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Kinetics of Hydrolysis of Type I, II, and III Collagens by the Class I and II *Clostridium histolyticum* Collagenases

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The kinetics of hydrolysis of rat tendon type I, bovine nasal septum type II, and human placental type III collagens by class I and class II *Clostridium histolyticum* collagenases (CHC) have been investigated. To facilitate this study, radioassays developed previously for the hydrolysis of these [^{3}H]acetylated collagens by tissue collagenases have been adapted for use with the CHC. While the CHC are known to make multiple scissions in these collagens, the assays are shown to monitor the initial proteolytic events. The individual kinetic parameters k_{cat} and K_M have been determined for the hydrolysis of all three collagens by both class I and class II CHC. The specific activities of these CHC toward fibrillar type I and III collagens have also been measured. In contrast to human tissue collagenases, neither class of CHC exhibits a marked specificity toward any collagen type either in solution or in fibrillar form. The values of the kinetic parameters k_{cat} and K_M for the CHC are similar in magnitude to those of the human enzymes acting on their preferred substrates. Thus, the widely held view that the CHC are more potent collagenases is not strictly correct. As with the tissue collagenases, the local collagen structure at the cleavage sites is believed to play an important role in determining the rates of the reactions studied.

KEY WORDS: *Clostridium histolyticum* collagenase; interstitial collagens; collagen specificity; kinetics of hydrolysis; collagen structure.

1. INTRODUCTION[3]

Interstitial collagens can be hydrolyzed at an appreciable rate only by specific tissue (EC 3.4.24.7) or bacterial (EC 3.4.24.3) collagenases (Seifter and Harper, 1971). The high resistance of these collagens to proteolytic attack has been attributed to the protective effect of their triple helical conformations. All known tissue collagenases attack native type I, II, and III collagens by the prototypic mechanism first delineated for tadpole collagenase (Gross and Nagai, 1965; Gross *et al.*, 1974) in which all three α chains of the tropocollagen (TC) monomers are hydrolyzed at a specific site approximately 3/4 from the N-terminus to yield the so-called TC^A and TC^B fragments. In order to provide insights into this important process, the kinetics of hydrolysis of these collagens by both known types of human collagenases have been extensively investigated (Hasty *et al.*, 1987; Horwitz *et al.*, 1977; Welgus *et al.*, 1981; Mallya *et al.*, 1990). Both the human fibroblast-type and neutrophil-type collagenases discriminate between these collagens in solution. Human fibroblast collagenase exhibits a strong preference for type II collagen (Welgus *et al.*, 1981; Mallya *et al.*, 1990), while human neutrophil collagenase (HNC) most rapidly hydrolyzes type I collagen (Hasty *et al.*, 1987; Horwitz *et al.*, 1977; Mallya *et al.*, 1990). These preferences are markedly diminished when the substrates are in their fibrillar forms (Welgus *et al.*, 1981; Hasty *et al.*, 1987; Mallya *et al.*, 1990).

Unlike tissue collagenases, bacterial collagenases are able to make multiple scissions within the triple helical domain of collagens and ultimately reduce

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[3] Abbreviations used: CHC, *Clostridium histolyticum* collagenase; Tricine, N-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TC, tropocollagen.

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them to a mixture of small, dialyzable peptides (Seifter and Harper, 1971). In the preceding paper (French *et al.*, 1990) and an earlier study (French *et al.*, 1987), the initial proteolytic events in the pathway of hydrolysis of type I, II, and III collagens by the class I and class II *Clostridium histolyticum* collagenases (CHC) were elucidated by carrying out the reactions under carefully selected conditions. The CHC were shown to attack these collagens at hyperreactive sites that are distinct from the tissue collagenase cleavage site. These initial proteolytic events are quite significant because they constitute the committed step in collagen catabolism (Woolley, 1984; Harris and Cartwright, 1977). Thus, once an interstitial collagen molecule is hydrolyzed within its central triple helical domain, it is destined to be completely catabolized. It is of interest to compare the efficiency of these initial proteolytic events for tissue and bacterial collagenases. However, no detailed kinetic studies of collagen hydrolysis have so far been reported for any bacterial collagenase. In this study, the kinetics of hydrolysis of type I, II, and III collagens by the class I and class II CHC have been investigated. It is shown that the CHC hydrolyze these collagens with kinetic parameters that are similar to those of the tissue collagenases, but that they exhibit less specificity toward these collagen types.

