The Mechanism of Inhibition of Collagenase by TIMP-1*

(Received for publication, February 26, 1996, and in revised form, June 10, 1996)

Kenneth B. Taylor‡‡§, L. Jack Windsor‡‡¶, Nancy C. M. Caterina‡, M. Kirby Bodden**, and Jeffrey A. Engler‡¶

From the Departments of ‡Biochemistry and Molecular Genetics, ¶Oral Biology, and **Restorative Dentistry, and the Research Center for Oral Biology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

Tissue inhibitor of metalloproteinase-I (TIMP-1) is a slow, tight-binding inhibitor of fibroblast-type collagenase. Time-course data from inhibition experiments were analyzed by graphic analysis, by nonlinear regression of the analytic integrals of the rate equations and by non-linear regression with numeric integration of the rate equations. With the same assumptions, approximations and data, all three methods of analysis produced the same model preferences and values for the kinetic parameters. The time-course data for the inhibition of fibroblast-type collagenase by TIMP-1 are best described by the equations for a noncompetitive two-step mechanism, in which an inactive, rapidly formed, reversible complex slowly forms an inactive, tight complex. However, from the analysis of data from experiments at concentrations of TIMP-1 comparable to that of collagenase, it is apparent that free TIMP-1 also functions in the breakdown of the tight complex. The rapidly formed complex has a dissociation constant of 8 nM and reacts to the tight complex with a first-order rate constant of 0.003 s⁻¹. The back reaction of the tight complex to the rapid complex has a second-order rate constant of 5 × 10⁴ M⁻¹ s⁻¹. The resulting global dissociation constant of the tight complex is 0.1 nM at 3 nM TIMP-1 and collagenase concentration.

Collagenase without the carboxyl-terminal domain (mini-collagenase) is inhibited by TIMP-1 according to a mechanism, in which the rapidly formed complex has such a high dissociation constant (247 nM) that it effectively constitutes a one-step mechanism, in which TIMP-1 binds with an apparent second-order rate constant of 9.6 × 10⁴ mol⁻¹·l⁻¹·s⁻¹ and the enzyme-TIMP-1 complex dissociates with a first order rate constant of 0.00026 s⁻¹. The apparent global dissociation constant for the tight complex (2.7 nM) is higher than that for the fibroblast-type collagenase. Participation of TIMP-1 in the dissociation is not demonstrable. Therefore, the carboxyl-terminal domain of fibroblast-type collagenase is important for the initial, rapid binding of TIMP-1 and the initial complex contributes to the overall binding.

Fibroblast-type collagenase (MMP-1) is a member of the family of matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, which play a major role in extracellular matrix remodeling as it occurs in health and disease (1, 2). The family also includes the stromelysins, gelatinases, matrilysin, membrane-type MMPs, and macrophage metalloelastase. Fibroblast-type collagenase is the prototype for several structural features shared by the MMPs, which include a series of distinct and highly conserved domains that include (i) a hydrophobic signal peptide that is cleaved off during secretion followed by (ii) a propeptide sequence that is removed during activation, (iii) a catalytic domain containing the active zinc binding site joined by (iv) a proline-rich hinge region to (v) a hemopexin-like carboxyl-terminal domain that possibly plays a role in substrate specificity. The other MMPs are variants of this prototype structure formed either by deletion of or addition to these domains.

“Tissue inhibitors of metalloproteinases” (TIMPs) constitute a family of three or more genetically distinct natural inhibitors of vertebrate matrix metalloproteinases (1)(2)(3)(4). TIMPs are thought to act as biological regulators of the turnover of extracellular matrix, a process that occurs in normal tissues during development and wound healing, as well as in inflamed tissues during rheumatoid arthritis and during tumor invasion and metastasis (5).

The inhibition of gelatinase by TIMP-1 (28 kDa) has been characterized as a slow, tight-binding inhibition (6). The dissociation of the stromelysin-TIMP-1 complex into active TIMP-1 demonstrates the noncovalent nature of the binding (7). The results below indicate that the inhibition of collagenase by TIMP-1 falls into the same kinetic classification. Willenbrock et al. (6) and Nguyen et al. (8) concluded from kinetic data that TIMP-1 inhibits gelatinase and stromelysin according to a single-step mechanism, on the basis of graphic analysis. Although the data were analyzed according to a competitive mechanism, the alternatives were not tested and the hypothesis by these workers did not include this as part of the mechanism. However, since Weligus et al. (9) produced evidence to indicate that TIMP-1 is a noncompetitive inhibitor of collagenase, these other mechanisms must also be considered.

Kinetic data from experiments involving other examples of this class of inhibitors have been analyzed according to the schemes reviewed and described by Morrison and Walsh (10) and by Williams and Morrison (11). Data that are most generally useful are from experiments in which the appearance of product (P) is measured as a function of time, after either the addition of enzyme to a mixture of substrate (A) and inhibitor (I) or the addition of substrate to an equilibrium mixture of

---

* This work was supported by United States Public Health Service Grants DE08228 (to S. Michalek), DE10631 (to J. A. E.), and DE00283 (to M. K. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, CH-19, Rm 509, UAB, University Station, Birmingham, AL 35294, Tel.: 205-934-4380; Fax: 205-934-0758; E-mail: kbtaylor@bmg.bhs.uab.edu.

