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Limited Proteolysis of Type I Collagen at Hyperreactive Sites by Class I and II *Clostridium histolyticum* Collagenases: Complementary Digestion Patterns†

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ABSTRACT: The initial proteolytic events in the hydrolysis of rat tendon type I collagen by the class I and II collagenases from *Clostridium histolyticum* have been investigated at 15 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis has been used to detect the initial cleavage fragments of both the α1(I) and α2 chains, which migrate at different rates in the buffer system employed. Experiments with the class I collagenases indicate that the first cleavage occurs across all three chains of the triple helix close to the C-terminus to produce fragments whose α chains have molecular weights of approximately 88 000. The second cleavage occurs near the N-terminus to reduce the molecular weight of the α chains to 80 000. Initial proteolysis by the class II collagenases occurs across all three chains at a site in the interior of the collagen triple helix to give N- and C-terminal fragments with α-chain molecular weights of 35 000 and 62 000, respectively. The C-terminal fragment is subsequently cleaved to give fragments with α-chain molecular weights of 59 000. These results indicate that type I collagen is degraded at several hyperreactive sites by these enzymes. Thus, initial proteolysis by these bacterial collagenases occurs at specific sites, much like the mammalian collagenases. These results with the individual clostridial collagenases provide an explanation for earlier data which indicated that collagen is degraded sequentially from the ends by a crude clostridial collagenase preparation.

The mechanism of degradation of collagens by specific collagenases and other proteolytic enzymes is of fundamental importance to an understanding of the biochemical basis for connective tissue catabolism. It is now widely accepted that there are two types of collagenases. Bacterial collagenases, such as those from *Clostridium histolyticum* (EC 3.4.24.3), are believed to degrade collagen into small peptides by hydrolysis at multiple sites along the triple helix (Seifter & Harper, 1971). Tissue collagenases (EC 3.4.24.7), on the other hand, cleave interstitial collagens into only two fragments by action at a specific locus approximately three-fourths of the length from the N-terminus (Wooley, 1984). In spite of this major difference, it is of interest to assess whether there is any similarity between the *initial* proteolytic cleavages made by the bacterial collagenases and the single cleavage made by the tissue collagenases.

The different mode of attack of collagen by these two classes of enzymes is, no doubt, largely due to the more restrictive sequence specificity of the tissue collagenases. One important question that has yet to be resolved adequately, however, is that of the influence that collagen has in directing its own degradation. Even for tissue collagenases, sequence specificity alone is insufficient to explain their highly selective mode of attack on native interstitial collagens. The resistance of collagen to proteolysis has been attributed to its tightly coiled, triple-helical structure. Thus, the possibility exists that there are local instabilities in the triple helix or sections of presently unrecognized secondary structure that could expose certain

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bonds, selectively rendering them susceptible to proteolysis. This could help explain the specificity of collagenases toward native collagens.

In this study, we show that type I collagen is initially hydrolyzed at a different set of distinct hyperreactive sites by the two classes of collagenases from *Clostridium histolyticum*. Since it is known from the sequence specificities of these enzymes that they are capable of hydrolyzing the great majority of the Y–Gly bonds that occur in type I collagen (Steinbrink et al., 1985; Van Wart & Steinbrink, 1986), this demonstrates the importance of local conformational features of the triple helix in directing collagenolysis. The present study clearly delineates a different sequence of events for the two classes of collagenases. The finding that type I collagen is initially hydrolyzed from the ends by the class I enzymes is generally in good agreement with earlier studies (Kühn & Eggl, 1966; Stark & Kühn, 1968) carried out using calf and rat skin collagen and a crude collagenase preparation. In contrast, however, it is shown here that the initial proteolysis of type I collagen by the class II clostridial collagenases is fundamentally similar to the tissue collagenases in that the first cleavage occurs at a highly specific, though different, locus in the interior of the collagen triple helix.

Materials and Methods

All *Clostridium histolyticum* collagenases (β, γ, ϵ, and ζ) were purified as described earlier (Bond & Van Wart, 1984a). Human neutrophil collagenase was purified from outdated blood by a modification of the methods of Macartney and Tschesche (1983) and Christner et al. (1982). Pronase was purchased from Calbiochem-Behring Corp. Type I collagen was isolated from rat tail tendons and purified by sequential salt precipitations (Miller & Rhodes, 1982). The telopeptides were removed from the collagen by treatment with Pronase as described by Helseth and Veis (1981). Briefly, a 2 mg/mL solution of collagen dissolved in 0.01 M acetic acid was brought to 50 mM tris(hydroxymethyl)aminomethane (Tris),[1] 10 mM $CaCl_2$, and 0.2 M NaCl, pH 7.5, by addition of concentrated buffer at 22 °C. Pronase was added to a weight ratio of 1:100 and the reaction left for 24 h, after which the collagen was recovered by precipitation with NaCl (17% w/v). The collagen was collected by centrifugation and redissolved in 0.01 M acetic acid, adjusted to pH 7.5, and the salt precipitation was repeated. The resulting collagen was redissolved in 0.01 M acetic acid, dialyzed against 4 L of 0.01 M acetic acid for 2 days, and stored at 4 °C. The concentration of all collagen solutions was determined by the biuret method (Gornall et al., 1949) using a standard curve constructed by using oven-dried collagen samples as primary standards.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by using a Tris/glycine running buffer as described by Laemmli (1970) using 6–12% gradient gels. Gels were stained with 0.25% Coomassie Blue R-250 in methanol/glacial acetic acid/water (50:10:40) and destained in methanol/glacial acetic acid/water (2:3:5). Gels were scanned by using a Hoefer Scientific Instruments Model GS300 densitometer and the bands integrated with a Hewlett-Packard Model 3390A integrator.