2. MATERIALS AND METHODS

2.1. Materials

The class I (β and γ) and class II (ε and ζ) CHC were purified from crude commercial preparations (Bond and Van Wart, 1984*b*) and their concentrations determined spectrophotometrically (Bond and Van Wart, 1984*a*). Rat tendon type I, bovine nasal septum type II, and human placental type III collagens were isolated and labeled with [^{3}H]acetic anhydride as described earlier (Mookhtiar *et al.*, 1986). [^{3}H]acetic anhydride (50 mCi/mmol) was purchased from New England Nuclear and fluorescamine from Fisher Scientific.

2.2. Kinetic Measurements

Assays for the hydrolysis of soluble collagens were performed as described in the text in a manner similar to a tissue collagenase assay by using the dioxane precipitation method to separate intact collagen from hydrolysis products (Mallya *et al.*, 1986). Assays for the hydrolysis of type I and III collagen fibrils were performed essentially as described earlier (Mallya *et al.*, 1990). Unlabeled collagen (50 μl, 1.5 mg/ml) dissolved in 50 mM Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, *p*H 7.5, was incubated overnight in microfuge tubes at 37°C to form firm, fibrillar gels. Eight tubes were used for each assay, each representing one time point. The assays were started by the addition of enzyme (50 μl) in the same buffer and the reaction mixtures were incubated at 37°C. At various time intervals, the microfuge tubes were centrifuged at 12,000 *g* at 23°C for 4 min, and an aliquot of the supernatant (50 μl) transferred to an empty microfuge tube. At the end of the assay, the collagen in these aliquots was denatured by incubating the tubes at 70°C for 20 min. After cooling to 23°C, a mixture of partially purified CHC (5 μl) was added and the mixture incubated for a further 2 hr at 37°C. Finally, 50 μl of fluorescamine (0.6 mg/ml in acetone) was added and the mixture diluted to 1 ml with assay buffer. The fluorescence of a diluted portion of this solution was measured on a Perkin Elmer LS-5 fluorimeter ($\lambda_{ex} = 385$ nm, $\lambda_{em} = 485$ nm). Data were plotted as % collagen hydrolyzed vs. time and specific activities, A_{sp}, were calculated as μg collagen degraded/min/mg enzyme.

2.3. Electrophoresis

Correlations between the extent of hydrolysis of the soluble collagens measured in the radioassays and by sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE) were carried out to validate the assays developed. The SDS-PAGE experiments were performed using 6–9% gradient slab gels using a Laemmli buffer system (Laemmli, 1970). Samples were diluted with a half volume of denaturing buffer (0.19 M Tris, *p*H 6.7, 8 M urea, 0.2 M EDTA, 6% SDS, 6% 2-mercaptoethanol, 20% glycerol, and 0.05% bromophenol blue) and heat denatured in a boiling water bath for 5 min before application to the gel. Proteins were visualized by the silver staining method (Merril *et al.*, 1981) and the gels scanned using a Hofer Scientific model GS300 scanning densitometer.

3. RESULTS

As an alternative to the labor-intensive SDS-PAGE experiments described in the preceding paper (French *et al.*, 1990), it is desirable to develop convenient assays that can be used to quantitate the rate of hydrolysis of soluble type I, II, and III collagens by the CHC over a wide variety of conditions (substrate concentration, temperature, etc.). Sensitive radio-