§ Current address: National Institute of Dental Research, National Institutes of Health, Bldg. 30, Rm. 404, Bethesda, MD 20892.

¶ The abbreviations used are: MMP, matrix metalloproteinase; APMA, 4-aminophenylmercuric acetate; FIB-CL, human gingival fibroblast-type collagenase; TIMP, tissue inhibitor of metalloproteinases; MSC, model selection criterion.
enzyme and inhibitor. Data from the former experiments is then fit to Equation 1 and values are estimated for the initial reaction velocity ($v_0$), the reaction velocity ($v_e$) after maximum inhibition, and the apparent first-order rate constant ($k_{ap}$) for the transition between the two. The values of these parameters from a series of experiments in which only the concentration of inhibitor is varied are then subjected to graphic analysis by several methods to exclude certain mechanisms and to estimate the values for the putative reaction parameters.

However, one of the approximations in the development of Equation 1, that the concentration of inhibitor does not change significantly during the course of the reaction, limits the possible experiments to those done with the inhibitor concentration much higher than the enzyme concentration. This approximation makes the estimation of the $k_{cat}$ and the $K_i$ almost impossible. Furthermore, graphic analysis of this data is subject to the same anomalies of statistical weighting and uncertainty about goodness of fit as is graphic analysis of steady-state enzyme kinetic data (12). Therefore, data analysis by nonlinear regression with the analytically integrated equation for each mechanism would seem to be preferable. Although a published integrated equation for a simple competitive mechanism circumvents these difficulties (11), analytically integrated expressions for modeling more complex mechanisms have been very few, presumably because the time and effort involved in analytic integration of a separate equation for each mechanism has been somewhat daunting. Alternatively, numeric integration of the differential equations has been used to simulate the mechanism for a boronic acid inhibitor of dipeptidyl peptidase IV (13), and have been suggested specifically for experiments in which the concentration of both inhibitor and substrate change appreciably during the reaction course (11, 14). Furthermore numeric integration in combination with nonlinear regression has been used for the analysis of pre-steady-state data (15). A comparison of these methods of data analysis for the same data and mechanism is presented below.

Among the schemes that describe data from kinetic experiments with slow, tight-binding inhibitors are those in which the binding of inhibitor occurs in a single slow step (Scheme 1) (16), and those in which binding occurs in two steps (Scheme 2). In the latter mechanism, a fast, reversible collision complex slowly reacts to form a tight complex (10). Moreover, since practically all of the inhibitors investigated to date have been substrate analogs, the kinetic data have been analyzed as competitive inhibition. Additional kinetic mechanisms are tested below.

**EXPERIMENTAL PROCEDURES**

**Fibroblast-type Collagenase**—Fibroblast-type collagenase was isolated from human gingival fibroblast conditioned media by affinity chromatography as described previously by Windsor et al. (17). Conditioned medium was passed over an affinity column prepared by coupling 28 mg of anti-collagenase monoclonal antibody VI$_2$ to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.). The bound material was eluted with 6 M urea in buffer (50 mM Tris, pH 7.5, 0.2 M NaCl, 5 mM CaCl$_2$, and 1 mM ZnCl$_2$) and dialyzed against the buffer to remove the urea.

Truncated fibroblast-type collagenase (mini-CL) lacking the heparin-like domain was expressed in Escherichia coli and extracted by 6 M urea as described by Windsor et al. (37). The mini-CL 6 M urea extract was passed over a Sephacryl S-200 HR (Pharmacia) column equilibrated with 6 M urea in 50 mM Tris, pH 7.5, 0.2 M NaCl, 5 mM CaCl$_2$, and 1 mM ZnCl$_2$ and dialyzed against the buffer to remove the urea.

**Tissue Inhibitor of Metalloproteinase-1 (TIMP-1)**—Native TIMP-1 was purified from human fetal lung cells by heparin-Sepharose chromatography followed by C4 reverse phase high performance liquid chromatography as described previously by Bodden et al. (18). Recombinant human TIMP-1 was purified from culture medium of HeLa cells infected with a recombinant vaccinia virus expressing the human TIMP-1 gene by the same procedure. The two products gave identical results and were used interchangeably. Protein concentrations were determined by the Bradford method as described by the supplier (Bio-Rad) using bovine serum albumin as a standard.

TIMP-1 protein (50 μg) was labeled with $^{125}$I by Bolton-Hunter reagent as described by the manufacturer (DuPont NEN) and resulted in a specific activity of 0.1 μCi/μg.