For the limited proteolysis experiments, an aliquot of collagenase was added to a sample of 2 mg/mL collagen in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, in a 15 °C water bath. In a typical experiment, the final collagenase concentrations were 300 and 83 nM for β- and ζ-collagenases, respectively. The collagenase concentrations were determined spectrophotometrically by using the extinction coefficients reported earlier (Bond & Van Wart, 1984b). Aliquots (25 μL) were withdrawn from the reaction tube as a function of time, quenched by addition of 1,10-phenanthroline to a final concentration of 10 mM, and mixed with SDS–PAGE denaturing buffer to a final volume of 40 μL. Twenty-five-microliter aliquots were applied to the gels.

Results

Type I collagen is a heterotrimer consisting of two α1(I) and one α2(I) chains coiled into a triple helix. For simplicity, these chains will be denoted α1 and α2 and the intact collagen triple helix [$(\alpha 1)_2\alpha 2$]. In native collagens, some of the α1 chains are cross-linked to form β_{11} components, and some of the α1 and α2 chains are cross-linked to form β_{12} components. The cross-links are localized primarily in the N-terminal, non-triple-helical telopeptides. The α1 and α2 chains have molecular weights of 95 000 and the β_{11} and β_{12} components molecular weights of 190 000. SDS–PAGE has been used to separate and detect these collagen chains and the fragments produced on proteolysis. In the buffer system used, the four intact collagen chains migrate at different rates and give rise to clearly resolved bands. In some experiments, the collagen used as substrate was pretreated with Pronase to remove the telopeptides, thus converting the β_{11} and β_{12} components into the constituent α1 and α2 chains. This simplifies the banding pattern on the gels and facilitates analysis of the reaction products.

In a series of initial experiments, type I collagen was incubated with variable concentrations of the collagenases at several different temperatures for various lengths of time in order to search for conditions under which the initial proteolytic events could be resolved. *Clostridium histolyticum* produces two classes of collagenases (Bond & Van Wart, 1984a,b), and experiments were carried out with two class I (β and γ) and two class II (ϵ and ζ) enzymes. Incubation of 2 mg/mL solutions of type I collagen with each of these four collagenases at 15 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at collagenase concentrations in the 80–300 nM range enables the initial proteolytic events to be resolved. Aliquots of the reaction mixture of collagen with these collagenases were withdrawn as a function of time and applied to SDS gels.

Densitometer tracings from a gel showing the products of the reaction with β-collagenase are shown in Figure 1. At the start of the reaction (0.5 min), only the α1 and α2 chains and β_{11} and β_{12} components (whose scans have been expanded in Figure 1 for clarity) are prominent, although the first evidence of proteolytic fragments has already started to appear. After 15 min, fragments F1 and F2 appear below the β_{12} band, F3 and F4 between the α1 and α2 bands, and F5 and F6 just below the α2 band. After 30 min, a whole series of lower molecular weight fragments is clearly evident. The disappearance of the α1, α2, β_{11}, and β_{12} bands and the corresponding appearance of about a dozen clearly resolved digestion products over a period of 90 min are depicted by the dashed lines in Figure 1.

While the densitometer tracings rapidly become complex, the initial proteolytic events can be deciphered by following the time dependence of the intensities of F1–F6. The α1 chain

[1] Abbrevations: unless stated otherwise, collagenase refers to *Clostridium histolyticum* collagenase, and collagen refers to rat tail tendon type I collagen; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; CNM-Leu^K-Gly-Pro-Ala refers to cinnamoyl-Leu-Gly-Pro-Ala in which the NH group of the Leu–Gly bond has been replaced by a CH_2 group to give a ketone.

