Characteristics of the Action of Human Skin Fibroblast Collagenase on Fibrillar Collagen

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The characteristics of the binding of pure human skin fibroblast collagenase to the in vivo form of its substrate, fibrillar collagen, has been studied under in vitro conditions. Collagenase binds tightly to reconstituted native collagen fibrils and appears to remain bound to the substrate throughout ongoing collagen degradation at 37°C independent of subsequent dilution with buffer or the addition of exogenous collagen as a competitor. Therefore, during the degradation of fibrillar collagen, no equilibrium appears to exist between collagenase molecules bound to fibrillar substrate and the buffer of the reaction mixture.

The binding of collagenase to fibrillar collagen at 25°C or 37°C displays saturation kinetics. At 25°C, binding is complete within 20 min, during which time collagen degradation is virtually absent. By analyzing the amount of bound enzyme as a function of collagenase concentration, the apparent Kd was determined to be 9.5 x 10^-7M. These same studies revealed that only 10% of the collagen molecules in the fibrillar substrate gel appear to be accessible to enzyme for binding, prior to degradation of the substrate. Despite the presence of a solid substrate, additional binding data indicate that, under appropriate volume conditions, equations derived from classical solution kinetics can be used as a model to rather accurately predict enzyme binding, provided that the number of substrate molecules available for binding is defined as equal to 10% of the total number of collagen molecules. It would, therefore, appear that collagen fibrils are composed of two sets of molecules: those that are initially available for collagenase binding, located on or near the surface of each individual fibril, and which comprise approximately 10% of the total, while the remaining 90% of molecules must become accessible to enzyme only during catalytic degradation of the substrate.

Finally, the turnover number of human skin fibroblast collagenase, with guinea pig type I fibrillar collagen as substrate, has been determined. Approximately 25 molecules of collagen are degraded per molecule of collagenase per h.

In recent years, a number of mammalian collagenases have been detected in a wide variety of tissues. These tissue collagenases appear to be zinc metalloenzymes, which also require exogenous calcium for activity (1). In addition, all such collagenases examined thus far cleave the native collagen triple helix at, or near, the same locus in the substrate molecule; between residues 775 and 776 in the α(I) chain (2). This cleavage in monomeric collagen results, at non-denaturing temperatures, in the appearance of ¾- and ¾-length derivatives of collagen, Tcα and TCβ (3-5). Studies have indicated that the cleavage site in type II and type III collagen is nearly identical to that of type I (6). Although the collagenase cleavage site in the substrate molecule has been identified, little information is available concerning the nature of the interaction of collagenase with its physiologic substrate, the collagen fiber.

Earlier studies of vertebrate collagenases led to the conclusion that previous failures to detect the enzyme in tissue extracts were due to tight binding of collagenase to endogenous collagen (7, 8). Thus, Ryan and Woessner developed an assay for rat uterine collagenase in which the enzyme appears to remain bound to, and act upon, the endogenous substrate over periods for as long as 18 h (9, 10). In this same system, Weeks et al. (11) have found that collagenase can only be dissociated from uterine collagen pellets by heating to the shrinkage temperature (Tc) of the fibrils, suggesting a very strong association between enzyme and substrate.

Precise studies on the interaction of collagenase with fibrillar collagen have been hampered by the lack of sufficient quantities of pure enzyme. Recent communications from this laboratory have described the production, purification, and characterization of a collagenase from human skin fibroblasts in monolayer culture (12, 13). The enzyme is secreted from fibroblasts as a set of twozymogens of molecular weights 60,000 and 55,000, respectively. The zymogen forms can be converted to fully active enzyme, either by incubation with trypsin and resultant loss of a 10,000-dalton peptide, or via an autoactivation process which occurs without a detectable change in molecular weight.

The dynamics of in vitro collagen degradation must be dependent, at least in part, upon the nature of the interaction of the physiologic substrate, the collagen fiber, and collagenase. However, parameters such as enzyme binding to collagen and the availability of substrate sites within a collagen fiber are presently undefined. As a result, values for the molecular activity or turnover number have not as yet been determined for any animal collagenase. The present communication describes studies designed to elucidate the characteristics of binding and subsequent activity of human skin fibroblast collagenase on native, reconstituted collagen fibrils.

MATERIALS AND METHODS

Reagents—Trypsin—Trypsin from porcine pancreatic trypsin, and soybean trypsin inhibitor were purchased from Sigma Chemical Co.

Culture Methods—Normal human skin fibroblasts (CRL 1187) were obtained from American Type Culture Collection. The cells

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were grown in the presence of 20% fetal calf serum and the medium harvested as described by Bauer et al. (14).