**Radiolabeled TIMP-1 Binding**—Fibroblast-type collagenase (4.8 nM), activated with APMA, was incubated with and without unlabeled TIMP-1 (4.6 nM) for 30 min at 23 °C. Radioactive $^{125}$I-labeled TIMP-1 (53.5 nM) was then added and incubated at 23 °C for indicated times before addition of sample buffer containing 0.1% SDS (20) to terminate the reaction. The samples were resolved by SDS-polyacrylamide gel electrophoresis by the methods of Laemmli (21). After electrophoresis the radioactive bands on the dried gel were identified and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Time-course Experiments**—In experiments in which the substrate was added last APMA-activated FIB-CL (6 nM) was incubated at 23 °C for 20 min with various concentrations of TIMP-1 in 1 ml of buffer, 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM CaCl$_2$, and 1 mM ZnCl$_2$. The reaction was initiated by the addition of 1 ml of the substrate, the coumarinyl peptide derivative described above (2.5 μM) was incubated in the buffer described above in the absence or in the presence of various concentrations of TIMP-1. The reaction was initiated by the addition of APMA-activated FIB-CL, 3 nM final concentration. The fluorescence was monitored as described above.

**Data Analysis**—The data from the time-course experiments in which enzyme was added last were averaged over each subsequent group of four points, corrected for quenching, and converted to units of product concentration in a spreadsheet program. The resulting data were then analyzed by one or more of three methods: nonlinear regression (Equation 1) followed by graphic analysis of the parameter values; nonlinear regression with a model-specific, integrated equation; and nonlinear regression with numeric integration of the differential equations for the model (all with the Scientist program, MicroMath Scientific Software, Salt Lake City, UT). The model selection criterion (MSC), calculated by the computer program (23), is a modification of the Akaike information criterion (24) and is proportional to the goodness of fit of the model. It is the natural logarithm of the sum of the residuals, normalized for the magnitude of the data and adjusted for the number of unknown parameters in the model.

Numeric integration, in those models in which it was used, was done by the episode method (25). Subsequent trials with other methods for numeric integration gave identical results as with the same data set and the same model with the former method.

**RESULTS**

**Reversibility of TIMP-1 Binding**

Labeled $^{125}$I-TIMP-1 was added to the unlabeled complexes, made in an incubation of fibroblast-type collagenase with unlabeled TIMP-1, and samples were analyzed for radioactivity in

---

2 N.C.M. Caterina, L. J. Windsor, M. K. Bodden, A. E. Yermovsky, K. B. Taylor, H. Birkedal-Hansen, and J. A. Engler, manuscript in preparation.
the collagenase-TIMP-1 complex at various times. The results (Fig. 1) show that radioactive TIMP-1 was exchanged into the TIMPzFIB-CL complex in a time-dependent manner. Thus, these results demonstrate that the TIMPzFIB-CL complex is a tight but reversible one.

Reactions Initiated by Substrate Addition

Evidence for competitive and noncompetitive mechanisms of slow, tight-binding inhibitors has resulted from experiments in which an equilibrium mixture of enzyme and inhibitor are diluted into a solution with a high concentration of substrate (26). If the shape of the time-course curve is concave upward, the substrate is displacing the inhibitor from the enzyme. In time-course experiments in which a stoichiometric equilibrium mixture of FIB-CL and TIMP-1 was added to substrate (15 μM), the rate of the reaction was constant with time (Fig. 2). This rate (5.83 × 10⁻² μM/s) was within 6% of the final steady-state rate (6.14 × 10⁻² μM/s) measured, when the same concentration of enzyme was added last to a mixture of substrate and TIMP-1 at the same concentrations. These results suggest that substrate does not displace TIMP-1 from the enzyme and the TIMP-1 must bind to both the free enzyme and the enzyme-substrate complex in a noncompetitive mechanism. Although similar results would be seen if only a fraction of the TIMP were active and participated in irreversible binding to the enzyme, the reversibility of TIMP-1 binding to the enzyme was demonstrated above. Furthermore, the binding of substrate at the concentration used may be insufficient to demonstrate a difference. Additional kinetic evidence for a noncompetitive mechanism is presented below.

Reactions Initiated by Enzyme Addition

Graphic Data Analysis—The time-course data, from experiments in which enzyme was added last to mixtures of substrate and various concentrations of TIMP-1, at least 10 times that of enzyme, were fit to Equation 1, and the values of ve and kobs were each estimated. The observation that the initial velocity, ve, decreases with increasing TIMP-1 concentration indicates that an inactive complex is formed rapidly before data collection begins (Scheme 2). Furthermore, the plot of 1/ve versus TIMP-1 concentration should be linear with a positive slope for a two-step mechanism such as Scheme 2 (10). Accordingly, the data (Fig. 3) are consistent with a mechanism in which TIMP-1 binds to fibroblast-type collagenase in a two-step mechanism. The dissociation constant, Kp, for the reversible complex calculated from the straight line is 19.5 nM (Table I). The two-step mechanism (Scheme 2) results in equations for kobs that predict a hyperbolic relationship versus TIMP-1 concentration (Equation 4), whereas a one-step mechanism predicts a linear plot. A plot of kobs versus TIMP-1 clearly shows a hyper-
The Mechanism of Inhibition of Collagenase by TIMP-1

