Studies on the Activation Energy and Deuterium Isotope Effect of Human Skin Collagenase on Homologous Collagen Substrates*

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The activation energy (E_a) and solvent-deuterium kinetic isotope effect (kH2O/kD2O) of human skin fibroblast collagenase were studied on the homologous human type I, II, and III collagens in both native and denatured states. Values for E_a on human type I and II collagens in solution were 47,000 and 61,000 cal, respectively. The Arrhenius plot for type III collagen, unlike that for the other types, was characterized by a break in E_a at approximately 26 °C. At temperatures below this point, E_a was 42,500 cal; at higher temperatures, E_a fell to 29,500 cal. This latter value, intermediate between type I collagen monomers and denatured random gelatin α chains, appears to result from a further opening in the already loosened helix of the type III collagen molecule in the region of the 3/4:3/4 collagenase cleavage site. The kcat of trypsin on native human type III collagen was also measured and found to be 70,000 cal. This high value calls into question the role of serine proteases in the physiologic degradation of this substrate; a much higher energy expenditure was required for trypsin to cleave type III collagen than for the fibroblast collagenase.

Reaction velocity on human collagen types I–III in solution was slowed 15–35% (kH2O/kD2O = 1.2–1.5) by the substitution of deuterium for hydrogen in the solvent buffer. This value was far lower than that observed following the aggregation of solution monomers into insoluble fibrils (kH2O/kD2O = 9). Denaturation of triple helical monomers into random gelatin α chains eliminated any slowing by deuterium, and kH2O/kD2O was 1.0 in all cases. The same peptide bond hydrolysis accompanies the cleavage of all of these forms of the collagen substrate, it would appear that the role of water at the rate-limiting step of collagen degradation may not reside in the hydrolysis of a peptide bond per se, but rather may reflect the difficulty in transporting water molecules to the site of such catalysis, especially following fibril aggregation.

In a previous study (1), the activation energy and solvent-deuterium kinetic isotope effect which characterize the action of human skin fibroblast collagenase on guinea pig type I collagen were examined. The E_a on collagen fibrils was measured to be 101,000 cal, extraordinarily high for an enzyme-catalyzed reaction (2). This value of E_a resulted in a 3-fold change in collagenase activity/°C change in temperature. This unusual dependence of fibrillar collagen degradation upon energy greatly exceeded the highest reported values for E_a of other enzymatic reactions. The energy of activation on collagen fibrils was accompanied by an extremely high solvent-deuterium kinetic isotope effect, as indicated by a value for the ratio (kH2O/kD2O) of 9. Thus, the degradation of fibrillar collagen by fibroblast collagenase exhibited a marked dependence upon both energy and hydrogen transfer. Substituting monomeric collagen in solution for fibrils as substrate, E_a was somewhat lower, 49,200 cal, still a very high value for enzymatic catalysis. The replacement of hydrogen by deuterium in the solvent buffer slowed the cleavage of this form of substrate by only 2-fold, a more commonly observed isotope effect for peptide bond hydrolysis. Finally, the E_a of fibroblast collagenase on denatured collagen, or gelatin, was 13,000 cal, further suggesting that the degree of substrate organization, specifically the presence of helical structure and particularly the aggregation of molecules into fibrils, was largely responsible for the observed water- and energy-dependence of catalysis.

The collagen specificity of human fibroblast collagenase with respect to both type and animal species of substrate origin has also been examined (3). Collagen types I (skin, bone, and tendon), II (cartilage), and III (skin, blood vessels, and GI tract) were all cleaved by the fibroblast enzyme. The K_m values, or affinities, of collagenase for these degraded collagens were similar, 1–2 × 10^-6 m. In contrast, catalytic rates varied markedly; the kcat for human type I was 53.4 h^-1, for human type II was 1.0 h^-1, and for human type III kcat was 565 h^-1. There were also significant, though more modest, differences attributable to species of substrate origin, the homologous human type I and III collagens being more susceptible to degradation than their respective nonhomologous counterparts.

