP-2 to the latent and active forms of MMP-2 and MMP-9 was examined at concentrations at or near the $K_d$ values for the high affinity site as determined by SPR using equimolar concentrations of enzymes and inhibitors. To account for the presence of the low affinity site, we also carried out similar experiments using a 3-fold molar excess inhibitor. Complex formation of the gelatinases and TIMPs at or near the $K_d$ value for the high affinity site would be expected to reflect TIMP-gelatinase ratios of 0.5:1 and 1:1, whereas binding in the presence of excess TIMP would be expected to show a stoichiometry greater than 1:1 due to the low affinity site. Binding of equimolar and 3-fold molar excess of either 125I-TIMP-1 (Fig. 4A) or 125I-TIMP-2 (Fig. 4B) to pro-MMP-9, pro-MMP-2, and MMP-2 (62 kDa) demonstrated a stoichiometry of 0.65–0.84:1 (equimolar) and 0.9–0.94:1 (3-fold molar excess), indicating a 1:1 stoichiometry. Under the same conditions, the active MMP-2 (45 kDa) species showed no detectable binding to either TIMP-1 or TIMP-2 in agreement with the $K_d$ value determined by SPR (Table II). Since the SPR data indicated the existence of a low affinity site with $K_d$ values in the micromolar range (Tables I and II), we asked whether the stoichiometry of the enzyme-inhibitor complex could be forced to a ratio approaching 1:2 in solution. To this end, binding was carried out at concentrations of enzyme and inhibitor 20-fold greater than the $K_d$ for the high affinity site and at either equimolar ratios or 3-fold molar excess inhibitor. Fig. 4, C and D, shows that at 1:1 molar ratios, the stoichiometry is 1:1, as expected from the $K_d$ values determined by SPR. Under conditions of 3-fold molar excess inhibitor, gelatin-agarose precipitation experiments showed an increase in 125I-TIMP-1 binding to the enzymes with enzyme:inhibitor ratios of 1:1.4 to 1:1.8. In addition, coprecipitation of 125I-TIMP-2 was observed with the active MMP-2 (45 kDa) species, consistent with a ~1:1 stoichiometry (Fig. 4D). In contrast, 125I-TIMP-1 failed to coprecipitate with the 45-kDa species, regardless of the enzyme and TIMP concentrations used (Fig. 4, A and C).

Analysis of the stoichiometry of pro-MMP-2-TIMP-2 and pro-MMP-9-TIMP-1 complexes was also performed by densitometric analysis of SDS-polyacrylamide gels of enzyme-inhibitor complexes subjected to gelatin-agarose chromatography, as de-
Kinetics of Gelatinase-TIMP Interactions

Table I
Kinetic and equilibrium constants of gelatinase forms with TIMP-1 determined by SPR analysis

| Analyte protein | $k_a$ (1) $M^{-1} s^{-1} \times 10^{-3}$ | $k_a$ (2) $s^{-1} \times 10^{3}$ | $k_d$ (1) | $k_d$ (2) $M^{-1} s^{-1} \times 10^{-3}$ | $K_d$ $\mu M$ | $K_d$ $nM$ |
|----------------|----------------------------------|-------------------|--------|----------------|---------|--------|
| MMP-9 species  |                                  |                   |        |               |         |        |
| 92 kDa         | 34.2 ± 0.2                       | 4.0 ± 0.3         | 29.7 ± 3.9 | 1.2 ± 0.2     | 7.4 ± 0.9 | 35.0 ± 5.8 |
| 82 kDa         | 51.8 ± 0.1                       | 7.4 ± 0.3         | 23.0 ± 3.2 | 1.2 ± 0.2     | 3.1 ± 0.4 | 23.9 ± 3.8 |
| MMP-2 species  |                                  |                   |        |               |         |        |
| 72 kDa         | NB*                             | NB               | NB      | NB            | 8.5 ± 0.7 | 28.6 ± 4.5 |
| 62 kDa         | 44.0 ± 0.3                       | 4.0 ± 0.7         | 33.9 ± 2.9 | 1.3 ± 0.2     | 25.3 ± 1.9 | 61.9 ± 11 |
| 45 kDa         | NB                              | NB               | NB      | NB            | 12.7 ± 1.3 | 57.9 ± 7   |
| CTD            | NB                              | NB               | NB      | NB            | 6.7 ± 0.9  | 61.6 ± 11  |

* NB, no binding.