Purification of Fibroblast Collagenase—Purification of human skin fibroblast collagenase from serum-containing medium was accomplished by carboxymethylcellulose and Ultrogel AcA-44 chromatography, as described by Stricklin et al. (12). Pure collagenase was utilized in all experiments performed in this study.

Activation of Procollagenase—Procollagenase was activated proteolytically at 25°C by the addition of approximately equimolar trypsin for 10 min. After this time period, a 4-fold molar excess of soybean trypsin inhibitor was added to prevent any further tryptic action. A range of trypsin concentrations, usually from 0.1 to 10.0 µg/50 µl enzyme sample was used to ensure that maximal collagenase activity was achieved.

Assay Procedures—Collagenase activity was measured at 37°C using native, reconstituted [14C]glycine-labeled guinea pig skin collagen as substrate (15). Twenty-five or fifty microliters of a 0.4% collagen solution in 0.4 M NaCl, having a specific activity of approximately 45,000 cpm/µg, was employed for each assay. The collagen was gelled at 37°C for at least 12 h to permit completion of the aggregation process to occur. Following incubation with enzyme, the reaction mixtures were centrifuged at 10,000 x g for 10 min, and the supernatant fraction was counted in a liquid scintillation spectrometer. The buffer used for all enzymatic assays was 0.05 M Tris-HCl, pH 7.5, containing 0.01 M CaCl2 and 0.15 M NaCl.

Collagenase remaining unbound to fibrillar collagen was quantitated by analysis of enzyme activity remaining in the buffer of the reaction mixture. Following the incubation of collagenase and [14C]-labeled fibrillar collagen at 25°C, aliquots of unbound collagenase in the suspending buffer were diluted to concentrations <50 µg/ml to ensure assaying on the linear portion of the activity versus enzyme concentration curve. The aliquots were then incubated with a new [14C]-collagen substrate gel at 37°C and collagenase activity was quantitated following subtraction of the activity of the appropriate blanks.

Protein concentrations were determined spectrophotometrically by the method of Groves et al. (16) using bovine serum albumin to establish the standard curve.

Electrophoresis—Polyacrylamide gel electrophoresis was performed, utilizing the method of Laemmli (17). Following electrophoresis, the protein bands were stained with 1% Coomassie blue.

RESULTS

Factors Determining Collagenase Activity on Collagen Fibrils—Collagenase activity on fibrillar collagen is ordinarily determined by a method that depends on the quantitative release of soluble [14C]glycine-containing peptides from native reconstituted collagen fibrils (15). As previously shown for a number of other collagenolytic enzymes (5), the activity of human skin fibroblast collagenase on collagen fibrils increased in a nearly linear fashion with enzyme concentration up to 50 to 100 µg/ml (Fig. 1A). Significant loss of linearity occurred at higher concentrations, consistent with the saturation of substrate with enzyme. Reaction velocity appeared to be primarily dependent upon enzyme concentration; Fig. 1B illustrates the resultant activity when 100 µg (25 µl) of fibrillar collagen was incubated with 50 µl, 100 µl, 200 µl, and 400 µl of a 15 µg/ml solution of collagenase. Enzyme activity remained essentially unchanged, despite increasing amounts of collagenase present, and a decrease in the nominal concentration of substrate, as the volume increased. Reaction velocity appeared to be primarily dependent upon enzyme concentration; Fig. 1B illustrates the resultant activity when 100 µg (25 µl) of fibrillar collagen was incubated with 50 µl, 100 µl, 200 µl, and 400 µl of a 15 µg/ml solution of collagenase. Enzyme activity remained essentially unchanged, despite increasing amounts of collagenase present, and a decrease in the nominal concentration of substrate, as the volume increased. Under conditions of apparent substrate excess (i.e., 100 µg of fibrillar collagen, 75 µl of collagenase, concentration <50 µg/ml), collagen degradation was linear with time (Fig. 1C) until approximately 70% of the substrate gel was solubilized. Therefore, for such enzyme concentrations, expected linearity of reaction velocity with both time and enzyme concentration was indeed observed.