assays for the hydrolysis of these collagen types by tissue collagenases have already been developed for this purpose (Mallya *et al.*, 1986). In these assays, the collagenases are incubated with [^{3}H]acetylated collagens at a temperature well below the denaturation temperature of the collagen. The acetylation both introduces the ^{3}H radiolabel and prevents fibrillogenesis, keeping the collagens soluble. Aliquots are withdrawn as a function of time and the reactions quenched by addition of 1,10-phenanthroline. Since the TC^A and TC^B fragments produced by tissue collagenases have a lower thermal denaturation temperature than the intact collagens, incubation of these reaction aliquots at a carefully selected temperature for each collagen selectively denatures the fragments (Mookhtiar *et al.*, 1986). Since the melting temperature of collagen and its fragments depend on their concentration, the incubation step is always performed after dilution to a low (66 μg/ml) concentration. Next, the addition of dioxane (50% v/v) selectively precipitates the undenatured collagen and the ^{3}H concentration in the supernatant is determined and used to quantitate the extent of the reaction.

These soluble radioassays for tissue collagenases can be readily adapted to quantitate the rate of hydrolysis of these same collagens by the CHC. The effect of salt concentration, labeling index, temperature, etc., on the properties of [^{3}H]acetylated type I, II, and III collagens has already been investigated (Mookhtiar *et al.*, 1986). The only change in the assay protocol that is required involves identifying conditions that maximize the separation of the initial products of hydrolysis from the intact collagens with the goal of monitoring the initial proteolytic event. The dioxane precipitation technique is once again applicable, provided that an appropriate temperature can be found at which the collagen fragments that are initially formed can be selectively denatured. The criterion for choosing this temperature is that it be one at which incubation of the reaction mixture produces the maximum concentration of dioxane nonprecipitable radiolabel *relative to* that formed in a solution containing only the intact collagen. This corresponds to the temperature at which maximum fragment melting relative to that of intact collagen occurs and is the incubation temperature of maximum sensitivity for the assay.

In order to determine the best incubation temperature for the hydrolysis of each collagen by each class of CHC, a series of reactions has been carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 30°C at a collagen concentration of 100 μg/ml. Aliquots of the reaction mixtures were quenched at various times with 1,10-phenanthroline (13 mM). These aliquots were diluted to a concentration of 66 μg/ml and incubated at various temperatures for 10 min, after which the dioxane (50% v/v) nonprecipitable concentration of radiolabel was measured. A parallel series of control experiments was carried out in which enzyme was omitted from the reaction tubes. A plot of cpm vs. reaction time was made for *each* incubation temperature and slopes obtained from the initial portions of these plots were used to calculate the reaction rates in tubes containing enzyme, v_E, and blank rates, v_B, from tubes containing no enzyme in units of μg/hr. Next, the apparent initial rate of the reaction, $v = v_E - v_B$, was plotted as a function of the temperature of incubation for each reaction.

The resulting plots for the hydrolysis of [^{3}H]acetylated rate type I, bovine type II, and human type III collagens by ζ-CHC, a class II enzyme, are shown in Fig. 1. The curves for type II and III collagens exhibit an initial increase in v as the incubation temperature is increased from 30°C into the region (34–35°C) where the collagen fragments produced in the reactions denature faster than the intact collagens. As the incubation temperature is increased further, v levels off and then falls sharply due to a rapid increase in the rate of denaturation of the intact collagens (v_B). Above 37°C, v goes to zero as the 10-min incubation fully denatures all of the collagen and fragments in both the reaction mixture and blank. The curve for type I collagen starts out high at 30°C and lacks the initial increase in v with temperature observed for the other two collagens. Apparently, the fragments are already fully melted at the assay temperature of 30°C. The optimum incubation temperature is taken to be the lowest value at which v is maximal, thereby maximizing sensitivity while minimizing the background in the assays. Based on the curves shown in Fig. 1, incubation temperatures of 30°, 34°, and 35°C have been chosen for assays of the hydrolysis of type I, II, and III collagens, respectively, by the class II CHC. A similar set of reactions has been carried out with β-CHC, a class I collagenase, and plots of v vs. incubation temperature for these reactions are shown in Fig. 2. The curve for each collagen has a shape that is similar to that shown in Fig. 1 for the reactions with ζ-CHC. Using the same criteria as above, 30°, 34°, and 35°C are also chosen as the optimal incubation temperatures for assays involving the hydrolysis