Table I

Parameter values associated with the equations derived from the mechanism (Scheme II) that best fits time-course data for the inhibition of fibroblast-type collagenase by TIMP-1

| Parameter | Analytical method | Graphic analysis | Nonlinear regression | Numeric integration |
|-----------|-------------------|------------------|---------------------|--------------------|
| $k_{cat}$ (s$^{-1}$) | N.D.$^a$ | 0.137 | 0.14 |
| $K_D$ (M) | $19.5 \times 10^{-9}$ | $20.9 \times 10^{-9}$ | $20.3 \times 10^{-9}$ |
| | | $(1.2 \times 10^{-4})$ | $(1.1 \times 10^{-4})$ |
| $k_1$ (M$^{-1}$ s$^{-1}$) | ID$^b$ | 3.68 x 10$^{-9}$ | 3.62 x 10$^{-9}$ |
| | | $(0.00034)$ | $(0.00028)$ |
| $k_2$ (s$^{-1}$) | ID$^b$ | $7.5 \times 10^{-5}$ | $7.5 \times 10^{-5}$ |
| | | $(3.8 \times 10^{-5})$ | $(3.3 \times 10^{-5})$ |

$^a$ Not determined.
$^b$ Indeterminate.

The differential models (Schemes 3–6) are available upon request.

Nonlinear Regression Analysis—In order to test further the hypothesis that the initial reversible binding of TIMP-1 is fast and noncompetitive, the time course of the reaction was measured in the absence of TIMP-1 and at low TIMP-1 concentration (6 nM) at various concentrations of substrate (2.5–15 μM). The initial velocity of the experiments without TIMP-1 was measured as the slope of the initial linear segment of the curve, whereas the initial velocity of the experiments in the presence of TIMP-1 ($V_0$) was determined from nonlinear regression of the time-course data with Equation 1. A plot of the reciprocal of the initial velocities versus the reciprocal substrate concentration (Fig. 5) appears to demonstrate further that the inhibition is noncompetitive. However, Morrison (27) showed that such plots of fast, reversible tight-binding inhibitors are nonlinear (concave down). Nonlinear regression analysis of the data for initial velocity, substrate, and inhibitor concentrations with equations for steady-state inhibition by such inhibitors$^3$ fit the noncompetitive model better than the competitive (model selection criteria: 4.1 versus 3.4). Although the estimates of the adjustable parameters were poor, presumably because of the paucity of experimental points, the value for the $K_*$ for substrate (11.6 μM) indicates that a significant effect of substrate (32%) on inhibition by a strictly competitive mechanism should have been detected at the highest substrate concentration. Thus, the data support the hypothesis that under the present experimental conditions TIMP-1 binds to either free enzyme or to the enzyme-substrate complex to form an initial, reversible, inhibited complex.

The fit by nonlinear regression to the same time-course data from experiments at high TIMP-1 concentration (30 nM) that were analyzed graphically above, but with equations from analytic integration of the differential equations associated with either Scheme 1 or Scheme 2, was better for the latter mechanism (MSC = 6.64 versus 6.28). From the fit to the equations associated with Scheme 2 (Equations 1–4),

$$P = v_0 + t \cdot k_{cat} \cdot (1 - e^{-kt_{obs}})k_{obs}$$

$$v_0 = E_{cat} \cdot \frac{K_D}{A} \cdot \frac{1}{1 + \frac{I_{cat}}{K_D}}$$

the values for $K_D$ and $k_1$ (Table I) are estimated with some precision and the value of $K_D$ agrees very well with that estimated by graphic analysis. The fact that $k_2$ is estimated poorly is a consequence of the required high TIMP-1 concentration in the experiments, which results in very little demonstrable reverse reaction. Therefore, the calculated value of the global dissociation constant, $K'_D$, will also be imprecise, and these same considerations call into question the precision with which the latter parameter was estimated by graphic analysis. Thus, the limitations imposed by the necessity for data gathering at high concentrations of TIMP-1 and the consequent limitations on graphic analysis and this method of nonlinear regression become apparent.

Nonlinear Regression with Numeric Integration—The fit of the same time-course data to the equation systems containing the differential equations from Scheme 1 and Scheme 2$^4$ (Equations 5 and 6) plus a conservation equation for enzyme (Equation 7) was also somewhat better for the latter mechanism (MSC = 6.64 versus 6.41).

$$\frac{dE_I}{dt} = k_1(E_I) - k_2(E_I)$$

$$\frac{dP}{dt} = E_I \cdot \frac{k_{cat}A}{(A + K_D)}$$

$$E_{cat} = E_I + EI_I + EI$$

The estimated values of the reaction parameters for the latter mechanism, $K_D$ and $k_1$, (Table I) are the same as those estimated by the previous methods. However, as with the previous
of the time-course data to Equation 1 (the presence of TIMP-1 the initial velocity was determined from the fit concentration). In the absence of TIMP-1 the initial velocity was determined from the fit of the time-course data to Equation 1 ($v_0$).

