Kinetic Analysis of the Mechanism of Interaction of Full-Length TIMP-2 and Gelatinase A: Evidence for the Existence of a Low-Affinity Intermediate†

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ABSTRACT: We have undertaken a detailed analysis of the mechanism of inhibition of matrix metalloproteinase-2 (gelatinase A) by tissue inhibitor of metalloproteinase-2 (TIMP-2). Quenched fluorescent substrates have been used to analyze the rate of inhibition of gelatinase A by TIMP-2 over a wide range of TIMP-2 concentrations. When the values of the observed rate constant for the inhibition are plotted against TIMP-2 concentration, saturation is observed at high concentrations, providing evidence for formation of an intermediate in the pathway. Rate constants for the formation and dissociation of the intermediate are 5.9×10^6 M^{-1} s^{-1} and 6.3 s^{-1} respectively, giving a K_i for the initial step of approximately 1 μM. The rate constant for the association of the final complex is 33 s^{-1}. By studying the dissociation of ^{125}I-labeled TIMP-2 from a gelatinase A−TIMP-2 complex using ligand exchange experiments, we obtained a rate constant for the dissociation of the final stable complex of 2×10^{-8} s^{-1}. This gives a value for the overall dissociation constant of approximately 0.6 fM.

The tissue inhibitors of metalloproteinases (TIMPs)[1] are specific protein inhibitors of the matrix metalloproteinases, a group of zinc-dependent enzymes that include the collagenases, gelatinases, and stromelysins and that degrade connective tissue at physiological pH and are therefore important in normal embryonic development, morphogenesis, and tissue remodeling (*1*). To date, four members of the family (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified and cloned from a number of species (*2*−*5*). The TIMPs are believed to be important in regulating matrix metalloproteinase activity, and an imbalance in the relative concentrations of enzyme and TIMP has been observed in many degradative diseases (*6*).

Individual TIMPs show about 40% sequence identity, but they share considerable higher structural similarity, notably the conservation of 12 cysteine residues that have been shown to form disulfide bonds in TIMP-1, giving a 6 loop structure (*7*). The correct tertiary structure is essential for activity, and reduced TIMPs and tryptic fragments are inactive (*8*). All the TIMPs inhibit active MMPs, binding in a 1:1 molar ratio to form tight complexes with K_i values of less than 10^{-10} M (*9*). Kinetic studies using quenched fluorescent substrates to follow the association of TIMP and enzyme have demonstrated that the mechanism is complex, involving at least two separate binding sites.

Recently NMR and crystallographic data for the complexes of the catalytic domain of stromelysin-1 (MMP-3) with a truncated form of TIMP-2 consisting of the three N-terminal loops (*10*) and full-length TIMP-1 (*11*), respectively, have provided invaluable information on the nature of the interaction between TIMPs and MMPs in the final complex. TIMP-1 binds in the active site cleft of stromelysin-1, blocking access to the catalytic site. The N-terminal α-amino and carbonyl groups of Cys-1 of TIMP-1 coordinate the catalytic zinc. The binding region is very large, covering about 1300 Å^2, and consists of six separate polypeptide segments of TIMP-1 (*11*).

To date, accurate analysis of K_i values lower than 200 pM has not been possible using steady-state kinetic techniques (*9*). This is due to both substrate insensitivity and the long incubation times that would be required to allow the reaction to reach equilibrium. We have therefore developed further methods to analyze the mechanism of TIMP interaction, and in the present work we characterize in detail the kinetics of the interaction between gelatinase A (EC 3.4.24.24; MMP-2) and TIMP-2. This reaction is of particular interest physiologically because the cell surface activation of pro-gelatinase A has been shown to involve a trimolecular complex of pro-gelatinase A, MMP-14, and TIMP-2 (*12*). It has also been shown that in mouse embryonic development MMP-14 (a membrane-bound MMP; MT1-MMP) expression correlates spatially and temporally with that of gelatinase A and TIMP-2 (*13*). Our work demonstrates that binding of gelatinase A and TIMP-2 is extremely tight and that it occurs by a two-step process involving formation of an initial complex followed by a slower complex stabilization step.