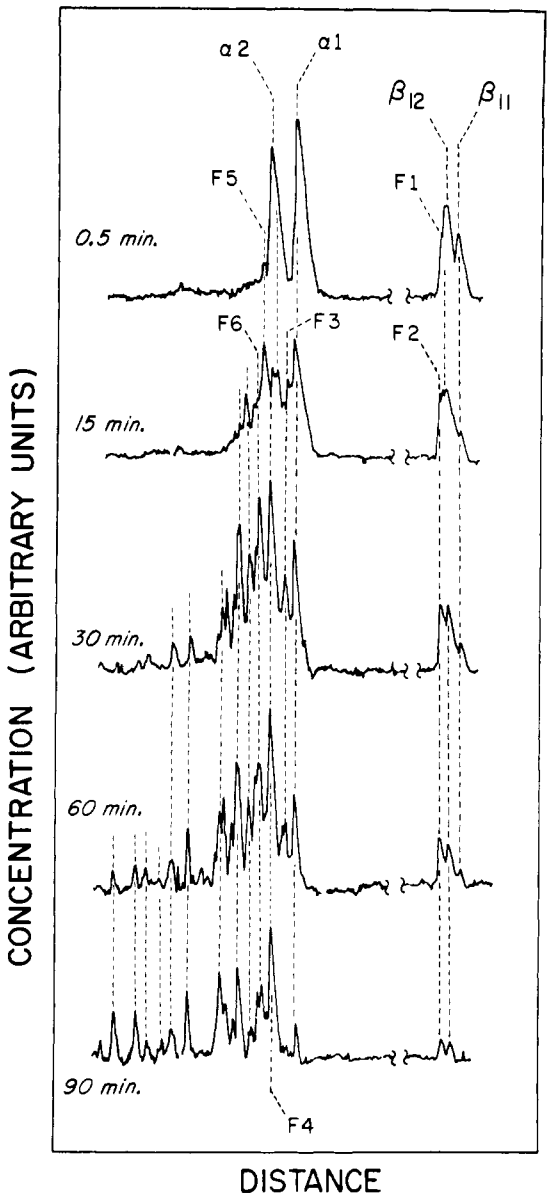

FIGURE 1: Densitometer tracings of an SDS gel showing the time course of reaction of type I rat tendon collagen with β-collagenase at 15 °C. The collagen and collagenase concentrations were 2 mg/mL and 300 nM, respectively. The top of the gel (high molecular weight) is on the right. The positions of the α1, α2, β_{11}, and β_{12} chains and fragments F1–F6 are indicated. The region of the scan showing the β_{11} and β_{12} chains has been expanded to better visualize the band profiles.

disappears to give rise first to F3 and then to F4 while the α2 chain is hydrolyzed at an apparently higher rate to give F5 and F6. The β_{12} band is first hydrolyzed to a closely spaced doublet (F1 and F2) and then to F2. The β_{11} band is also cleaved, but the products are not resolved by the densitometry. Interestingly, F2 subsequently decays, but no fragments with molecular weights above those of the α1 and α2 chains are produced. It is not clear from which higher molecular weight chains the unlabeled series of lower molecular weight fragments shown in Figure 1 is formed.

The mobilities exhibited by the fragments on the SDS gels can be used to estimate their molecular weights, provided that appropriate standards are available. It is important to note, however, that fragments derived from α1 vs. α2 chains have different inherent mobilities and require separate calibration curves, as do dimers such as β_{12}, which consist of one of each type of chain. Thus, plots of log molecular weight vs. mobility (not shown) have been constructed for species derived from α1 chains, from α2 chains, and from β_{12} chains. For α1 chains, the α1 and β_{11} chains themselves and the $TC^A(\alpha1)$, $TC^A(\beta_{11})$, and $TC^B(\alpha1)$ fragments produced on reaction with human neutrophil collagenase were used as molecular weight standards. For the α2 chains, the α2 chain itself and the $TC^A(\alpha2)$ and $TC^B(\alpha2)$ chains were used as standards. For the β_{12} chains, the β_{12} and $TC^A(\beta_{12})$ chains were used, and the calibration curve was drawn equidistant between the α1 and α2 curves.

In order to use these curves for molecular weight estimates, it must be known which type of chain each fragment is derived from. The only bands in Figure 1 for which this is the case are F1 and F2 (from β_{12}), F3 and F4 (from α1), and F5 and F6 (from α2). When these calibration curves are used, the molecular weights of F3 and F5 are both 88 000, those of F4 and F6 are both 80 000, and those of F1 and F2 are 185 000 and 175 000, respectively. Collectively, this information strongly suggests that the initial proteolytic event is the hydrolysis of triple-helical collagen across all three chains to produce a new triple-helical fragment with α chains that have molecular weights of 88 000. Thus, $[(\alpha1)_2\alpha2]$ is hydrolyzed to $[(F3)_2F5]$, and the β_{12} components are hydrolyzed first to F1, which is a dimer of F5 and an undegraded α1 chain, and then to F2, which is a dimer of F3 and F5. The partially hydrolyzed fragment F1 is observed because the α2-chain scission occurs faster than that in the α1 chain. The molecular weights of 185 000 and 175 000 for F1 and F2 are in reasonable agreement with the values of 183 000 and 176 000, respectively, obtained by summing the values for the monomeric species. Since this initial cleavage is near one end of the triple helix, but leaves the cross-links in the β_{12} components intact, it is clear that it occurs near the C-terminus.

The second cleavage of the α chains produces F4 and F6 with molecular weights of 80 000. Thus, there is apparently hydrolysis across all three chains of $[(F3)_2F5]$ to produce $[(F4)_2F6]$. However, the removal of the M_r 8000 piece in this case must occur from the N-terminus since no new bands with molecular weights of 160 000 or greater are evident due to the corresponding cleavage of β_{12} or F2. After these first 2 sets of cleavages, about 10 new fragments with molecular weights in the 30 000–75 000 range are produced. However, the complexity of the densitometer pattern precludes further detailed analysis. Very similar gel patterns are found when the same experiments are carried out with γ-collagenase. Thus, the class I enzymes appear to hydrolyze type I collagen by a common mechanism.