Detailed information on the activation energy and the solvent deuterium isotope effect have been reported for human skin fibroblast collagenase only with guinea pig skin type I collagen as a substrate. Such studies have not been available for other collagen types, especially the homologous human collagens. Of particular interest is the question of whether a relationship exists between these energetic parameters and the relative susceptibility of collagen types to the enzyme. In the present study, we have examined this question, using human types I, II, and III collagens in both the native and denatured states.

MATERIALS AND METHODS

Reagents

Acrylamide and bisacrylamide were purchased from Eastman. Sodium dodecyl sulfate (99% pure) and deuterium oxide (99.8%) were purchased from Eastman.
obtained from Gallard-Schlesinger. Tris base, bovine pancreatic trypsin (type III), and soybean trypsin inhibitor were procured from Sigma. All other chemicals were reagent-grade.

Culture Methods

Normal human skin fibroblasts were grown in the presence of 10% fetal calf serum and the medium harvested as described by Bauer et al. (4).

Purification of Collagenase

Human skin fibroblast procollagenase was purified to homogeneity from serum-containing medium by a combination of ammonium sulfate precipitation, carboxymethylcellulose chromatography, and Ultrigel Aca-44 chromatography, as described by Stricklin et al. (5, 6).

Activation of Collagenase

The activation of fibroblast procollagenase was accomplished photochemically by the addition of trypsin at 25 °C for 10 min. Further trypsinic action was prevented by adding an 8-fold molar excess of soybean trypsin inhibitor. Maximal collagenase activity was ensured by performing a trypsin titration.

Preparation of Collagens

Human collagen types I and III were isolated from human chorionicamniotic membranes as previously described (7). Human type II collagen was isolated from neonatal hyaline cartilage of the femoral head as previously reported by Burgess and Hollister (8). Matrix collagens were solubilized by limited pepsin digestion and partially purified by differential salt precipitation. Final purification was obtained following ion exchange chromatography on DEAE-cellulose under nondenaturing conditions. Each collagen was better than 95% pure as determined by polyacrylamide gel electrophoresis and amino acid analysis.

Assay Procedures

**Determination of EA on Collagens in Solution—**Values for the $E_A$ of human skin collagenase on the different collagen preparations studied were determined in solution as described previously (1). Briefly, collagen degradation was measured in 1-2 °C increments over the temperature range of 14-34 °C. Because of the very high values for $E_A$, the same enzyme concentration could not be used at all temperatures, varying only the time of incubation. Rather, for each collagen examined, one enzyme concentration was utilized between 14 and 24 °C, and a lower one employed to ascertain the data points from 24-34 °C. At each temperature, collagenase was incubated with 12 μg of collagen in a total reaction mixture volume of 100 μl. Degradation of collagen was always <25% of the initial amount present, thereby enabling measurement of a true initial velocity (3). The lengths of incubation at each temperature were chosen, following preliminary experiments, to give approximately equal substrate degradation, thus permitting the most accurate possible measurement of $E_A$. Following incubation, the reaction mixtures were stopped with EDTA, applied to a polyacrylamide slab gel, and reaction velocity quantitated following spectrophotometric scanning of the stained 3% length products (1, 3). The results were then graphed as an Arrhenius plot and the $E_A$ calculated by the equation

$$\ln \frac{k_0}{k_1} = \frac{E_A}{RT} - \frac{1}{T_1} - \frac{1}{T_2}.$$  

The $E_A$ of trypsin on human type III collagen was similarly determined between 16 and 32 °C. In the case of trypsin, however, the reaction mixtures were stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM.

The buffer used for reaction mixtures involving collagen types I and II was 0.05 M Tris, 0.01 M CaCl$_2$, 0.25 M NaCl, pH 7.5. The buffer utilized for reaction mixtures containing type III collagen was 0.05 M Tris, 0.01 M CaCl$_2$, 1.0 M NaCl, 1.0 M glucose, pH 7.5. This latter buffer was chosen in order to minimize the propensity of the type III substrates for microscopic aggregation.