However, there were indications that the interaction of collagenase with fibrillar collagen was not a simple one. When enzyme concentrations, which clearly appeared to be saturating the substrate (i.e. 100 to 500 µg/ml from Fig. 1A), were incubated with fibrillar collagen (100 µg), substrate degrada-
denoted "premixed" enzyme. Of particular interest, the activity of prebound enzyme remained nearly constant as reaction volume increased, whereas premixed enzyme displayed the expected dependence on concentration. The time course of volume increased, whereas premixed enzyme displayed the tration 6808 also suggested that essentially when present in high concentration during the prebinding period at 25°C. This linearity of bound enzyme activity with time also suggested that essentially all of the additional enzyme remained bound to the gel throughout incubation at 37°C, independent of the volume of suspending buffer.

Several experiments were performed to confirm these observations. Fig. 3A demonstrates the difference in reaction velocity when 1.0 μg of pure collagenase, premixed in either 20 μl or 200 μl of buffer, was incubated with a collagen gel at 37°C. As expected, a large difference in reaction rates was observed. Fig. 3B shows the enzymatic breakdown of fibrillar collagen which was first prebound with 1.0 μg of collagenase in 20 μl of buffer at 25°C for 20 min. The reaction mixtures were then centrifuged at 10,000 × g for 10 min, and the resultant pellets were washed with buffer and resuspended in either 20 μl or 200 μl of the same buffer, prior to incubation at 37°C. Under these conditions, the observed reaction rates at 37°C were essentially identical. Enzyme activity reflected prebinding to collagen and was essentially independent of the volume of buffer subsequently added. Thus, no equilibrium appeared to exist between enzyme molecules bound to collagen following centrifugation and the buffer of the reaction mixture.

Finally, 1.0 μg of pure collagenase was prebound to equal amounts of either 14C- or 12C-labeled fibrillar collagen at 25°C for 20 min. The gels were then washed and transferred into tubes containing buffer and a second collagen gel—14C if enzyme was prebound to a 14C-gel, 12C if prebinding was to a 12C-gel. In a control test tube, an amount of enzyme approximately equal to that actually bound to the other samples was allowed to compete equally for [14C]- and [12C]collagen. All reaction mixtures were then incubated at 37°C for 75 min (Fig. 4). If dissociation of enzyme from fibrillar substrate occurred after enzymatic activity, all gels would be expected to release radioactively labeled peptides at a rate identical to the equal competition control sample after a single collagenolytic event at 37°C. However, as can be seen, despite active collagen breakdown at 37°C, most collagenase remained bound to the collagen gel to which it had been initially prebound.

Thus, the results of the foregoing experiments indicate that the binding of collagenase to collagen at 25°C is clearly

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**Fig. 2.** Effect of dilution on collagenase prebound to fibrillar collagen. A, in the prebound samples, 50 μl of collagenase (concentration = 40 μg/ml) was incubated at 25°C for 20 min with 200 μg of fibrillar collagen. Increasing volumes of buffer were subsequently added to the reaction mixtures. In the premixed samples, 2.0 μg of collagenase was appropriately diluted to the same final enzyme concentration as the prebound samples and then added to 200 μg of fibrillar collagen. Both prebound and premixed samples were then incubated at 37°C for 60 min, B, time course of prebound versus premixed samples prepared as described in A and suspended in a final volume of 400 μl.

**Fig. 3.** Effect of dilution on the activity of prebound collagenase following centrifugation. A, 1 μg of pure collagenase was suspended in either 20 μl (●) or 200 μl (○) of buffer and then added to 200 μg of fibrillar collagen. Comparison of the time course of subsequent collagen degradation at 37°C is shown. B, 1 μg of collagenase in 20 μl of buffer was prebound to 200 μg of fibrillar collagen at 25°C for 20 min. The reaction mixture was then centrifuged at 10,000 × g for 10 min, and the resultant collagen pellet was washed, resuspended in either 20 μl (●) or 200 μl (○) of buffer, and then incubated at 37°C.

**Fig. 4.** Substrate competition for prebound enzyme. Seventy-five microliters of collagenase (13 μg/ml) was prebound to 200 μg of either 14C (●) or 12C (○) fibrillar collagen at 25°C for 20 min. The gels were then washed, transferred into tubes containing 100 μl of buffer plus a second collagen gel (14C if prebinding was to a 14C-gel, 12C if prebinding was to a 12C-gel), and then incubated at 37°C. In a control test tube (○), an amount of enzyme approximately equal to that actually bound to the other samples (0.5 μg) was allowed to compete equally for [14C] and [12C]collagen.
dependent on initial enzyme concentration, and once bound to fibrillar collagen, most collagenase molecules remain bound to this substrate throughout ongoing enzyme activity and collagen degradation at 37°C.