Figure 2 shows the gel patterns that are observed when type I collagen is reacted with ζ-collagenase under conditions similar to those described above. After 9 min, six fragments designated F′1, F′2, F′3, F′5, F′7, and F′8 are apparent as over half of the α1, α2, β_{11}, and β_{12} bands have disappeared. At 9 min, F′7 and F′8 have already started to be degraded further, and both F′3 and F′5 show some evidence of splitting to form F′4 and F′6, respectively. After 38 min, none of the α1, α2, β_{11}, or β_{12} bands remain, and the splitting of F′3 and F′5 to give F′4 and F′6, respectively, is clearly evident. New bands have now appeared in the region containing F′7 and F′8. Over the next 2 h, F′1–F′6 decay to produce a series of new bands with lower molecular weights. After 3 h, a pair of bands denoted F′9 and F′10 accumulate at the bottom of the gel. It should be noted that no new fragments from the β_{11} or β_{12} bands appear above the positions of the α1 or α2 bands.

The variation in the concentrations of F′1–F′8 identified in Figure 2 as a function of time obtained from a separate ex-

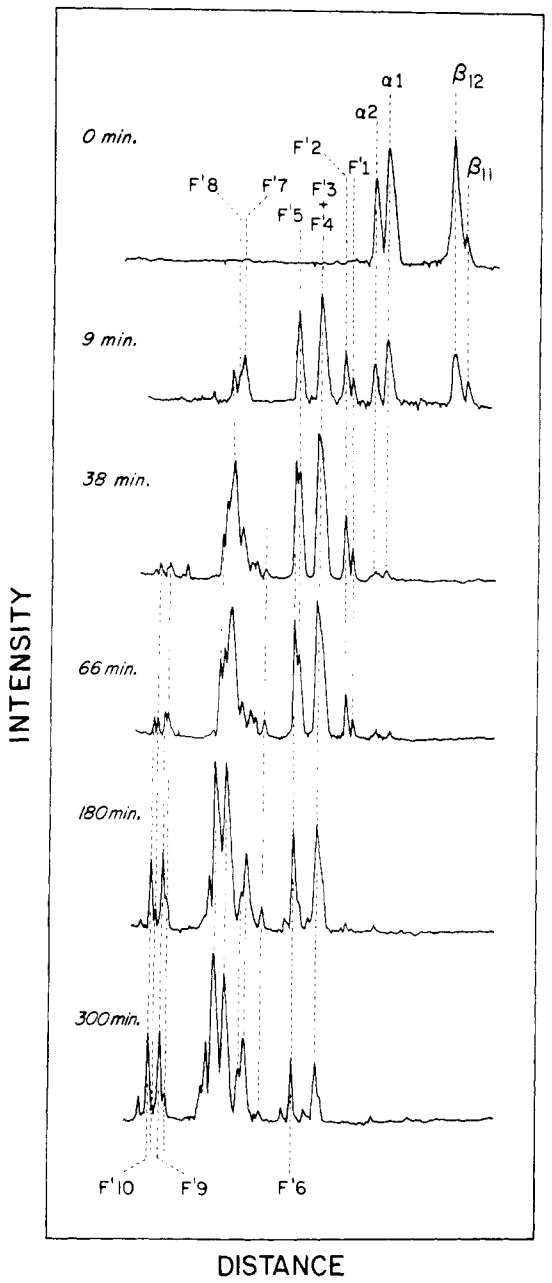

FIGURE 2: Densitometer tracings of an SDS gel showing the time course of reaction of type I rat tendon collagen with ζ-collagenase at 15 °C. The collagen and collagenase concentrations were 2 mg/mL and 83 nM, respectively. The top of the gel (high molecular weight) is on the right. The positions of the α1, α2, β_{11}, and β_{12} chains and fragments F'1–F'10 are indicated.