**Solvent-Deuterium Kinetic Isotope Effect—**Values for the deuterium isotope effect ($k_{H_2O}/k_{D_2O}$) were determined as described previously (1). In all cases, a final D$_2$O:H$_2$O ratio equal to 9.0 was utilized in the solvent buffer of the reaction mixture. For reactions involving gelatin, the native substrate was exposed to a temperature of 70 °C for 20 min in order to achieve thermal denaturation. Measurements of $k_{H_2O}/k_{D_2O}$ were performed at 20 °C for the native collagen preparations and at 57 °C on the denatured substrates.

Protein concentrations were determined spectrophotometrically by the method of Groves et al. (9). Albumin was used to establish a standard curve.

The hydroxyproline content of the various collagens studied was determined by the method of Bergmann and Loxley (10).

**RESULTS**

The activation energy of human skin fibroblast collagenase on human type I collagen in solution was measured between 14 and 34 °C in 2 °C increments. A linear Arrhenius plot was obtained (Fig. 1), from the slope of which was derived an $E_A$ of 47,000 cal. This value for the homologous type I collagen is very similar to the value of 49,200 cal for the activation energy of fibroblast collagenase on monomeric guinea pig type I collagen (1). The activation energy on human type II collagen was next measured in 2 °C intervals over the same range of temperatures (14-34 °C). Again, a linear Arrhenius plot resulted (Fig. 2), with an apparent value for $E_A$ of 61,000 cal. The difference in activation energy between human type I and II collagen degradation implies that cleavage of the native type II substrate is a more energy-dependent process than for type I. The above values indicate that human fibroblast collagenase activity on its homologous type I collagen increases approximately 13.5-fold for each 10 °C increase in reaction mixture temperature, whereas for the homologous type II collagen, the magnitude of this increase is 29-fold.

In contrast to the determinations of $E_A$ on human collagen types I and II, the Arrhenius plot of human type III cleavage clearly was not linear throughout its entire length (Fig. 3). Rather, when measured in 1 °C increments, the observed plot is seen to be the result of the intersection of two straight lines, each characterized by a different activation energy. In effect, there is a break in the Arrhenius plot at a temperature of approximately 26 °C; at temperatures below 26 °C, $E_A$ is equal to 42,500 cal, above 26 °C, this value falls to 29,500 cal.

In order to provide further insight into the reaction mechanism of human skin fibroblast collagenase on its homologous collagen substrates, the solvent-deuterium kinetic isotope effect was examined. Values for $(k_{H_2O}/k_{D_2O})$ were determined.
between different levels of substrate organization in Table I, Tables 11, and II is not present for different collagen types at the same level of substrate order. The magnitude of the deuterium isotope effect is diminished even further following substrate denaturation. These results are also shown in Table II. Once collagen was thermally denatured into random α chains, the substitution of deuterium for hydrogen in the solvent buffer of the reaction mixture completely failed to affect the rate of substrate degradation. No measurable deuterium isotope effect on fibroblast collagenase-catalyzed gelatinolysis was observed for any substrate preparation examined.

Type III collagen, unlike types I and II, is known to be susceptible to enzymatic attack by certain noncollagenolytic proteases such as trypsin (11) and thermolysin (12). This susceptibility has been suggested to be due to the presence of a looser helix in the region of the ¾ collagenase cleavage site in the native molecule. In this study, the activation energy of trypsin on human type III collagen was determined in the same manner as described for fibroblast collagenase.

Values for the deuterium isotope effect (k_D/k_H) of human fibroblast collagenase were determined on human collagen types I, II, and III in solution as described in Figures 1–3. The value for k_D/k_H of trypsin on human type III collagen was determined as described in Figure 4.

| Collagen     | Enzyme         | k_D/k_H  |
|--------------|----------------|----------|
| Human type I | Fibroblast collagenase | 47,000   |
| Human type II| Fibroblast collagenase | 61,000   |
| Human type III| Fibroblast collagenase | 29,500 (fibris) |
| Guinea pig type I | Fibroblast collagenase | 49,500 (monomers)² |
| Human type III| Trypsin         | 101,000  |
| Human type III| Trypsin         | 13,000 (denatured)² |

² The values shown for guinea pig type I collagen are from Welgus et al. (1).