Table II
Kinetic and equilibrium constants of gelatinase forms with TIMP-2 determined by SPR analysis

| Analyte protein | $k_a$ (1) $M^{-1} s^{-1} \times 10^{-3}$ | $k_a$ (2) $s^{-1} \times 10^{3}$ | $k_d$ (1) | $k_d$ (2) $M^{-1} s^{-1} \times 10^{-3}$ | $K_d$ $\mu M$ | $K_d$ $nM$ |
|----------------|----------------------------------|-------------------|--------|----------------|---------|--------|
| MMP-9 species  |                                  |                   |        |               |         |        |
| 92 kDa         | NB*                             | NB               | NB      | NB            | 8.5 ± 0.7 | 28.6 ± 4.5 |
| 82 kDa         | 22.8 ± 0.2                       | 2.6 ± 0.2         | 33.1 ± 3.5 | 1.3 ± 0.2     | 12.7 ± 1.3 | 57.9 ± 7   |
| MMP-2 species  |                                  |                   |        |               |         |        |
| 72 kDa         | 140.6 ± 0.6                      | 24.7 ± 1.9        | 4.7 ± 0.4  | 0.7 ± 0.1     | 0.19 ± 0.02 | 5.2 ± 0.4 |
| 62 kDa         | 32.6 ± 0.3                       | 4.8 ± 0.8         | 12.7 ± 0.6 | 0.8 ± 0.1     | 2.7 ± 0.1  | 23.1 ± 4.1 |
| 45 kDa         | 3.2 ± 0.2                        | 1.0 ± 0.1         | 5.7 ± 0.9  | 1.3 ± 0.2     | 13.5 ± 1.7 | 61.9 ± 11  |
| CTD            | 92.1 ± 3.9                       |                   |        |               | 6.7 ± 0.9  | 61.6 ± 11  |

* NB, no binding.

Fig. 4. Coprecipitation of gelatinase-TIMP complexes. 125I-TIMP-1 and 125I-TIMP-2 were allowed to bind to MMP-9 (latent and active species) and to MMP-2 (latent and active species) for 30 min at 25 °C, and the resulting complexes were subjected to precipitation with gelatin-agarose as described under “Experimental Procedures.” A, gelatinases (25 nM) incubated with 25 nM (solid bars) or 75 nM (dotted bars) 125I-TIMP-1. B, gelatinases (25 nM) incubated with 25 nM (solid bars) or 75 nM (dotted bars) 125I-TIMP-2. C, gelatinases (450 nM) incubated with 450 nM (solid bars) or 1.4 μM (dotted bars) 125I-TIMP-1. D, gelatinases (450 nM) incubated with 450 nM (solid bars) or 1.4 μM (dotted bars) 125I-TIMP-2. The error bars represent the standard deviation from three independent determinations.
MMP-2 and MMP-9 in the picomolar range (9, 10, 25). Since $K_d$ is equal to $K_s$, when inhibition is studied, the data derived from SPR analysis indicated $K_s$ values in the nanomolar range. Therefore, binding and affinity of the TIMPs for the gelatinases were evaluated using enzymatic activity assays with the peptide substrate MOAcPLGLA2pr(Dnp)-AR-NH2 (21). As shown in Fig. 6A, the enzymes show saturation kinetics in hydrolysis of the peptide substrate. Double-reciprocal analysis of the data (Fig. 6B) allowed for determination of the $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values (Table III). Insofar as $K_m$ may approximate $K_s$, and as such give an expression of affinity, these enzymes show essentially the same affinity for the substrate with $K_m$ values in the range of 1.5 to 3 μM. In addition, the correlation coefficient ($r^2$) values for the fitted lines are ~0.99 (Fig. 6B). Thus, the results indicate that all these enzymes are equally competent as catalysts in hydrolysis of the synthetic substrate with $k_{cat}/K_m$ values equivalent to or greater than values obtained previously (10, 11).