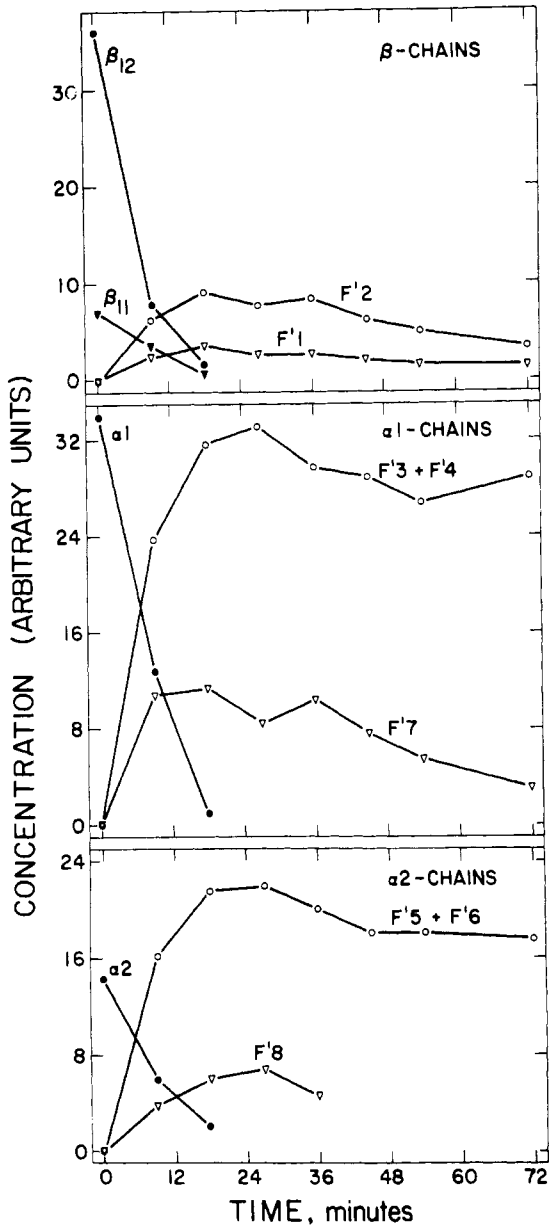

FIGURE 3: Variation of the concentration of the α1, α2, β_{11}, and β_{12} chains and fragments F'1–F'8 as a function of time during the reaction of type I rat tendon collagen with ζ-collagenase at 15 °C. The F'3, F'4 and F'5, F'6 pairs were not resolved by the densitometry and, thus, their sum is plotted.

periment carried out at a lower collagenase concentration to slow down the initial cleavage is plotted in Figure 3. Since the F'3, F'4 and F'5, F'6 pairs are not resolved, only their sum has been plotted. The appearance of all eight fragments correlates with the decay of the α1, α2, β_{11}, and β_{12} chains. The uniform spacings between the five pairs of bands in the 9-min tracing in Figure 2 strongly suggest that α1 chain is hydrolyzed to give F'3 and F'7 and that the α2 chain is hydrolyzed to give F'5 and F'8. It also suggests that β_{12} and β_{11} are degraded to F'1 and F'2, respectively. On the basis of this assumption, the molecular weights of F'3 and F'5 are both found to be 62 000, and those of F'7 and F'8 are both found to be 35 000. The sum of the molecular weights of F'3 and F'7, and also of F'5 and F'8, is 97 000 which is in good agreement with the value of 95 000 for the intact α1 and α2 chains.

The time course of the hydrolysis of the β components is similar to that of the α chains in that hydrolysis of β_{11} and β_{12} gives F'1 and F'2, respectively. The molecular weights of F'1 and F'2 are both estimated to be 70 000 by using the calibration curve described earlier. Thus, F'1 consists of two cross-linked F'7 chains while F'2 is a dimer of F'7 and F'8. The molecular weight of 70 000 for F'1 and F'2 agrees well with the sum of the molecular weights of the constituent fragments. Thus, the initial proteolytic event is the hydrolysis of triple-helical collagen at a single site across all three chains to produce two new triple-helical fragments with α chains that have molecular weights of approximately 62 000 and 35 000. For un-cross-linked chains, this corresponds to $[(\alpha1)_2\alpha2]$ being hydrolyzed to two fragments that have the structures $[(F'7)_2F'8]$ and $[(F'3)_2F'5]$, respectively. For the collagen molecule containing cross-linked chains, $[\beta_{11}\alpha2] \rightarrow [F'1, F'8] + [(F'3)_2F'5]$ while $[\beta_{12}\alpha1] \rightarrow [F'2, F'7] + [(F'3)_2F'5]$.

In a second round of proteolysis, F'4 and F'6 are derived from F'3 and F'5, respectively, by removal of a 3000 molecular