The reaction was carried out in a cuvette (3 ml total volume) containing substrate (2.5–15 µM) and TIMP-1 (0 and 6 nM) in buffer. It was initiated by the addition of collagenase (3 nM, final concentration). In the absence of TIMP-1 the initial velocity was determined from the initial, linear segment of the time course, whereas in the presence of TIMP-1 the initial velocity was determined from the fit of the time-course data to Equation 1 ($v_0$).

The Mechanism of Inhibition of Collagenase by TIMP-1

methods, the limitations on the experimental conditions result in a poor estimate of the value of $k_x$. However, the good agreement of both the model selection and the parameter values with the previous methods of data analysis demonstrate the validity of numeric integration.

Model Selection—Fits of six additional data sets from experiments with both low (1.5–30 nM) TIMP-1 and high TIMP-1 concentrations, 60–150 nM, to the two models described above (Scheme 1 and Scheme 2) by nonlinear regression with numeric integration of a system of the differential equations plus the conservation equations for TIMP-1 (Equation 8) and substrate (Equation 9), as well as one for enzyme which were indistinguishable from each other.

$$I_{tot} = I + EI + EI_t$$  \hspace{1cm} (Eq. 8)

$$A_{tot} = A + P$$  \hspace{1cm} (Eq. 9)

Therefore, fits of one or more of the data sets were attempted to the corresponding system of equations from at least five additional mechanisms. These included: Scheme 3 (a slow enzyme conformation change followed by rapid binding to TIMP-1), Scheme 4 (a slow TIMP-1 conformation change followed by rapid binding to enzyme), Scheme 5 (a rapidly formed enzyme-TIMP-1 complex followed by slowly formed enzyme-TIMP-1 and enzyme-TIMP-TIMP complexes), Scheme 6 (a two-step binding of TIMP-1 to enzyme (Scheme 2) in competition with the rapid dimerization of TIMP-1), and Scheme 7 (a two-step binding of TIMP-1 to enzyme (Scheme 2) in which the rate of breakdown of the tight complex ($EI^*$) is proportional to free TIMP-1 (Scheme 7).

The fit of the experimental data from the inhibition of collagenase by TIMP-1 to the equations derived from various mechanisms

Time-course data were collected and fit to the differential equations derived from the various mechanisms both as described under "Experimental Procedures." The model selection criterion parameter (MSC) was estimated by the computer program used for regression analysis.

| Model | MSC average |
|-------|-------------|
| One-step (Scheme 1) | 4.59 |
| Two-step sequence (Scheme 2) | 4.85 |
| A slow enzyme conformation change followed by rapid binding to TIMP-1 | 5.30 |
| A slow TIMP-1 conformation change followed by rapid binding to enzyme | 3.65 |
| Independent, parallel, rapidly-formed enzyme-TIMP-1 complex and slowly formed enzyme-TIMP-1 and enzyme-TIMP-TIMP complex | 4.60 |
| A two-step binding of TIMP-1 to enzyme (Scheme 2) in competition with the rapid dimerization of TIMP-1 | 6.08 |
| A two-step binding of TIMP-1 to enzyme in which the rate of breakdown of the tight complex ($EI^*$) is proportional to free TIMP-1 | 5.99 |

Of these mechanistic models, one or the other of the last two fit each of the data sets clearly better than any of the others. Consequently the average MSC is clearly higher for these models than for any of the others (Table II). Furthermore, the MSC is clearly higher for both of these models with each of the individual data sets. However, the dimerization model is less attractive because the very low values of the regression analysis estimates of the dissociation constant for the rapid, reversible binding of TIMP-1 to enzyme (10$^{-4}$ nM) are inconsistent with the rough estimates above (e.g. 10 nM) by graphic analysis and analytic integration. Furthermore, the very low estimates of the dissociation constant for the TIMP-1 dimer (10$^{-7}$ nM) would predict that TIMP-1 exists only as a very stable dimer. Although TIMP-1 dimers have been demonstrated (30), their stability is much lower than predicted by the dissociation constant above. Therefore, the most feasible model of those tested to date is that in which the dissociation of the enzyme-TIMP-1 complex requires the participation of a molecule of free TIMP-1. It is hypothesized that the free TIMP-1 forms a dimer with the bound TIMP-1 and causes its release from the enzyme, and that the dimer subsequently is in equilibrium with TIMP-1 monomers.

Parameter Estimates—The average parameter estimates for $K_p$ and $k_1$ from the simple two-step model fit to data from experiments with high TIMP-1 concentration (Table I) compare with those from the fit of the two-step, noncompetitive model in which the dissociation of the enzyme-TIMP-1 complex is proportional to the concentration of free TIMP-1 from the latter data sets (Table III) from experiments with a wide range of TIMP-1 concentrations. However, the estimates for $k_2$ are expected to be somewhat more divergent, since in the latter case it is a second order rate constant, whereas in the first case it is a first order constant. According to the data and model reported here, the global dissociation constant of the tight enzyme-TIMP-1 complex is about 0.1 nM at 3 nM TIMP-1.