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[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; APMA, *p*-aminophenylmercuric acetate; FPLC, fast protein liquid chromatography.

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MATERIALS AND METHODS

Materials

All materials were purchased from Sigma−Aldrich unless otherwise stated. Bolton and Hunter reagent was purchased from Amersham Life Science.

Methods

Recombinant human pro-gelatinase A was purified from conditioned medium from transfected mammalian cell lines as described previously (*14*). Active gelatinase A was prepared by incubating pro-gelatinase A with 2 mM APMA for 120 min at 25 °C, followed by a 10-fold dilution in 50 mM Tris/HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl, 0.025% Brij 35. Recombinant human TIMP-2 was prepared as (*15*), modified with a final purification step using a Sephacryl S200 gel filtration column equilibrated with 25 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.02% NaN_3. The concentrations of TIMP-2 and gelatinase A were determined by active site titration as described previously (*9*).

Radiolabeling. TIMP-2 was labeled with ^{125}I using Bolton and Hunter reagent (*16*), and the ^{125}I-labeled protein was separated from other labeled products of the conjugation reaction using Sephadex G-15 spin columns equilibrated with 50 mM Tris/HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl, 0.025% Brij 35. The specific activity of ^{125}I-TIMP-2 was calculated to be 0.094 μCi/μg.

Kinetic Studies. (1) Dissociation of Enzyme−Inhibitor Complex. Unless otherwise stated, all reactions were performed in 50 mM Tris/HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl, 0.025% Brij 35. The dissociation was followed by mixing a complex of ^{125}I-TIMP-2 with gelatinase A (prepared by preincubating equimolar concentrations of enzyme and inhibitor for 18 h) with a 10-fold excess of unlabeled TIMP-2 at 25 °C. At time intervals for up to 6 weeks, small samples were removed, and TIMP-2 that was complexed to enzyme was separated from free TIMP-2 by gel filtration using a Superdex 75 FPLC column. The two pools of radioactive TIMP-2 were quantified, and the rate of release of ^{125}I-TIMP-2 was determined as the gradient of a plot of ln [complexed TIMP-2] against time. The experiment was also performed using unlabeled TIMP in the initial complex and by incubating this complex with a 10-fold excess of ^{125}I-TIMP to check whether labeling of the TIMP-2 with ^{125}I affected the stability of the complex.

(2) Association of Enzyme and Inhibitor. Inhibition of enzyme activity was monitored continuously using the substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (*17*) at a concentration of 1 μM in a fluorometric assay as described previously (*15*). The assays were performed with an enzyme concentration of 40 pM and a TIMP concentration in the range of 100−250 pM. The data were fitted to the equation:

$$P = (v_i - v_s)(1 - e^{(-k't)})/k' + v_s t \qquad (1)$$

in which P is the product at time t, v_i and v_s are the initial and steady-state rates, respectively, and k' is the apparent rate constant for the reaction (*18*). The data were collected over a range of TIMP-2 concentrations, and k, the association rate constant, was obtained as the gradient of the plot k' against TIMP-2 concentration.

Stopped-flow analysis was carried out on an Applied Photophysics stopped-flow spectrofluorometer (slit widths = 10 nm) using an excitation wavelength of 328 nm and monitoring fluorescence emission above 360 nm. Four thousand data points were recorded over the course of each reaction, and at least five runs were analyzed for each measurement by fitting the data to eq 1 to obtain values for k'.