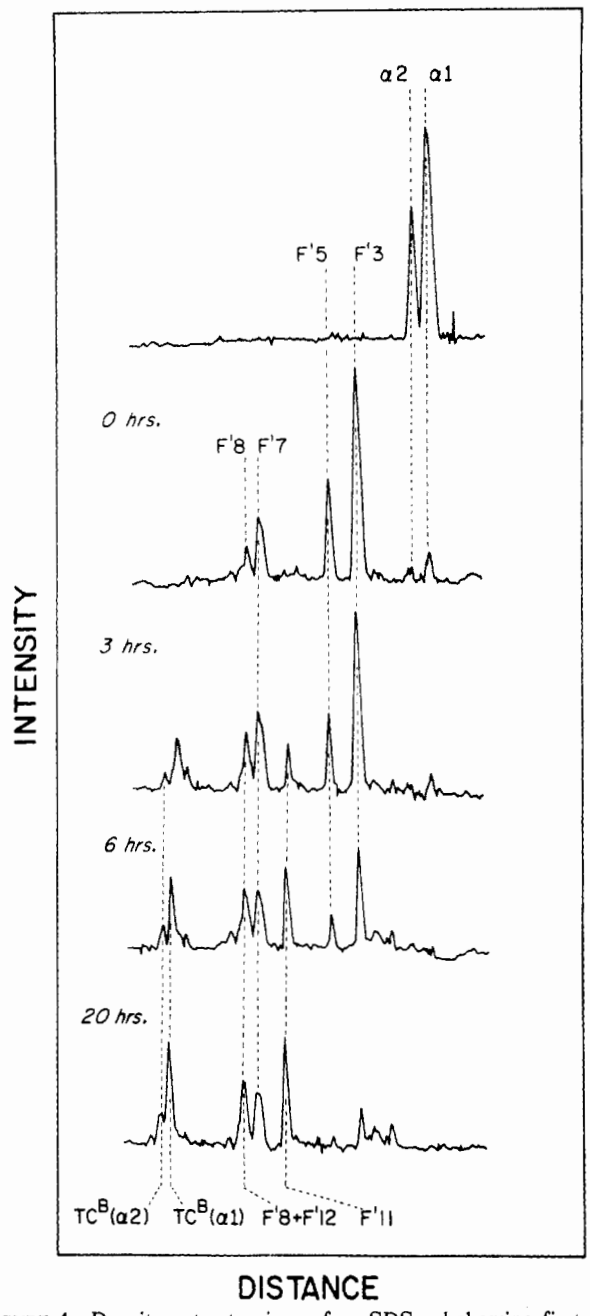

FIGURE 4: Densitometer tracings of an SDS gel showing first the reaction of Pronase-treated type I rat tendon collagen with ζ-collagenase at 15 °C to form F′3, F′5, F′7, and F′8. The reaction was quenched with a specific inhibitor of ζ-collagenase, and the fragments were digested at 20 °C with neutrophil collagenase. The top of the gel (high molecular weight) is on the right. The positions of the α1 and α2 chains and fragments F′3, F′5, F′7, F′8, F′11, F′12, $TC^B(\alpha 1)$, and $TC^B(\alpha 2)$ are indicated.

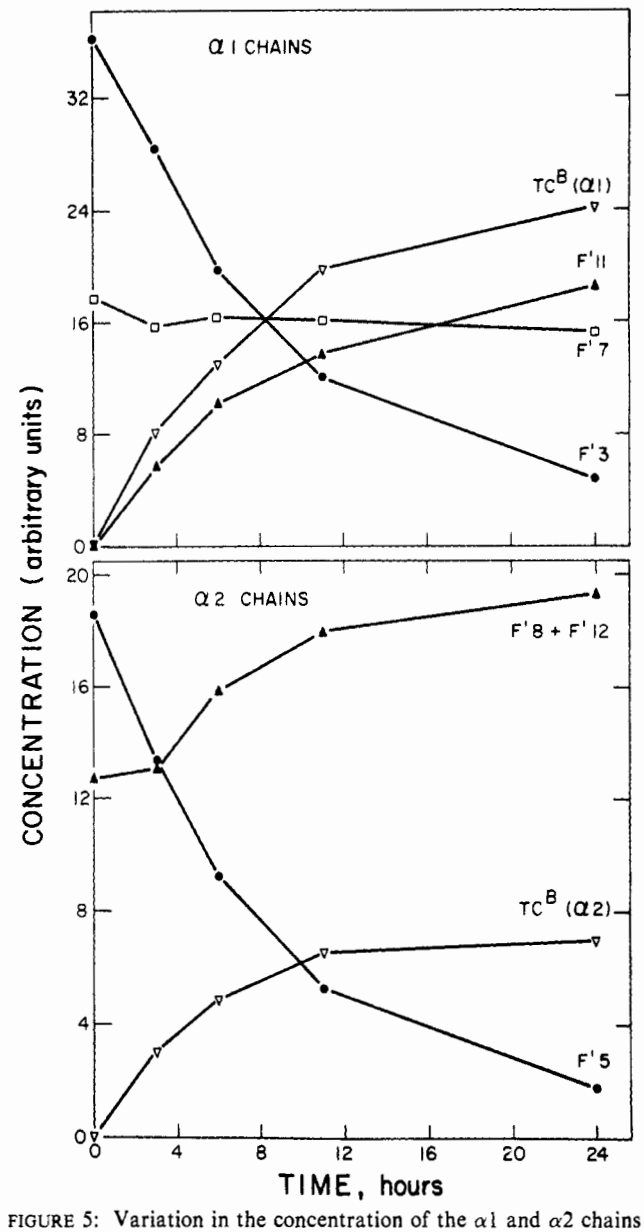

FIGURE 5: Variation in the concentration of the α1 and α2 chains and fragments shown in Figure 4 as a function of time after addition of neutrophil collagenase.

weight peptide from each fragment. After this, it becomes almost impossible to trace the fate of individual chains because of the complexity of the patterns. However, after 3 h, F′9 and F′10 accumulate, and assuming that they arise from parallel cleavages of α1 and α2 chains, they both have molecular weights of 24 000. From their intensities, it is clear that they can only be derived from F′3–F′6. Essentially the same digestion patterns shown in Figure 2 are found in experiments with ϵ-collagenase. Thus, the class II enzymes also hydrolyze type I collagen by a common mechanism.