TIMP-1 Inhibits COOH-terminally Truncated Collagenase in One Step

Recombinant fibroblast-type collagenase in which the COOH-terminal hemopexin domain (starting with amino acid 251) is absent (mini-collagenase) was described by Windsor et al (35). Although the $K_m$ of the enzyme for the fluorogenic substrate was too high to be estimated with meaningful preci-
**TABLE III**

Parameter values associated with the equations derived from a two-step mechanism fit to a data set for the inhibition of collagenase by TIMP-1

| Parameter | Value (S.D.) |
|-----------|-------------|
| $K_D$ (mM) | $7.7 \times 10^{-9}$ (0.18 $\times 10^{-9}$) |
| $k_1$ (s$^{-1}$) | 0.00342 (9.42 $\times 10^{-5}$) |
| $k_2$ (m$^{-1}$ s$^{-1}$) | $5.44 \times 10^{4}$ (1.8 $\times 10^{4}$) |

**TABLE IV**

Parameter values associated with the equations derived from a one-step and a two-step mechanism fit to data for the inhibition of mini-collagenase by TIMP-1

| Parameter | One-step | Two-step |
|-----------|----------|----------|
| Model fit | 4.74 | 4.75 |
| (correlation) | (0.996) | (0.996) |
| $k_{2a}$ (sec$^{-1}$) | 0.156 | 0.156 |
| $K_p$ (mM) | $3.1 \times 10^{-4}$ | $3.1 \times 10^{-4}$ |
| $k_1$ (s$^{-1}$) | $9.6 \times 10^{4}$ (1.8 $\times 10^{4}$) | 0.0215 s$^{-1}$ (0.007) |
| $k_2$ (s$^{-1}$) | 0.000926 | 0.000131 |
| | (2.5 $\times 10^{-5}$) | (3.7 $\times 10^{-5}$) |

Time-course data were collected and fit as described under “Experimental Procedures” to the differential equations derived from a two-step mechanism in which the reactivation of the collagenase-TIMP-1 complex requires a molecule of free TIMP-1 (Scheme 7). The reaction was carried out in a cuvette (3 ml total volume) containing substrate (2.5 $\mu$m), and various concentrations of TIMP-1 (0, 3, 6, 15, 30, and 150 nM) in buffer. It was initiated by the addition of fibroblast-type collagenase (3 nM final concentration). The parameter values and their respective standard deviations (in parentheses) were estimated by the computer program used for regression analysis.

The data and analysis presented above support the hypothesis that fibroblast-type collagenase binds TIMP-1 rapidly and reversibly and the resulting complex slowly changes conformation to a tight complex, in which some other domain (e.g. the active site) is involved in binding. The tight complex can bind a second molecule of TIMP-1 to the hemopexin domain to form a ternary complex that dissociates to active enzyme. The contribution of the carboxyl-terminal domain to this reversible binding is consistent with a similar contribution proposed by others for collagenase (32), stromelysin (33), and gelatinase-A (6). Howard et al. (34) reported stabilization of gelatinase to autodigestion by TIMP-2, but not TIMP-1, binding at the carboxy-terminal domain to form a complex that still catalyzes gelatinolysis; they distinguished this complex from an inhibited complex in which TIMP-2 is bound to the active site (35). Kleiner et al. (36) demonstrated in cross-linking studies that the binding of TIMP-2 to the carboxy-terminal domain of gelatinase A inhibited binding to the active site. In addition, Ogata et al. (19) demonstrated TIMP-1 binding to the carboxy-terminal domain of progelatinase B and the conversion of this complex to the stable, inhibited complex upon activation. The hypothesis presented above in the present paper requires that the stable inhibited complex, in the case of fibroblast-type collagenase forms a ternary complex with a second molecule of free TIMP-1 and converts the inactive complex to active enzyme at a significant rate. However, this route for the formation of active enzyme does not preclude the parallel formation by the reverse of the steps by which it was formed. Models reflecting these two parallel routes of formation were fit to the data, but the goodness-of-fit was no better than the simpler model with fewer adjustable parameters. It seems doubtful that the data are sufficiently precise to distinguish between these two possibilities.

The present data also indicate that TIMP-1 forms an initial inhibited complex with fibroblast-type collagenase in a manner that is noncompetitive with the substrate used in these experiments. However, it is possible that the macromolecular physiological substrates might compete more effectively with TIMP-1 binding. Experiments to test the latter idea will be of interest.

Willenbrock et al. (6) and Nguyen et al. (8) analyzed the results of similar time-course experiments of the inhibition by TIMP-1 and TIMP-2 of gelatinase-A and stromelysin by the graphic method and by nonlinear regression of the analytic integral equation, after simplification of the latter by several approximations. With their method of data analysis, these investigators were prevented by substrate solubility of and quenching by the substrate from using a sufficiently high substrate concentration to distinguish a competitive mechanism
The Mechanism of Inhibition of Collagenase by TIMP-1

from noncompetitive (also called mixed inhibition). However, the linearity of $k_{obs}$ with TIMP-1 concentration and the independence of $v_i$ with TIMP-1 concentration led to the conclusion of a single-step binding mechanism. It is possible that they were prevented from detecting the rapid, collision complex, because their methods of data analysis required that the concentrations of TIMP-1 be restricted to those less than 2 nm. Although the difference in mechanisms renders direct comparison difficult, generally the binding rate and the global dissociation constants of the tight complex found by the latter investigators are lower, by about an order of magnitude, than the ones determined in the present work.