The formation of gelatinase A−TIMP-2 complex was also followed using ^{125}I-TIMP-2 and quantifying the concentrations of labeled TIMP-2 associated with gelatinase A for set time intervals. Enzyme and ^{125}I-TIMP were incubated in buffer at 25 °C. At time intervals, the reaction was stopped by the addition of a large excess of unlabeled TIMP-1 (approximately 1000-fold) which bound to free enzyme and to the enzyme that was loosely bound to ^{125}I-TIMP-2 and released during the subsequent purification step. To the reaction mixture was added 1 mL of gelatin−agarose, and this was mixed at 25 °C for 30 min, after which time the mixture was centrifuged at 3000*g* for 3 min and the number of counts in the pellet and the supernatant were quantified in a Canberra Packard Cobra Multi-Prias gamma counter. The determination was performed at a number of enzyme and inhibitor concentrations. As the reaction was effectively irreversible over the time scale of the experiment, the results were analyzed by fitting the data to the equation for a second-order irreversible reaction using Grafit (*19*).

RESULTS

Effect of Iodination on TIMP-2 Activity. Labeled TIMP-2 was tested initially by titration of the TIMP-2 against active gelatinase A of known concentration. This confirmed that the labeled preparation consisted of fully active TIMP-2. The effect of iodination was further investigated by comparing the rate of association of labeled and unlabeled TIMP-2 with gelatinase A. The concentration range of TIMP-2 used for this analysis was 50−200 pM. In this concentration range, the dependence of the observed rate constant for the association, k', on TIMP-2 concentration is linear (*15*) for unlabeled TIMP-2. The same dependence was observed for the ^{125}I-TIMP-2, with association rate constants of 2.6×10^7 and 2.1×10^7 M^{-1} s^{-1} for labeled and unlabeled TIMP-2, respectively. Thus, TIMP-2 activity appears to be unaffected by the degree of iodination used in the present work. The results described below further support this conclusion.

Dissociation of the TIMP-2−Gelatinase A Complex. This was studied by following the exchange of free unlabeled TIMP-2 with complexed ^{125}I-TIMP-2 over a period of time. The release of ^{125}I-TIMP-2 from the ^{125}I-TIMP-2−gelatinase A complex was followed for 40 days, during which time only a small proportion (≅5%) of the labeled TIMP dissociated from the complex. The half-life of the complex is considerably longer than the duration for which the experiment could be practically performed, and the estimated rate constant obtained from such data of 2×10^{-8} s^{-1} (i.e., $t_{1/2} \cong 1$ year) is thus subject to large error. The experiments were also performed using unlabeled TIMP-2−gelatinase A complex which was incubated with excess ^{125}I-TIMP-2, and also by incubating ^{125}I-TIMP-2−gelatinase A complex with

Table 1: Effect of the Competing Ligand on the Rate of Dissociation of the TIMP-2−Gelatinase A Complex

TIMP in complex	competing TIMP	k_{off} (s^{-1})
^{125}I-TIMP-2	TIMP-2	3×10^{-8}
TIMP-2	^{125}I-TIMP-2	2×10^{-8}
^{125}I-TIMP-2	TIMP-1	2×10^{-7}

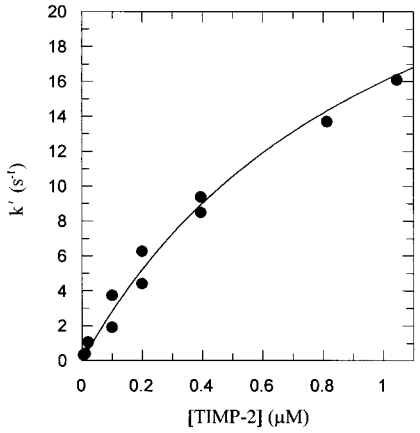

FIGURE 1: Dependence of the observed first-order rate constant, k', on TIMP-2 concentration. The data were fitted to eq 4 with k_{-2} fixed as 2×10^{-8} s^{-1}. Parameters for the fitted curve are $k_1 = 5.9 \times 10^6$ M^{-1} s^{-1}, $k_2 = 33$ s^{-1}, and $k_{-1} = 6.3$ s^{-1}.

excess cold TIMP-1. The results, summarized in Table 1, demonstrate that iodination of TIMP-2 does not affect the rate of complex dissociation. A 10-fold increase in the rate is observed when TIMP-1 is used as the competing ligand, suggesting that excess TIMP-2 may exert a small influence on the stability of the enzyme−inhibitor complex. The significance of such an effect is uncertain, however, due to the large margin of error inherent in the experiment.