Availability of Substrate Binding Sites—We next investigated whether all molecules in a fibrillar collagen gel are equally accessible to collagenase for binding. In the first experiment, 50 μl of three different enzyme concentrations (100 μg/ml, 60 μg/ml and 15 μg/ml) were bound to collagen gels for increasing periods of time at 25°C. The gels were then washed, resuspended in buffer, and allowed to incubate at 37°C. As shown in Fig. 5, collagenase binding was apparently complete at 25°C within 20 min. Enzyme binding at 37°C was also studied. At this temperature, binding was complete within 12 to 15 min and maximum enzyme bound always approached a level identical to that observed at 25°C. However, significant substrate degradation ensued during the binding process at the higher temperature, and, therefore, all subsequent binding studies were performed at 25°C.

Increasing amounts of pure collagenase were then bound to 200 μg of fibrillar collagen at 25°C for 20 min. Following completion of the binding process, the amount of enzyme bound was determined by analysis of enzyme activity remaining in the supernatant after appropriate dilution to ensure assaying on the linear portion of the enzyme concentration activity curve. Bound enzyme calculated in this manner corresponded exactly with the rates of collagenolysis observed when the gels themselves were washed, resuspended in buffer, and collagen degradation by bound enzyme was measured at 37°C. As shown in Fig. 6, plotting bound versus free collagenase resulted in a typical saturation curve. When subsequently assayed on the linear portion of the enzyme concentration activity curve, the y intercept represents the maximum amount of enzyme bound when 75 μl of four collagenase concentrations (1 pg/ml, 15 pg/ml, 100 pg/ml, and 500 pg/ml) were bound to 200 μg of fibrillar collagen. Predicted values were derived from the equation

\[
K_d = \frac{X}{A}
\]

where \(K_d\) is the dissociation constant, \(X\) is the amount of enzyme bound, and \(A\) is the number of initially available collagenase binding sites.

An experiment was now performed to determine why such a large proportion of the collagen molecules within a collagen gel appear to be inaccessible to the enzyme prior to substrate degradation. This was designed to distinguish between two possible explanations: 1) that all collagen molecules within a given fibril are equally available to collagenase for binding, but that the nature of the semispherical substrate gel allows only those fibrils at or near the surface of the gel itself access to the enzyme, or 2) that all fibrils within this gel are equally available to the enzyme, but that the close packing of collagen monomers allows the enzyme to bind only to those molecules on the surface of each fibril. In this experiment, human fibroblast procollagenase, which is totally inactive and has been shown not to bind to fibrillar collagen (13), was mixed with [14C]collagen in solution. Gel formation at 37°C for 30 min was allowed to occur and buffer was subsequently added. The polymerization process was apparently >95% complete at that time, as determined by comparison to the blank obtained after gelation for 24 h without included proenzyme. At various times after the addition of buffer, samples were removed and assayed for the appearance of procollagenase in the suspending medium. The results (Fig. 7) indicated that by 1 min, approximately 30% of the zymogen had diffused from the gel into the buffer, and by 15 min, equilibration was complete. Therefore, collagen fibrils within the interior of a collagen gel must be available to enzyme prior to substrate degradation, since the procollagenase molecule can readily diffuse from the interior of a gel into the external buffer.

Reassessment of Factors Determining Collagenase Activity on Collagen Fibrils—Despite the limitations imposed by the use of a solid substrate, by analogy with membrane-bound receptor systems (19, 20), we have determined an apparent dissociation constant, \(K_d\) (Fig. 6). The value of this \(K_d\) and the measurement of the number of available binding sites in fibrillar collagen provides an explanation of our initial observation of the seeming coexistence of substrate excess and saturation when measuring collagenase activity on collagen fibrils. Utilizing the experimentally derived value, \(K_d = 9.5 \times 10^{-7} M\). Table I illustrates the predicted and observed amounts of enzyme bound when 75 μl of four collagenase concentrations (1 μg/ml, 15 μg/ml, 100 μg/ml, and 500 μg/ml) were bound to 100 μg and 300 μg of fibrillar collagen. Predicted values were derived from the equation

\[
K_d = \frac{X}{A}
\]

where \(K_d\) is the dissociation constant, \(X\) is the amount of enzyme bound, and \(A\) is the number of initially available collagenase binding sites. Examination of the table shows that actual values of bound enzyme coincide with the predicted values over a range of enzyme concentrations and substrate quantities, provided that only 10% of the total number of collagen molecules is taken as the number of initially available...
binding sites. Alternatively, if all collagen molecules are assumed to be accessible, the predicted values fail to reflect accurately the resultant bound enzyme in any of the cases presented in Table I.