It is interesting that Willenbrock et al. (6) reported no competition of binding to gelatinase-A by the COOH-terminal domain of collagenase. However, it is doubtful that the rapid, reversible complex between TIMP-1 and the COOH-terminal domain of collagenase suggested in the present work would have been detected at concentrations of the latter present in their experiments (less than 2 nm), even if it does form in the absence of the remainder of the collagenase molecule. The report by Baragi et al. (33) that COOH-terminal truncated stromelysin bound TIMP-1 but that full-length stromelysin bound preferentially in competition experiments may be explained by the hypothesis that the full-length molecule rapidly formed a reversible complex, which left a diminished supply of TIMP-1 for the slow formation of a tight complex with the attenuated molecule.

Although Welgus et al. (9) reported evidence for noncompetitive inhibition of collagenase cleavage of collagen substrate by TIMP-1, uncertainties associated with the determination of initial velocity and with the assumption that the free inhibitor concentration is the same as the total inhibitor concentration raise some questions regarding the strict interpretation of the data. Nevertheless, the estimated value of the inhibition constant ($K_i < 1 \times 10^{-9}$ m) agrees well with the value of the global inhibition constant ($K_{global} = 0.1 \times 10^{-9}$ m) determined above as well as the value of the dissociation constant ($K_{D} = 4 \times 10^{-9}$ m) determined by Bodden et al. (18). Although Morrison and Walsh (10) reviewed a number of low molecular weight inhibitors of proteinases that are slow, tight-binding inhibitors, practically all are substrate analogs and are analyzed as competitive inhibitors. Most of the proteinaceous molecules that are slow, tight-binding inhibitors of proteinases inhibit the serine or cysteine proteinases. The serpins, protein inhibitors of serine proteases, might seem somewhat analogous to TIMP-1, but at least some of those that have been analyzed kinetically inhibit by a competitive mechanism (16) and apparently bind to the active-site cleft of the enzyme (29). Indeed they form a tetrahedral intermediate with the enzyme and a portion of the serpin is actually cleaved by the enzyme (38). Although data for the inhibition of factor Xa by both the tick anticoagulant peptide (29) and by tissue factor pathway inhibitor (31) were analyzed as competitive mechanisms, evidence for such a mechanism was reported only in the former case. Both inhibitors functioned by a two-step mechanism. The inhibition by TIMP-1 appears to be uniquely noncompetitive by kinetic analysis.

The data presented above were analyzed by graphic methods, by nonlinear regression of the integral equations and by nonlinear regression combined with numeric integration of the differential equations. The fact that analysis of the same data under the same assumptions and approximations as previous workers produced the same results of model selection and values of kinetic parameters demonstrates the accuracy and utility of the latter method. In addition, the use of the latter method led to the identification of some mechanistic features not possible with the previously used methods. Moreover, the analysis of data by nonlinear regression with numeric integration provides several additional advantages over previously used methods. It is applicable to data gathered under the widest range of experimental conditions, and it requires the fewest mechanistic assumptions and approximations. Furthermore, the associated speed and convenience encourage the analysis of the data with a larger series of mechanistic models than possible by graphic methods or by analytic integration.

APPENDIX

The equations for rapid, tight-binding inhibitors, competitive and noncompetitive, are as follows. For competitive inhibition,

$$v_i = \frac{k_i}{2} \left[ \frac{1}{K_i + \frac{K_a}{A}} \left( \frac{1}{K_a + \frac{1}{A}} \right) \right]^{1/2} - \frac{1}{K_i + \frac{K_a}{A}} - \frac{I - E_i}{1 + \frac{K_a}{A}}$$

For noncompetitive inhibition,

$$v_i = \frac{k_i}{2} \left[ \frac{1}{K_i + \frac{K_a}{A}} \left( \frac{1}{K_a + \frac{1}{A}} \right) \right]^{1/2} - \frac{1}{K_i + \frac{K_a}{A}} - \frac{I - E_i}{1 + \frac{K_a}{A}}$$

$E_i$ is the total concentration of enzyme; the experimental variables are $v_i$ = initial velocity, $A$ = substrate concentration, $I_i$ = total inhibitor concentration; and the adjustable parameters are: $k_i$ = catalytic constant for the enzyme, $K_a$ = Michaelis constant for the enzyme and substrate, $K_{is}$ = dissociation constant of the inhibitor from enzyme not bound to substrate, and $K_{dis}$ = dissociation constant of the inhibitor from the enzyme-substrate complex.