Values for native guinea pig type I collagen are from Welgus et al. (1).

| Collagen     | Enzyme         | k_D/k_H  |
|--------------|----------------|----------|
| Human type I | Fibroblast collagenase | 1.5      |
| Human type II| Fibroblast collagenase | 1.2      |
| Human type III| Fibroblast collagenase | 1.3      |
| Guinea pig type I | Fibroblast collagenase | 1.9 (solution)² |
| Human type III| Trypsin         | 1.7      |

² The values for native guinea pig type I collagen are from Welgus et al. (1).
**TABLE III**

**Catalytic rates on homologous collagens versus temperature**

| Temperature | I | II | III |
|-------------|---|----|-----|
| 20          | 13.7 | 0.17 | 168 |
| 25          | 53.4 | 1.0  | 565 |
| 30          | 198  | 5.5  | 1238 |

Values of $k_{cat}$ at 25°C for human collagen types I, II, and III in solution were determined in a previous study (3). Values at 20 and 30°C were then calculated based on the respective values of $E_a$. 

**DISCUSSION**

In this study, we have examined the activation energies and solvent-deuterium kinetic isotope effects that characterize the action of human skin fibroblast collagenase on its homologous type I, II, and III collagens in solution. Despite large differences in catalytic rates (3), the observed differences in activation energy were more modest. The collagen least susceptible to fibroblast collagenase cleavage, human type II, was marked by the highest value for $E_a$, 61,000 cal. Cleavage of this collagen in solution is therefore a very energy-dependent process, an increase in reaction velocity of nearly 30-fold accompanying each 10°C change in temperature. In comparison, human type I degradation was characterized by an activation energy of 47,000 cal. This value is very similar to the $E_a$ reported for fibroblast collagenase cleavage of monomeric guinea pig type I collagen (42,200 cal), suggesting that activation energy does not vary appreciably between type I collagens as a function of animal species of origin. In addition, human type III collagen, although far more susceptible than type I to collagenase, displayed an $E_a$ of 42,500 cal, only slightly lower than that of Type I. Furthermore, as seen in Table I, the level of collagen substrate organization (i.e. gelatin chains, monomers in solution, reconstituted fibrils) appears to be the single most crucial factor governing activation energy, more important than either collagen type or animal species of substrate.

In contrast to the Arrhenius plots for fibroblast collagenase cleavage of human collagen types I and II, the corresponding plot for human type III displayed a distinct departure from linearity. This Arrhenius plot, shown in Fig. 3, resulted from the intersection of two linear portions characterized by different activation energies. Below the intersection point, which occurs at approximately 26°C, $E_a$ is equal to 42,500 cal; above this temperature, $E_a$ falls to 29,500 cal. Nonlinearity of the Arrhenius plot generally results from a change in the structure of the chemical reactants or a change in the rate-limiting step of the reaction (2). A structural alteration in fibroblast collagenase at 26°C is not a tenable possibility, in view of the relative stability of this enzyme (13) and the lack of any such break in the Arrhenius plots of the degradation of native human type I and II collagens. On the other hand, as previous studies have shown, the activation energy of fibroblast collagenase exhibits a marked dependence on the level of substrate organization (11), Table I. Therefore, the break in $E_a$ would seem best explained by a change in the structure of human type III collagen at temperatures above 26°C. At temperatures below 26°C, $E_a$ (42,500 cal) is similar to that of type I collagen; above 26°C, $E_a$ falls to 29,500 cal, a value intermediate between triple helical type I collagen monomers and denatured random gelatin α-chains. It would seem reasonable then, that the break in the Arrhenius plot for native type III collagen would result from a further loosening in the triple helix of this molecule in the region of the 3/4% collagenase cleavage site. It must be emphasized that such a loosening does not represent a generalized denaturation or widespread loss in triple helical structure; circular dichroism studies have clearly demonstrated that the type III collagen molecule, like type I, does not denature at neutral pH until a temperature of approximately 40°C (14). Furthermore, only a single proteolytic cleavage by fibroblast collagenase was observed between 26 and 34°C, which resulted in the typical TCα and TCβ products. In contrast, complete thermal denaturation of the type III substrate has been shown to render the resultant gelatin molecule susceptible to proteolytic attack by collagenase at multiple loci (15).