At all enzyme concentrations, even those <50 µg/ml, significantly more collagenase was bound to fibrillar collagen when the substrate was increased above 100 µg (Table I). This behavior was accurately predicted by the above equation, and is the direct consequence of the fact that available binding sites are present in lower concentration than Kd. As seen in Table I, if all collagen molecules were initially accessible to enzyme, such increases in substrate would not result in enhanced collagenase binding. Thus, for example, when 75 µl of collagenase of concentration 15 µg/ml was bound to 100 µg of fibrillar collagen, already representing a nominal 17-fold molar excess of substrate, the amount of enzyme bound nevertheless increased significantly with a higher quantity of substrate present (Fig. 1D, Table I). Since only 10% of total substrate molecules are initially accessible to collagenase, this enhanced enzyme binding occurred, in large measure, because of an effective substrate concentration (3.46 × 10⁻² M) which is less than Kd (2.5 × 10⁻⁵ M), and additionally, because 100 µg of fibrillar collagen actually constitutes only a 1.7-fold molar excess of substrate.

When assaying collagenase concentrations >100 µg/ml on 100 µg of fibrillar collagen, substrate saturation was clearly evidenced from the reaction velocity versus enzyme concentration plot depicted in Fig. 1A. As expected, most available substrate molecules (>50%) were occupied by a collagenase molecule at the above enzyme concentrations (Table I). Therefore, fibrillar substrate is indeed saturated with enzyme. However, since a maximum of 10% of the total number of substrate molecules are accessible to collagenase for initial binding, degradation of collagen always occurs in real substrate excess and will proceed linearly with time until approximately 70% gel lysis, no matter how high the collagenase concentration used.

**Determination of the Turnover Number of Human Skin Fibroblast Collagenase on Collagen Fibris**—With the knowledge of the time course of enzyme binding to fibrillar collagen and the apparent nonsociable nature of this binding, the turnover number characterizing the degradation of guinea pig type I collagen fibrils by human skin fibroblast collagenase could now be calculated. Following incubation of several concentrations of pure collagenase with collagen fibrils at 25°C, bound enzyme was determined by supernatant analysis. The gels were then washed with buffer, transferred to a second test tube, collagen degradation was measured at 37°C, and the number of collagen molecules degraded per collagenase molecule was determined (Table II). Approximately 25 molecules of collagen were degraded per molecule of collagenase per h. Alternatively, a single collagenase molecule catalyzed the enzymatic cleavage of one α chain of a collagen molecule in 45 sec. This turnover number was constant at all concentrations of enzyme used (Table II).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the supernatant fraction of the transferred gels, following 50% collagen degradation at 37°C, is shown in Fig. 8. The supernatant contains only solubilized TCA and TC² fragments, confirming that the same catalytic event characterizing the enzymatic cleavage of collagen molecules in solution at 25°C also occurs with fibrillar collagen as substrate.

Finally, knowing both the turnover number and the number of initially available binding sites in fibrillar collagen, the

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![Graph](image)

**Table I**

<table>
<thead>
<tr>
<th>Initial enzyme concentration (µg/ml)</th>
<th>Initial enzyme amount (µl)</th>
<th>Predicted enzyme bound if available [substrate] (µl)</th>
<th>Observed enzyme bound (µl)</th>
<th>Observed % of substrate sites occupied if available [substrate]</th>
<th>Predicted enzyme bound if available [substrate] (µl)</th>
<th>Observed enzyme bound (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg/ml</td>
<td>0.075</td>
<td>0.659</td>
<td>0.202</td>
<td>0.022</td>
<td>3.46 × 10⁻¹ M</td>
<td>3.69 × 10⁻¹ M</td>
</tr>
<tr>
<td>15 µg/ml</td>
<td>1.13</td>
<td>0.88</td>
<td>0.27</td>
<td>0.23</td>
<td>3.46 × 10⁻¹ M</td>
<td>3.69 × 10⁻¹ M</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>7.50</td>
<td>5.47</td>
<td>1.09</td>
<td>1.00</td>
<td>3.46 × 10⁻¹ M</td>
<td>3.69 × 10⁻¹ M</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>37.5</td>
<td>15.87</td>
<td>1.72</td>
<td>1.40</td>
<td>3.46 × 10⁻¹ M</td>
<td>3.69 × 10⁻¹ M</td>
</tr>
</tbody>
</table>
TABLE II

Turnover number of human skin fibroblast collagenase

Fifty-microliter quantities of several concentrations of pure collagenase were incubated at 25°C for 20 min with 200 µg of fibrillar collagen to ascertain maximal binding. The gels were then washed with buffer, transferred to a second test tube, resuspended in buffer, and collagen degradation was measured at 37°C. Bound collagenase was determined as described in Fig. 6.