Enzymatic Determination of the Inhibition Constant of TIMP-1 and TIMP-2 for the Gelatinases—We examined the binding of TIMP-1 and TIMP-2 to the active forms of MMP-2 and MMP-9 by enzyme inhibition assays as described under "Experimental Procedures." As shown in Fig. 7, TIMP-1 and TIMP-2 inhibit each enzyme. However, TIMP-1 failed to inhibit the active MMP-2 (45 kDa) species even at concentrations of 200 nM (Fig. 7A). The pattern of inhibition was consistent with a slow binding process (22). This type of behavior is characterized by the formation of curves that display a time-dependent onset of inhibition within the period that substrate turnover is linear in the absence of inhibitor. For slow binding inhibition, the first-order rate constant ($k$) is equal to the rate of product formation that is derived from the asymptote, where the $k$ value is determined from the intersection point of the tangent at $t = 0$, as given by the expression $P = v_0 + (v_{max} - v_0)/k$. The asymptote of the curve is given by the equation $P = v_0 + (v_{max} - v_0)/k$. At this point, $k = 1/t$ (22). The association rate constant ($k_a$) for the formation of enzyme-inhibitor complexes is determined from these progress curves of substrate hydrolysis. The second-order rate constant ($k_{cat}$) is provided by linear regres-
sion of $k$ as a function of inhibitor concentration. The off-rate ($k_{off}$) was determined by the recovery of enzyme activity (Fig. 8).

Tables IV and V show the $k_{on}$ and $k_{off}$ values using the analysis described above. TIMP-1 inhibits both MMP-9 (82 kDa) and MMP-2 (62 kDa) enzymes with comparable rate constants for inhibition onset ($k_{on}$) and recovery of activity ($k_{off}$), and by consequence result in similar $K_i$ values (8.5 and 9.7 nM, respectively). The $k_{on}$ is fast ($>10^5$ M$^{-1}$ s$^{-1}$) and $k_{off}$ is slow ($<10^{-3}$ s$^{-1}$), resulting in effective inhibition. The same trend is true for TIMP-2 with MMP-9 (82 kDa) and MMP-2 (62 kDa) enzymes ($K_i$ values of 43.4 and 7.2 nM, respectively). For the active MMP-2 (45 kDa) species, the $k_{on}$ (1.4 x $10^4$ M$^{-1}$ s$^{-1}$) was considerably slower resulting in a $K_i$ value of 275 nM, indicative of a relatively poor affinity of the inhibitor for the truncated enzyme. The $k_{on}$ values (Tables IV and V) of TIMP-1 for MMP-2 (62 kDa) and MMP-9 (82 kDa) and of TIMP-2 for MMP-2 (62 and 45 kDa) and MMP-9 (82 kDa) species were consistently greater (2.5 to 7-fold) than those determined by SPR (Tables I and II). Likewise, the $k_{off}$ values (Tables IV and V) were 1.5 to 3-fold higher than those determined by SPR (Tables I and II). The calculated $K_i$ values of TIMP-1 and TIMP-2 for the MMP-2 (62 and 45 kDa) and MMP-9 (82 kDa) (Tables IV and V) species were in the nanomolar range and within 3-fold of the $K_i$ values determined by SPR (Tables I and II).

DISCUSSION

We have carried out a comprehensive study to determine the kinetic parameters for the binding of TIMP-1 and TIMP-2 to the latent and active forms of MMP-2 and MMP-9. The results of the SPR analyses were consistent with previous studies (4, 5, 9–11) demonstrating binding of TIMP-1 to the latent and active MMP-9 and MMP-2 (62 kDa) species, and binding of TIMP-2 to the latent and active MMP-2 species, and the active form of MMP-9 (82 kDa). In addition, TIMP-1 and TIMP-2 failed to interact with pro-MMP-2 and pro-MMP-9, respectively (for reviews see Refs. 2 and 3), as expected. We have also shown that TIMP-1, in contrast to TIMP-2, did not bind to the CTD of MMP-2 or to the MMP-2 45-kDa species. SPR methodology for analysis of TIMP-MMP interactions was used in a previous study by Bodden et al. (26) which demonstrated $k_{on}$, $k_{off}$, and $K_i$ values of 8.9 x $10^4$ M$^{-1}$ s$^{-1}$, 3.6 x $10^{-4}$ s$^{-1}$, and 4.1 nM, respectively, for the complex of TIMP-1 with active MMP-1. The $K_i$ values of the MMP-1/TIMP-1 interaction (determined by SPR) are 7- and 14-fold lower than those for TIMP-1 with the active MMP-9 (82 kDa) and MMP-2 (62 kDa) species, respectively, as reported here. This suggests that TIMP-1 may be a more efficient inhibitor of MMP-1 than of MMP-9 and MMP-2. Previous studies demonstrated that binding of TIMP-1 and
TIMP-2 to MMP-9 and MMP-2 is mediated by two distinct domains of the inhibitor molecules and two domains of the enzymes (3, 9–11, 25, 27). The N-terminal domains of TIMPs were shown to bind to the enzymes within the active site of the catalytic domain. Alternatively, the low affinity site in the MMP-2 62-kDa enzyme may be different from that in the 45-kDa species, and its accessibility is only possible after removal of the CTD. The lack of any measurable binding of TIMP-1 to both the 45-kDa active species and the CTD were unexpected since the 62-kDa form bound TIMP-1 with high affinity. Since the binding of the 62-kDa species to TIMP-1 also showed biphasic binding, it is possible that the CTD of MMP-2 may work synergistically with the active site in promoting TIMP-1 binding.