Since the first cleavage of type I collagen by ζ-collagenase produces dimer chains (F′1 and F′2) with molecular weights of 70 000, it can be concluded that these as well as the fragments with α-chain molecular weights of 35 000 arise from the N-terminus. To test this hypothesis, a double digestion experiment was carried out with Pronase-treated collagen. As shown in the top tracing of Figure 4, Pronase-treated collagen shows bands due to only α1 and α2 chains. First, this collagen was reacted with ζ-collagenase at 15 °C until the reaction to form F′3, F′5, F′7, and F′8 was essentially complete (tracing 2, labeled 0 h). It is immediately obvious that bands F′1 and F′2 are absent, confirming that they do indeed arise from digestion only of β_{11} and β_{12}, respectively. At this point, CNM-Leu^K-Gly-Pro-Ala (final concentration 0.44 mM) was added to completely inhibit the ζ-collagenase (K_I = 110 nM; K. A. Mookhtiar, H. E. Van Wart, and R. E. Galardy, unpublished results), the temperature was raised to 20 °C, and human neutrophil collagenase was added to cleave the fragments that contain the mammalian collagenase cleavage site. Over the next 20 h, F′3 and F′5 decay, and four new bands appear. F′3 is hydrolyzed to give F′11 and $TC^B(\alpha 1)$ with molecular weights of 40 000 and 21 000, respectively. F′5 undergoes a similar cleavage to give F′12 (which falls on top of F′8) and $TC^B(\alpha 2)$, which also have molecular weights of 40 000 and 21 000, respectively. The concentrations of all

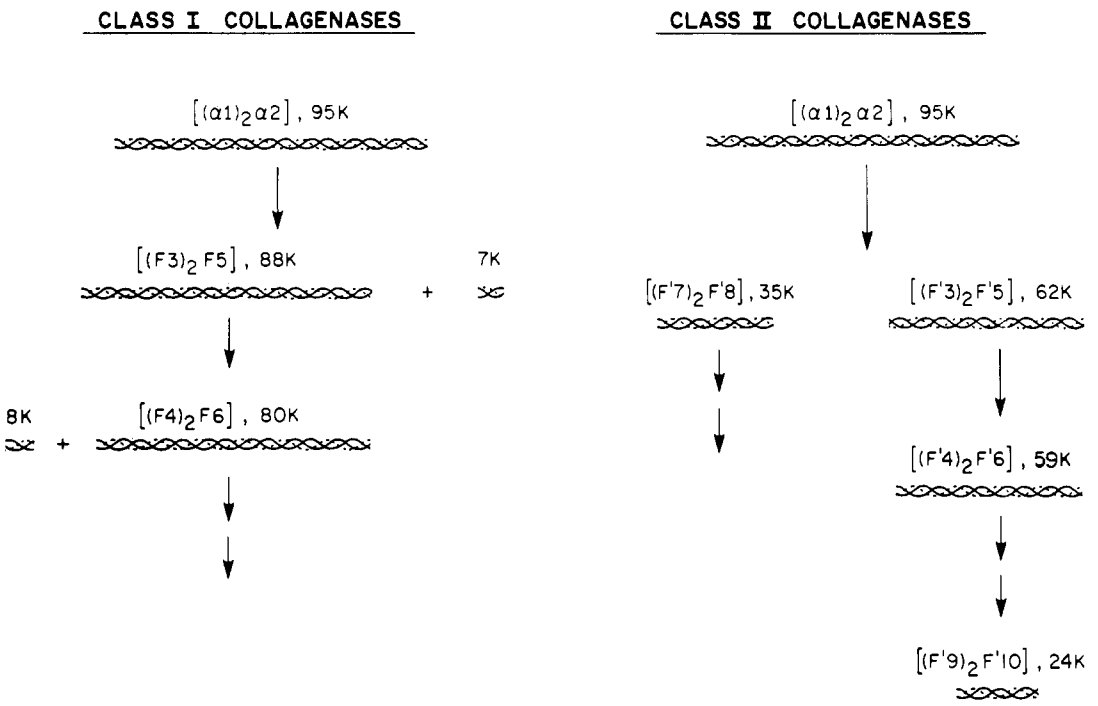

FIGURE 6: Schematic showing the sequence of cleavages of type I collagen by the class I and II clostridial collagenases. The solid lines are the $\alpha 1$ chains and the broken lines the $\alpha 2$ chains. Only un-cross-linked chains are shown as defined in the text.

Table I: Approximate Molecular Weights of Collagen Chains and Digestion Fragments

chain	mol wt	chain	mol wt
β_{11}, β_{12}	190000	$TC^A(\alpha 1,\alpha 2)$	74000
F1	185000	F'3, F'5	62000
F2	175000	F'4, F'6	59000
$TC^A(\beta_{11},\beta_{12})$	148000	F'11, F'12	40000
$\alpha 1$, $\alpha 2$	95000	F'7, F'8	35000
F3, F5	88000	F'9, F'10	24000
F4, F6	80000	$TC^B(\alpha 1,\alpha 2)$	21000
F'1, F'2	70000		

species are plotted as a function of time after addition of neutrophil collagenase in Figure 5. This plot shows quite clearly that only F'3 and F'5 are hydrolyzed by neutrophil collagenase. Since they contain the mammalian collagenase cleavage site, this confirms that they are the C-terminal fragments from the first cleavage by ζ-collagenase. A schematic summary of the initial events in the proteolysis of type I collagen by the class I and II collagenases is shown in Figure 6, and a summary of the molecular weights of all collagen chains and fragments is given in Table I.