<table>
<thead>
<tr>
<th>Enzyme concentration (µg/ml)</th>
<th>Total enzyme recovered</th>
<th>Collagenase bound</th>
<th>Collagen degraded per h (molecules of collagen degraded/molecule of collagenase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>6.25</td>
<td>1.50</td>
<td>177.7</td>
</tr>
<tr>
<td>62.5</td>
<td>31.12</td>
<td>0.93</td>
<td>106.4</td>
</tr>
<tr>
<td>125</td>
<td>62.5</td>
<td>0.51</td>
<td>64.3</td>
</tr>
</tbody>
</table>

Fig. 8. Collagenase cleavage products on fibrillar collagen. Fifty microliters of pure collagenase (31 µg/ml) was prebound to 200 µg of 14C-labeled fibrillar collagen at 25°C for 20 min. The collagen gel was then washed, transferred to a second test tube, resuspended in buffer, and incubated at 37°C until 40 to 50% gel lysis. The sample was then centrifuged, and the supernatant fraction was applied to a sodium dodecyl sulfate polyacrylamide slab gel. Slot A, undegraded fibrillar collagen. α refers to the constituent 100,000-dalton polypeptide chains of the native collagen molecule. β denotes the intramolecular dimers formed from these chains; Slot B, supernatant fraction of fibrillar collagen, following 30% degradation as described above. Note the appearance of typical ¼-length (α⅓, β⅓) and ½-length (α½) products of collagenase activity. The enhanced protein band, present at the front of slot B compared to slot A, represents trypsin and SBTI used to activate the collagenase.

fastest possible rate of collagen gel lysis at 37°C can be theoretically calculated. This rate would be predicted never to exceed 50% gel lysis in 10 min, regardless of how high the collagenase concentration employed (i.e. 10% of total substrate molecules accessible for enzyme binding × 5 turnover times). Indeed, utilizing enzyme concentrations as high as 600 µg/ml, we have never observed reaction velocities in excess of this for either 100 µg or 200 µg of total fibrillar substrate.

DISCUSSION

In an attempt to gain insight into in vivo collagen-degrading processes, we have examined the interaction of human skin fibroblast collagenase with its physiologic substrate, the collagen fiber. The results of this study indicate that the large majority of collagenase molecules, once bound to collagen, remain bound to that substrate throughout extensive degradation of the collagen. Persistence of binding of collagenase to fibrillar collagen is independent of the volume of suspending reaction buffer, or the presence of another collagen gel in the reaction mixture. Two possible explanations for this apparent lack of equilibrium between bound collagenase and the external buffer during ongoing collagen degradation are: 1) that, once bound to a collagen molecule within the fibrillar collagen gel, enzyme molecules are in such close proximity to other substrate molecules, that they appear not to be in equilibrium with buffer, or 2) that a mechanism exists whereby a collagenase molecule binds directly to a second molecule of substrate prior to its dissociation from the products of a previous catalytic event. In this way, a molecule of collagenase could move from one substrate molecule to another without the existence of a free-solution intermediate.

The studies reported here cannot distinguish between these two alternatives. Whatever the mechanism, however, the phenomenon of apparent nondissociable binding of active enzyme to fibrillar collagen may have important in vivo implications. Our in vitro data indicate that, once bound to fibrillar collagen, collagenase continues to degrade the substrate nearly to completion, without leaving the substrate gel. This raises the question of how in vivo collagenolysis is terminated. In this respect, the collagenase inhibitor synthesized by human skin fibroblasts (21) which appears to inhibit the enzyme in the presence of substrate, may play a crucial role in the precise regulation and termination of collagenase activity in vivo.