As reported earlier (3), human type III collagen in solution at 25°C is degraded more rapidly than its homologous type I and II substrates ($k_{cat}$ III = 565 h⁻¹, $k_{cat}$ I = 53.4 h⁻¹, $k_{cat}$ II = 1.0 h⁻¹). It is tempting to speculate that part of this susceptibility may be attributable to either a lower energy of activation or the presence of a labile helical structure in the catalytic region of this collagen. However, when catalytic rates are compared at 20, 25, and 30°C (Table III), temperatures both above and below the break in the Arrhenius plot, the rather pronounced preference for type III collagenolysis persists at all points. Thus, the observed type III specificity is most probably due to an easier access by collagenase and/or water to this substrate's catalytic site because of an intrinsically looser helix in this region at all temperatures, and the existence of such specificity cannot be attributed to the further loss of helical integrity which only appears at temperatures above approximately 26°C.

Hayashi et al. (16) have reported the activation energy of tadpole collagenase on bovine collagen types I-III in solution. These values ($E_a$(I) = 41,000 cal, $E_a$(II) = 39,000 cal, and $E_a$(III) = 63,000 cal) differ significantly from the values of $E_a$ which characterize the cleavage by human fibroblast collagenase of its homologous collagen substrates. One explanation for these data are known differences between the tadpole and human enzymes, e.g. the tissue of origin (epithelial versus mesodermal (17)), chemical structure and specific activity (18), and cooperativity of type II and III collagen degradation (19). The absence of a discernible break in the Arrhenius plot of bovine type III collagen cleavage could be explained by...
that fibroblast collagenase cleavage of chick skin type III collagen is not characterized by a break in $E_A$. As reported earlier for guinea pig type I collagen (1), both $E_A$, and $k_{H}/k_{D}$ are crucially dependent upon the level of substrate organization. Thus, there is a correlation between the energy dependence of catalysis and the utilization of water molecules at the rate-limiting step of collagen degradation. The data in this present investigation concerning the solvent-deuterium kinetic isotope effect on gelatinolysis certainly supports such a relationship. While values for $k_{H}/k_{D}$ on native human type I–III collagen in solution were all approximately 1.2–1.5, the thermal denaturation of these collagens to gelatin resulted in catalytic rates which were not affected by the substitution of deuterium oxide for water in solvent buffer of the reaction mixture (Table II). Since extensive peptide bond hydrolysis accompanies the cleavage of gelatin as well as collagen (15), it would appear that the role of water at the rate-limiting step of collagen degradation may not reside in the participation of water in hydrolysis of a peptide bond per se, but rather may reflect the difficulty in translocating water molecules to the site of such catalysis. The native collagen molecule exists as a triple helix with a hydrophobic core; the transport of water molecules to the inside of this helical structure may become increasingly difficult as the substrate assumes higher orders of organization. Thus, in the case of the collagen fibril, the water of hydrolysis must traverse a liquid-solid phase boundary in addition to gaining access into the interior of the triple helix. In the case of random gelatin polypeptide chains, however, access by water to the site of peptide bond hydrolysis is unimpeded, and $k_{H}/k_{D}$ becomes negligible.