Kinetics of the Association of TIMP-2 with Gelatinase A. Complex formation was studied using two methods: (i) by following the rate of inhibition of gelatinase A activity and (ii) by measuring the rate of formation of complex directly using ^{125}I-TIMP-2.

(i) Inhibition of Gelatinase A Activity. Our previous studies (*15*) involved following the rate of inhibition of gelatinase A over a concentration range of up to 200 pM TIMP-2. In the present work, we are able to extend the range of concentrations of TIMP-2 considerably by using rapid reaction techniques. TIMP-2 concentration was 50 pM−1 μM, and the gelatinase A concentration was in the range 5 pM−5 nM. The concentration of TIMP-2 was always at least 10-fold greater than the gelatinase A concentration, and the observed rate constant for the inhibition of enzyme activity, k', was determined by fitting data for each progress curve as described under Methods. A plot of k' against [TIMP-2] is shown in Figure 1. The saturation effect seen at high TIMP-2 concentrations provides evidence for an intermediate in the pathway.

The simplest model to fit the data in Figure 1 is shown in Scheme 1:

Scheme 1

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

where E and I denote the enzyme and inhibitor, respectively,

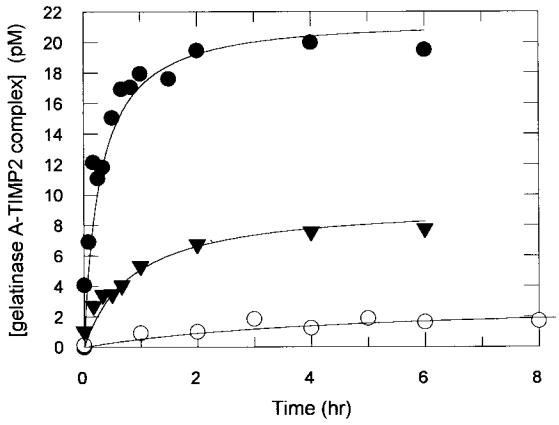

FIGURE 2: Rate of binding of gelatinase A to ^{125}I -TIMP-2. Data were obtained at several TIMP-2 and gelatinase A concentrations and analyzed to obtain the apparent second-order rate constant. Concentrations used in the figure are 4 pM (○), 10 pM (▼), and 21 pM (●), giving values of 4.8×10^7, 2.5×10^7, and 5.1×10^7 M^{-1} s^{-1}, respectively.

and EI and EI* are two isomers of the enzyme−inhibitor complex. Equations 2−4 characterize the mechanism:

$$K_i = k_{-1}/k_1 \quad (2)$$

$$K_i^* = K_i k_{-2}/(k_2 + k_{-2}) \quad (3)$$

$$k' = k_{-2} + k_2[k_1 I/(k_{-1} + k_1 I)] \quad (4)$$

in which K_i and K_i^* are the dissociation constants for the initial complex and final complex, respectively (*18*). When the data in Figure 1 are fitted to eq 4 with k_{-2} fixed at 2×10^{-8} s^{-1}, values for the remaining rate constants are as follows: $k_1 = 5.9 \times 10^6$ M^{-1} s^{-1}, $k_2 = 33$ s^{-1}, and $k_{-1} = 6.3$ s^{-1}. This gives a K_i value of 1.1 μM for the initial complex and of 0.6 fM for the overall dissociation constant.

(ii) Binding of ^{125}I-TIMP-2 to Gelatinase A. This was studied by incubating equimolar concentrations of ^{125}I-TIMP-2 and gelatinase A. Samples were removed at time intervals, and free and complexed TIMP-2 were separated from each other and quantified. Each data set was fitted as a second-order reaction as described under Methods. Some typical results are shown in Figure 2, and the average value for the apparent second-order rate constant was $(3.1 \pm 0.6) \times 10^7$ M^{-1} s^{-1}.