The contribution of the N-terminal prodomain of the gelatinase for the binding of TIMPs was also made apparent by the SPR analysis, particularly with MMP-2. Here, removal of the N-terminal prodomain resulted in a decreased affinity of TIMP-1 for both the 45-kDa active species (315 nM) was 8.5-fold lower than that for the low affinity site of TIMP-2 for the MMP-2 62-kDa form (2.7 μM), which presumably resides within the catalytic domain. Thus, removal of the CTD appears to increase the affinity of TIMP-2 for the catalytic domain. Alternatively, the low affinity site in the MMP-2 62-kDa enzyme may be different from that in the 45-kDa species, and its accessibility is only possible after removal of the CTD. The lack of any measurable binding of TIMP-1 to both the 45-kDa active species and the CTD were unexpected since the 62-kDa form bound TIMP-1 with high affinity. Since the binding of the 62-kDa species to TIMP-1 also showed biphasic binding, it is possible that the CTD of MMP-2 may work synergistically with the active site in promoting TIMP-1 binding.

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We have also observed that binding of gelatinases to the TIMPs, at concentrations near or below the $K_v$ values, for the high affinity sites showed sub-stoichiometric enzyme-inhibitor complexes consistent with $K_v$ values in the nanomolar range. Likewise, binding at concentrations greater than the $K_v$ values for the high affinity sites resulted in the generation of near-stoichiometric complexes. Taken together, these data suggest that the two sites of interaction are unique and can bind inhibitor independently. It is difficult to envision the evolutionary reason for conservation of the second TIMP binding site, if it were irrelevant in vivo. The existence of the second site awaits resolution of the crystal structures of the gelatinase-TIMP complexes. Furthermore, regarding pro-MMP-2, the interaction of this enzyme with TIMP-2 will have to be addressed in the context of a current model describing the association of pro-MMP-2 with a MT1-MMP-TIMP-2 complex (7).

We determined the $k_{on}$, $k_{off}$, and $K_v$ values by enzyme inhibition analysis and demonstrated that both TIMP-1 and TIMP-2 behave as slow binding inhibitors. Using kinetic treatment for slow binding inhibition (22), the results indicated that the association of TIMPs and gelatinases is rapid ($k_{on}$, $10^{-4}$ $M^{-1}$ s$^{-1}$). Furthermore, the dissociation of the enzyme-inhibitor complexes was slow ($k_{off}$, $10^{-3}$ s$^{-1}$), resulting in a very effective inhibition of activity. The $k_{off}$ values were determined from recovery of enzyme activity as a function of time, which allowed for the determination of the $K_v$ value. This value was calculated to be in the nanomolar range, similar to the results obtained by the SPR analysis. Also, in agreement with the SPR results, the enzyme-inhibition studies demonstrated that the 45-kDa species of MMP-2 exhibited a 35-fold reduction in affinity (275 nM) for TIMP-1, and Taylor et al. (28) recently reported that pro-MMP-2

It is significant that the SPR analysis, which evaluates protein-protein interactions without regard for inhibition of enzymatic activity, indicated biphasic behavior and provided $K_v$ values for the high affinity site similar to the $K_v$ values determined from enzyme inhibition assays. The fact that the kinetics of inhibition are monophasic clearly indicates that one binding event accounts for the onset of enzymatic inhibition. However, the presence of a second gelatinase-TIMP interaction site in the latent and active forms was clearly evident in the SPR experiments with the 45-kDa species of MMP-2 and the CTD fragment. The role of the second site in manifestation of enzyme inhibition is unclear. We wish to underscore that the kinetic parameters for enzyme inhibition corresponded closely (within 3-fold) to those for the high affinity phase of the protein-protein interaction determined by SPR analysis. Thus, two entirely distinct analyses provided essentially similar results.

The ultimate structural information should await elucidation of the crystal structures for the gelatinases and gelatinase-inhibitor complexes.

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