Trypsin is known to cleave the native type III collagen molecule at an Arg-Gly bond located only one triplet COOH-terminal to the $\frac{4}{3}$ collagenase cleavage site (11). Since the same bond is also present in collagen types I and II but is not cleaved, it is widely believed that the trypsin susceptibility of native type III collagen is due to a looser helix in this region of the molecule. The activation energy of trypsin on human type III collagen, 70,000 cal, indicates that this catalysis is far more energy-dependent than degradation of the same substrate by fibroblast collagenase. This is not surprising since activation energy seems to be closely correlated in the presence of triple helical and fibrillar structure, and the resulting difficulties posed to enzymatic catalysis (1). The cleavage of type III collagen by trypsin has been shown to be so slow as to question the physiologic role of this serine protease in the degradation of this collagen (3). It is thus likely that the higher energy of activation simply reflects the action of an enzyme on a substrate which it is not designed to attack. Since the biologic function of trypsin is not to degrade the collagen triple helix, a higher energy expenditure might be expected from this enzyme to accomplish this end than would be demanded of fibroblast collagenase. The lack of a discernible break in the Arrhenius plot of trypsin’s cleavage of type III collagen likely indicates that the additional helix loosening that occurs at 26°C, though sufficient to affect the action of fibroblast collagenase, is insufficient to affect trypsin’s degradation of this substrate. It may well be, then, that collagenase is a more sensitive probe of small changes in the collagen helix than is trypsin.

Many questions remain unanswered regarding the role of water and energy in collagenolysis. Likewise, the role of a loosened helix in determining the susceptibility of native type III collagens to cleavage by mammalian collagenase requires further investigation. Studies designed to address these issues are currently in progress.

REFERENCES

1. Welgus, H. G., Jeffrey, J. J., and Eisen, A. Z. (1981) J. Biol. Chem. 256, 9516–9521
2. Gutfreund, H. (1975) Enzymes: Physical Principles, pp. 157–175, John Wiley and Sons, New York
3. Welgus, H. G., Jeffrey, J. J., and Eisen, A. Z. (1981) J. Biol. Chem. 256, 9511–9515
4. Bauer, E. A., Stricklin, G. P., Jeffrey, J. J., and Eisen, A. Z. (1975) Biochem. Biophys. Res. Commun. 64, 232–240
5. Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z. (1977) Biochemistry 16, 1607–1615
6. Stricklin, G. P., Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J. (1978) Biochemistry 17, 2531–2537
7. Burgeson, R. E., Adli, E. L., Kaitila, I. I., and Hollister, D. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2579–2583
8. Burgeson, R. E., and Hollister, D. W. (1979) Biochem. Biophys. Res. Commun. 87, 1124–1131
9. Groves, W. E., Davis, P. C., Jr., and Sells, B. (1968) Anal. Biochem. 22, 195–210
10. Bergmann, I., and Lockley, R. (1963) Anal. Chem. 35, 1961–1965
11. Miller, E. J., Fisch, J. E., Jr., Chung, E., and Butler, W. T. (1976) Arch. Biochem. Biophys. 173, 631–637
12. Wang, H.-M., Chan, J., Pettigrew, D. W., and Sodek, J. (1978) Biochim. Biophys. Acta 533, 270–277
13. Bauer, E. A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4646–4650
14. Peltonen, L., Palomo, A., Hayashi, T., and Prockop, D. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 162–166
15. Welgus, H. G., Jeffrey, J. J., Stricklin, G. P., and Eisen, A. Z. (1982) J. Biol. Chem. 257, 11534–11539
16. Hayashi, T., Nakamura, T., Hori, H., and Nagai, Y. (1980) J. Biochem. 87, 809–815
17. Eisen, A. Z., and Gross, J. (1965) Dev. Biol. 12, 408–418
18. Hori, H., and Nagai, Y. (1979) Biochim. Biophys. Acta 566, 211–221
19. Hayashi, T., Nakamura, T., Hori, H., and Nagai, Y. (1980) J. Biochem. 87, 993–995

2 H. G. Welgus and J. J. Jeffrey, unpublished observations.
Studies on the activation energy and deuterium isotope effect of human skin collagenase on homologous collagen substrates.

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