DISCUSSION

As part of a long-term study into the mechanism of TIMP action, we have analyzed the kinetic properties of a number of TIMP mutants in order to define the roles of individual amino acid residues in the TIMP−enzyme interaction (*20*−*23, 15*). We have previously studied MMP−TIMP binding by characterizing the kinetics of enzyme inhibition. For several systems, however, the interaction is so tight that we have been unable to determine dissociation constants using these methods. In the present work, we have overcome these restrictions and have determined the kinetic constants which characterize both the formation and the stabilization of the TIMP-2−gelatinase A complex. We have extended our previous studies on the kinetics of gelatinase A inhibition by TIMP-2 by using radiolabeled TIMP-2 to study the kinetics of dissociation of the complex and by increasing

the range of concentrations over which complex association is analyzed using rapid reaction techniques. We have avoided the use of any techniques which require immobilization of either the enzyme or TIMP as our previous work has indicated that multiple binding sites are involved in the interaction (*23)* and therefore nonspecific binding of either protein may mask or distort a binding site and therefore give misleading results. However, it has been necessary to label TIMP-2 in a process which has involved chemical modification and which could affect an enzyme binding site. Therefore, we have been careful to compare the properties of our preparation of ^{125}I-TIMP-2 with unlabeled TIMP-2 throughout the study and have demonstrated that the degree of radiolabeling of the TIMP-2 used in the present work does not appear to affect the inhibitory activity of TIMP-2.

The rate of dissociation of the gelatinase A−TIMP-2 complex determined in the present work by ligand displacement is remarkably slow with a half-life of approximately 400 days. Thus, in practical terms the reaction is effectively irreversible. This result is consistent with our practical observations on the stability of the complex; e.g., we cannot dissociate the complex without denaturing the enzyme. One possible artifact in this experiment could be due to the fact that gelatinase A can bind two TIMP-2 molecules (*24, 25*). Thus, the ^{125}I-TIMP-2−gelatinase A complex could be stabilized in the presence of excess cold TIMP-2 by the binding of the second TIMP-2, and this effect would give an artificially low value for k_{-2}. However, since gelatinase A is unable to bind two TIMP-1 molecules (*26*), we also performed the experiment in the presence of an excess of cold TIMP-1. The results were similar, suggesting that the binding of a second TIMP-2 molecule does not affect the stability of the inhibitory complex.

Analysis of the rate of inhibition of gelatinase A by TIMP-2 shows curvature in the dependence of the observed rate constant, k', on TIMP-2 concentration. This is diagnostic of the mechanism described in Scheme 1. In this mechanism, the rate-limiting step at low TIMP-2 concentrations involves binding of TIMP-2 to gelatinase A, characterized by the rate constants k_1 and k_{-1}, whereas at high TIMP-2 concentrations the rate-limiting step is independent of free TIMP-2 concentration and therefore involves an isomerization reaction, with rate constants k_2 and k_{-2}. Equation 4 relates k' to the individual rate constants with the intercept of the curve giving the value for k_{-2}. However, in this case the value of k_{-2} is so low that the curve appears to intercept the *y*-axis at the origin. An accurate value for k_{-2} cannot be obtained from this experiment therefore, but it can be concluded that k_{-2} is the rate-limiting step in the dissociation of the gelatinase A−TIMP-2 complex and is therefore the rate constant determined independently in the ligand displacement experiment.