Our studies also indicate that, despite the tightness of association of collagenase with its substrate, less than 10% of the molecules in a fibrillar collagen gel are initially accessible to enzyme prior to substrate degradation. When the collagenase zymogen, which does not bind to collagen, is itself incorporated into the substrate gel during fibril aggregation, a significant fraction equilibrates with the reaction mixture buffer within 1 min, and full equilibrium is achieved by 15 min. Thus, the substrate gel itself is not a barrier for macromolecular diffusion. Therefore, all collagen fibrils contained within the gel appear to be accessible to enzyme, but only a small fraction of the total number of molecules within each fibril, presumably those located on its surface, seem initially able to bind collagenase. Scanning electron microscopic examination of the collagen substrate gels utilized in these studies (data not shown) reveal the presence of fibrils, the bulk of which have diameters of 50 to 100 nm. A simple geometric model of an idealized cylindrical collagen fibril of diameter 50 nm, assuming the area of a cross-section to be filled with 1.5 nm collagen molecules in closest packing arrangement, indicates that approximately 12% of the total number of molecules present will occupy the surface of the fibril. For a fibril of 100 nm in diameter, this calculated percentage drops to 6%. Thus, our experimentally derived
value of 10% is in good agreement with the predictions of such a geometric model. It should be noted that, in the foregoing discussion, it is assumed that all collagen molecules can be bound by only a single enzyme molecule. While no definitive evidence exists to support this assumption, our data indicate that essentially all binding that occurs is of the same affinity. Nonlinearity of the Lineweaver-Burke plots of bound versus free enzyme has never been observed. This fact, coupled with the correspondence of our values of actual maximum binding to those predicted from spatial considerations, makes it unlikely that significant fractions of observed binding is of a nonspecific nature.

The finding that only limited numbers of substrate molecules are available for enzyme binding, and the suggestion that these molecules are located on the surface of the collagen fibrils, can be used to explain our initial puzzling findings of apparent coexistence of both substrate excess and substrate saturation at the same enzyme concentration. Our studies indicate that, functionally, an individual collagen fibril is composed of two distinct sets of substrate sites. The first set, constituting approximately 10% of all molecules present within the fibril, are presumably located on or near its surface, and are the only collagen molecules available for enzyme binding prior to substrate degradation. The second set, comprising the remaining 90% of molecules within a fibril, must become accessible to collagenase only following enzymatic degradation of the first set. Since only those molecules of the first set can ever be occupied by collagenase during initial enzyme binding, catalytic degradation of the substrate always proceeds linearly with time.

Therefore, when enzyme concentrations >100 µg/ml are utilized, saturation of the available substrate with collagenase is approached, as evidenced by only marginal increases in enzyme activity, despite large increases in collagenase concentration (Fig. 1A). Yet, collagen degradation proceeds linearly with time, characteristic of substrate excess, since 90% of the molecules of a substrate gel always remain unoccupied following maximal enzyme binding to collagen. On the other hand, when using enzyme concentrations <50 µg/ml, even 100 µg of total substrate represents appreciable nominal molar excess of fibrillar collagen to enzyme. Nevertheless, collagen molecules available for binding are in considerably less excess, and indeed, represent an effective substrate concentration which is considerably greater than $K_d$. As a direct consequence of this, additional substrate results in enhanced collagenase binding at these enzyme concentrations, despite a significant molar excess of total substrate and a total substrate concentration which is considerably greater than $K_d$.

When small volumes of enzyme (<75 µl) are assayed on fibrillar collagen, thus effectively surrounding the 25-µl, 50-µl, or 75-µl collagen gel with only a thin rim of buffer, resultant equilibrium conditions are reasonably well described by standard equations derived from classical solution kinetics. Thus, using the experimentally determined value $K_d = 9.5 \times 10^{-7}$ M, the equation $K_d = [enzyme]_{free} \times [substrate]_{free}/[enzyme-substrate]_{bound}$ predicts at several enzyme concentrations and substrate quantities, the actual amount of enzyme-substrate complex formed (Table I). This occurs, provided that substrate concentration is defined in terms of fibrillar collagen molecules accessible to collagenase, that is, as equal to $\frac{1}{2}$ the total number of collagen molecules present/volume of the reaction mixture.

Application of the above equation reveals that for any enzyme-substrate assay performed in solution, when enzyme concentration is lower than $K_d$, substrate concentrations considerably greater than $K_d$ are required to achieve true substrate excess. Thus, virtually all described enzymatic assays utilize substrate concentrations much greater than $K_d$, thereby assuring that essentially all enzyme present is bound to substrate. However, since only 10% of collagen molecules in fibrillar form are accessible for initial binding, total substrate concentrations in excess of $10 \times K_d$ would be needed to meet these requirements in the case of fibrillar collagen. Unfortunately, this is not practicable, due to the viscosity of collagen, which largely prohibits the use of final reaction mixture concentrations higher than $6.7 \times 10^{-7}$ M. Therefore, when assessing collagenase activity on collagen fibrils, initial enzyme binding occurs at an available substrate concentration of $6.7 \times 10^{-7}$ M, significantly less than $K_d$; consequently, even at enzyme concentrations much less than $K_d$, large percentages of collagenase remain unbound, as shown in Table I.