REFERENCES

1. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. (1993) Crit. Rev. Oral Biol. Med. 4, 197–250
2. Woessner, J. F., Jr. (1991) J. Biol. Chem. 266, 17321–17326
3. Emonard, H., and Grimaud, J.-A. (1990) Biochem. J. 263, 261–266
4. Liotta, L. A., and Stetler-Stevenson, W. (1989) Natl. Cancer Inst. 81, 556–557
5. Willenbrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, I. R., and Murphy, G. (1993) Biochemistry 32, 4330–4337
6. Murphy, G., Koklitis, P., and Carne, A. F. (1989) Biochem. J. 261, 1031–1034
7. Nguyen, Q., Willenbrock, F., Cockett, M. I., O'Shea, M., Docherty, A. J. P., and Murphy, G. (1994) Biochemistry 33, 2089–2095
8. Welgus, H. G., Jeffrey, J. J., Eisen, A. Z., Roswit, W. T. & Stricklin, G. P. (1985) Collagen Relat. Res. 2, 167–189
9. Morrison, J. F., and Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301
10. Williams, J. W., and Morrison, J. F. (1979) Methods Enzymol. 63, 437–467
11. Wilkins, G. B. (1961) Biochem. J. 80, 324–332
12. Gauthier, W. G., and Bachovchin, W. W. (1993) Biochemistry 32, 8723–8731
13. Waley, S. G. (1993) Biochem. J. 294, 195–200
14. Chamberlain, A. P., Harkness, F. E., and Spivey, H. O. (1972) Comp. Biomed. Res. 5, 515–534
15. Morgenstern, K. A., Spricher, C., Helth, L., Foster, D., Grant, F. J., Ching, A., and Kiziel, W. (1994) Biochemistry 33, 3432–3441
16. Windsor, L. J., Bodden, M. K., Birkedal-Hansen, B., Engler, J. A., and Birkedal-Hansen, H. (1994) J. Biol. Chem. 269, 26201–26207
17. Bodden, M. K., Harbor, G. J., Birkedal-Hansen, B., Windsor, L. J., Caterina, N. C. M., Engler, J. A., and Birkedal-Hansen, H. (1994) J. Biol. Chem. 269, 18943–18952
18. Ogata, Y., Itoh, Y., and Nagase, H. (1995) J. Biol. Chem. 270, 18506–18511
19. DeClerck, Y. A., Yuan, T.-J., Lu, H. S., Ting, J., and Langley, K. E. (1991) J. Biol. Chem. 266, 3881–3899
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Knight, C. G., Willenbrock, F., and Murphy, G. (1992) FEBS Lett. 296, 263–266
The Mechanism of Inhibition of Collagenase by TIMP-1

23. MicroMath Scientific Software (1993) Scientist Handbook, Rev. 7F51, pp. 207–208, MicroMath Scientific Software, Salt Lake City, UT
24. Akaike, H. (1976) Math. Sci. 14, 5–9
25. Byrne, G., and Hindmarsh, A. (1976) EPISODE: An experimental package for the integration of systems of ordinary differential equations with banded Jacobians, Lawrence Livermore National Laboratory Report UCID-30132, Livermore, CA
26. Bakker, A. V., Jung, S., Spencer, R. W., Vinick, F. J., and Faraci, W. S. (1990) Biochem. J. 271, 559–562
27. Morrison, J. F. (1989) Biochim. Biophys. Acta 185, 269–286
28. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
29. Jordan, S. P., Waxman, L., Smith, D. E., and Vlasuk, G. P. (1990) Biochemistry 29, 11095–11100
30. Mercer, E., Cawston, T. E., De Silva, M., and Hazelman, B. L. (1985) Biochem. J. 231, 505–510
31. Huang, Z.-F., Wun, T.-C., and Broze, G. J., Jr. (1993) J. Biol. Chem. 268, 26950–26955
32. Bigg, H. F., Clark, I. M., and Cawston, T. E. (1994) Biochim. Biophys. Acta 1298, 157–165
33. Baragi, V. M., Fliszar, C. J., Conroy, M. C. Ye, Q.-Z., Shipley, J. M., and Welgus, H. G. (1994) J. Biol. Chem. 269, 12692–12697
34. Howard, E. W., Bullen, E. C., and Banda, M. J. (1991) J. Biol. Chem. 266, 13064–13069
35. Howard, E. W., and Banda, M. J. (1991) J. Biol. Chem. 266, 17972–17977
36. Kleiner, D. E., Jr., Unsworth, E. J., Krutzsch, H. C., and Stetler-Stevenson, W. G. (1992) Biochemistry 31, 1665–1672
37. Windsor, L. J., Steele, D. L., LeBlanc, S. B., and Taylor, K. B. (1996) Biochim. Biophys. Acta, in press
The Mechanism of Inhibition of Collagenase by TIMP-1
Kenneth B. Taylor, L. Jack Windsor, Nancy C. M. Caterina, M. Kirby Bodden and Jeffrey A. Engler

J. Biol. Chem. 1996, 271:23938-23945.
doi: 10.1074/jbc.271.39.23938

Access the most updated version of this article at http://www.jbc.org/content/271/39/23938

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 15 of which can be accessed free at
http://www.jbc.org/content/271/39/23938.full.html#ref-list-1