The value for k_1 of 5.9×10^6 M^{-1} s^{-1} obtained from the study of the rate of inhibition of gelatinase A activity appears to differ somewhat from that obtained from our previous work of 2.5×10^7 M^{-1} s^{-1} (*15*) and from the average value of 3.1×10^7 M^{-1} s^{-1} obtained by fitting the data obtained from following the binding of ^{125}I-TIMP-2 to gelatinase A to a second-order reaction. However, both the latter values were obtained by assuming a simple bimolecular collision mechanism for the reaction and therefore ignore the contribution of k_{-1} and k_2 to the apparent rate of association. To correct the values to give the true k_1, a simplification of eq 4 is used: the assays were performed at low concentrations of TIMP-2 so that $k_1 I \ll k_{-1}$, and under these conditions the denominator of eq 4 simplifies to k_{-1}, giving eq 5:

$$k' = k_{-2} + k_2 k_1 I / k_{-1} \quad (5)$$

The gradient of the linear portion of k' vs [TIMP-2] is therefore equivalent to $k_2\, k_1/k_{-1}$, so the apparent value for k should be divided by k_2/k_{-1} (i.e., 5.24) to give the true value, k_1. Division of 2.5×10^7 M^{-1} s^{-1} and 3.1×10^7 M^{-1} s^{-1} by 5.24 gives a value for k_1 of 4.77×10^6 M^{-1} s^{-1} and 5.92×10^6 M^{-1} s^{-1}, respectively, which is in good agreement with the value of 5.9×10^6 M^{-1} s^{-1} obtained from the complete curve in Figure 2.

The agreement between the values obtained using inhibition of enzyme activity and ligand binding gives further support to the assumption that labeling of the TIMP-2 has not affected its activity. It also confirms that binding of a second TIMP-2 molecule to gelatinase A, which can occur in the inhibition experiments but not in the ligand binding experiment as the reagents are at equimolar concentrations, does not affect inhibition of gelatinase A activity.

It is not possible, on the basis of the present work, to ascertain whether the first step of the reaction forms a complex which inhibits enzyme activity. As the initial complex is "loose" and the reagent concentrations used in the present work are well below its K_i, the efficiency of the inhibition of enzyme activity under these conditions is due to the rapid rate of isomerization which favors the formation of the complex where it is effectively trapped. From our observations on other MMP−TIMP systems [many require a preincubation period to form stable complexes during titration experiments (*9*), and the complexes formed after this preincubation period are extremely stable], we anticipate that this reaction pathway applies to each system and that in each case k_{-2} is sufficiently low to make the reaction effectively irreversible. The ratio of k_2:k_{-1} is therefore critical in determining the outcome of an enzyme−TIMP encounter, and differences in this ratio could be primarily responsible for different specificity characteristics of the TIMPs. Other workers have reported K_i values for TIMP−MMP reactions which are significantly higher than ours [e.g., 10^{-7}−10^{-9} M (*27*)], and it is possible that this discrepancy is due to the fact that they could be observing the initial step of the reaction only whereas our values are for the final stabilized complex.

Our data contrast somewhat to recently reported values for rate constants for the inhibition of gelatinase A by TIMP-2 (*28*). Using surface plasmon resonance, biphasic kinetics for binding were also observed but the affinity of TIMP-2 for gelatinase A appeared to be considerably lower than that observed in the present work. Olson et al. reported that inhibition of gelatinase A by TIMP-2 contrasts with binding in that it is a single-step process. However, they used a lower TIMP-2 concentration range and would therefore not detect the second step in the process. It is not clear why our K_i values differ significantly from theirs (K_i 10^{-7}−10^{-9} M). It is possible that the immobilization of TIMP for the surface plasmon resonance disrupts one or more binding sites and therefore the affinity of the TIMP for gelatinase A.

The identification of two steps in the binding of TIMP to gelatinase A suggests that a significant conformational change may occur during the complex stabilization step. Circular dichroism studies of the TIMP-1−gelatinase A interaction demonstrating that inhibition is accompanied by a conformational change (*29*) support this hypothesis. Future work involving detailed kinetic analysis of TIMP mutants to identify residues important in each step of the reaction should indicate the extent to which the free enzyme and TIMP differ in conformation from the enzyme−TIMP complex.

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