It should be emphasized that equations derived from solution kinetics are only satisfactory as a model for describing equilibrium conditions between collagenase and fibrillar collagen, when enzyme volume is very small. At collagenase volumes in excess of 75 µl, equilibrium concentrations are no longer predicted with sufficient accuracy, presumably due to the solid nature of the substrate gel, whose molecules available for enzyme binding must no longer be equally accessible to each collagenase molecule present in solution. Therefore, equations from solution kinetics provide a valuable analytic tool only under the above limited set of conditions. However, as shown in Fig. 1B, collagenase concentration is always the crucial determinant of enzyme binding to fibrillar substrate. Thus, at any volume of enzyme employed, even those in considerable excess of the volume occupied by fibrillar substrate, a given enzyme concentration will always specify an amount of binding to a fixed quantity of solid substrate.

Measurements of specific activity on fibrillar collagen have been utilized to assess catalytic rates for many of the different collagenases studied to date (12, 22, 23). Implicit in the interpretation of specific activity is the assumption that increasing the number of enzyme molecules present results in proportionally enhanced substrate binding and reaction velocity. However, collagenase concentration, not the absolute number of enzyme molecules, is the major determinant that dictates binding to fibrillar collagen. Thus, when increasing volumes of a constant enzyme concentration were assayed on fibrillar collagen (Fig. 1B), the rate of observed collagenolysis did not change appreciably. Yet, the calculated specific activity of fibrillar collagenase resulting from this maneuver would dramatically decrease due to the greater number of enzyme molecules present at the higher volumes. Additionally, as discussed above, although reaction velocity on fibrillar collagen may be linear with both time and enzyme concentration, large percentages of enzyme remain unbound at all collagenase concentrations. These facts underscore the need for caution when the specific activity of any collagenase is assessed on collagen fibrils. Clearly, determination of precise turnover numbers, dependent solely on the activity of bound enzyme, would provide a more valid comparison among reaction velocities on the fibrillar form of substrate of the many collagenases examined thus far.

An important conclusion of these studies is that the turnover number of collagenase on guinea pig type I fibrillar collagen is indicative of a very slow enzymatic process—on the order of 25 molecules of collagen are cleaved per enzyme molecule per h. Indeed, this is certainly one of the lowest turnover numbers observed for an enzyme-catalyzed reaction to date. Although we have no data at present indicating the reasons for this low turnover number, it is interesting to note the trend, as reviewed by Sols and Gancedo (24), towards far slower turnover numbers, when both enzyme and substrate are macromolecules, as compared to the velocities character-
izing the interaction of enzymes with low molecular weight substrates. It is also worthwhile to speculate that perhaps additional properties of the collagen substrate itself contribute further to the low turnover number observed. First, since the collagen molecule is large, extremely asymmetric, and contains only a single catalytic site for human fibroblast collagenase, there may be difficulty in obtaining effective collisions between enzyme and substrate. Second, if sequential hydrolysis of all three polypeptide chains of the collagen triple helix were required for successful completion of a single substrate cleavage, three binding, hydrolysis, and dissociation steps would have to occur before collagenase could then proceed to another substrate molecule. Finally, the catalytic degradation of a highly structured, solid substrate might be expected to proceed at a rate far slower than that which is typically observed for protein molecules in solution. Studies designed to elucidate which, if any, of these mechanisms are responsible for the slow turnover number of human skin fibroblast collagenase on fibrillar collagen are currently in progress.

Nevertheless, despite the solid nature of the fibrillar collagen substrate, the site of collagenase cleavage appears to be identical to that previously reported for vertebrate collagenases, using collagen molecules existing freely in solution as substrate (4). As seen in Fig. 8, a single cleavage in the fibrillar collagen molecule at a locus approximately 1/3 from its NH₂-terminal end results, at 37°C, in the appearance of TC⁶ and TC⁸ products in the supernatant of the reaction mixture.

It should be noted that in vivo collagen-degradative processes are slow. Normal cutaneous collagen turnover, as well as phenomena such as wound healing and amphibian metamorphosis, occur in biological time frames of days or months. In the postpartum involution of the human uterus, which represents perhaps the most rapid process of connective tissue resorption known, less than 0.1 mmol of collagen is degraded in approximately 10 days (25). The results obtained in this study indicate that this amount of degradation would require approximately 1 mg of collagenase. The low turnover number of human skin fibroblast collagenase on fibrillar collagen is, therefore, compatible with, and may be partially responsible for, the slow rate at which collagen degradative processes occur in vivo.

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REFERENCES