A series of proteolysis experiments with denatured collagen was also carried out in order to see whether the same fragments could be observed. By lowering the enzyme:collagen ratio by 10-fold, the initial cleavages could be detected. However, in all reactions, a very large number of bands, many of almost equal intensity, were observed. Because of the complexity of the product mixtures obtained, it was not possible either to analyze the pathway of hydrolysis of the gelatins or to determine whether any of the bands observed were the same as those discussed above for collagen.

Discussion

The limited proteolysis experiments described here establish that both the class I and class II clostridial collagenases initially hydrolyze rat tendon type I collagen at distinct hyperreactive sites. These collagenases are ideal tools for this objective, since they have a broad specificity for collagen-like sequences (Steinbrink et al., 1985; Van Wart & Steinbrink, 1986) and are capable of hydrolyzing almost all of the Y–Gly bonds in collagen chains. Thus, the sites that are hydrolyzed preferentially are likely to be those at which the structure of the collagen differs locally from that of the majority of the chain, though the specific sequences at these sites will also obviously influence the hydrolysis rates.

The class I collagenases initially hydrolyze type I collagen close to the C-terminus followed by a second cleavage close to the N-terminus. This is followed by a series of further cleavages that consecutively reduce the fragments in size. It is of interest to compare the results presented here for β-collagenase with some limited proteolysis experiments carried out on calf skin collagen by Kühn and associates (Kühn & Eggl, 1966; Stark & Kühn, 1968). These workers reacted crude *Clostridium histolyticum* collagenase with calf skin collagen at 10 °C and precipitated the fragments produced at various stages of the reaction as segment long spacing aggregates. By electron microscopy, the lengths and molecular weights of the different fragments were determined. The pattern of fragments formed showed that the digestion started with cleavage close to the A end (or N-terminus) followed by cleavage very close to the B end (or C-terminus). Next, a series of five cleavages appeared to occur consecutively from the N-terminus (Kühn & Eggl, 1966).

From these observations, it was concluded that clostridial collagenase digests calf skin collagen from the ends. The fragments isolated had α-chain molecular weights of approximately 88000, 85000, 72000, 62000, 41000, and 26000. These findings, which were reported before it had been established that this bacterium produces two classes of collagenases, are in qualitative agreement with the result found here that the class I collagenases initially degrade rat tendon collagen from the ends. One difference, however, is that we find that the collagen is hydrolyzed first from the C-terminus and next from the N-terminus to sequentially give the 88000 and 80000 molecular weight fragments, whereas Kühn and associates found that these events occur in the reverse order. It is possible that this difference is related to the source (calf or rat skin vs. rat tendon) of the collagen, to a difference in

reaction conditions, or to the fact that an impure preparation was used in the earlier studies. We are unfortunately unable to resolve the subsequent proteolytic events, although fragments with molecular weights close to 72000 are definitely observed.

The initial action of the class II collagenases differs markedly from that of class I enzymes. In fact, it is qualitatively similar to that of mammalian collagenases in that the primary event is the hydrolysis of collagen across all three chains at a site located well within the interior of the molecule to produce two large fragments. While mammalian collagenases produce N- and C-terminal fragments with molecular weights of 74000 and 21000, the class II clostridial collagenases give corresponding fragments with molecular weights of 35000 and 62000, respectively. The latter fragment is subsequently hydrolyzed first to a 59000 and ultimately to a 24000 molecular weight fragment. This mode of hydrolysis contrasts markedly with that of the class I enzymes. Since all clostridial preparations that we have examined contained both classes of collagenases, it is possible that the fragments with molecular weights of 62000 and 26000 observed by Kühn and associates arose from a class II enzyme in their preparation. In any event, the two classes are complementary in their action in that the class I enzymes digest collagen from the ends while the class II enzymes start from the middle.

The results presented here provide strong evidence for the existence of several new hyperreactive sites in rat tendon type I collagen that are determined by some local conformational feature of the molecule. If the identical experiments are carried out on type I gelatin, a large number of fragments are produced from the outset, indicating that many more sites are susceptible to proteolysis. This indicates that the hyperreactivity of the cleavage sites in native collagen is determined in large part by their conformation. Mammalian collagenases exhibit similar behavior in that they hydrolyze native interstitial collagens at a single site approximately three-fourths from the N-terminus (Miller et al., 1976). These collagenases hydrolyze the peptide bond following the Gly residue of Gly-Ile-Ala or Gly-Leu-Ala sequences at the cleavage site in types I, II, and III collagens, but other sites in the native collagens containing these sequences are not cleaved. Interestingly, Welgus and co-workers (Welgus et al., 1982) have found that human skin fibroblast collagenase hydrolyzes multiple sites in the gelatins of these collagens. Thus, it is apparent that collagen directs its own degradation by some unknown conformational feature or sequence-dependent instability in its local secondary structure. As the sequences of the collagen chains in the vicinity of the new hyperreactive sites detected here become available, it will be interesting to see if they provide a common basis for their susceptibility to these collagenases.

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