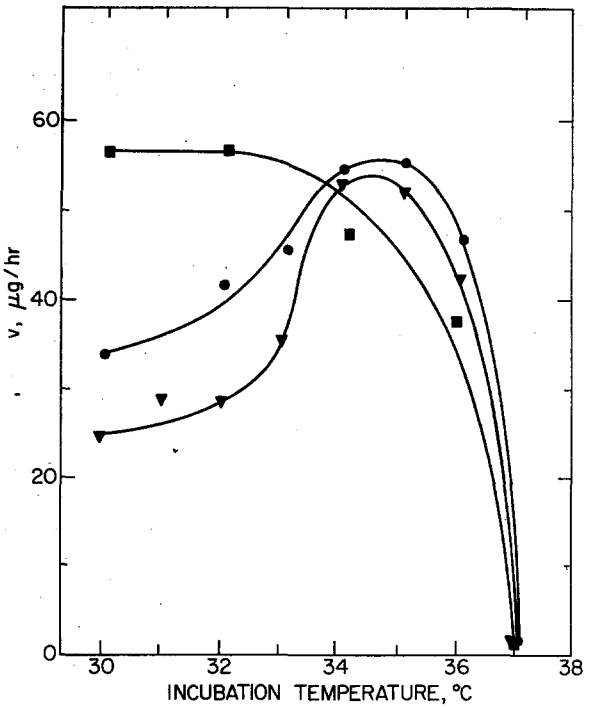

Fig. 1. Effect of temperature of incubation of quenched aliquots of the reaction mixture of [^{3}H]acetylated (■) rat type I, (▼) bovine type II, and (●) human type III collagens with ζ-CHC on the apparent initial rate of the reaction, v. Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, pH 7.5, at 30°C.

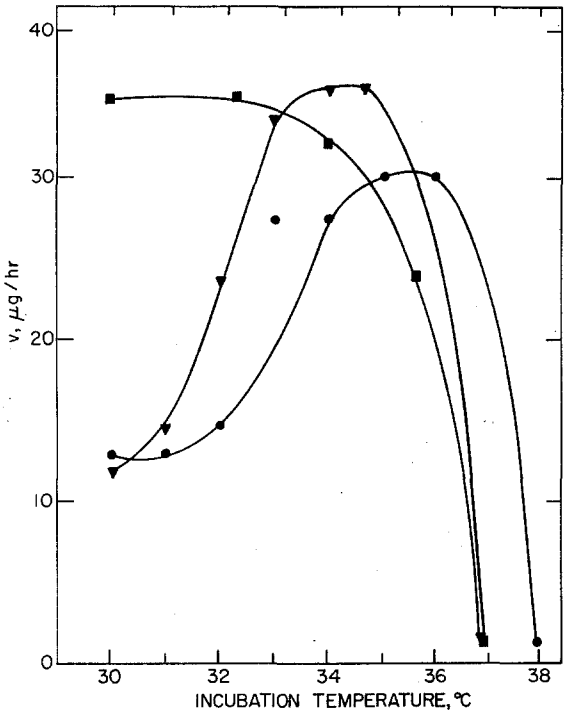

Fig. 2. Effect of temperature of incubation of quenched aliquots of the reaction mixture of [^{3}H]acetylated (■) rat type I, (▼) bovine type II, and (●) human type III collagens with β-CHC on the apparent initial rate of the reaction, v. Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, pH 7.5, at 30°C.

of type I, II, and III collagens, respectively, by the class I CHC.

Having determined the incubation conditions that give assays with maximum sensitivity for these reactions, a series of experiments has been carried out to specifically assess whether the rates measured in the radioassays reflect the initial proteolytic events that were described in the previous paper (French *et al.*, 1990) for each class of CHC. A series of assays has been carried out for each collagen with a representative class I (β) and class II (ζ) CHC at a substrate concentration of 100 μg/ml at 15°C. Aliquots of each reaction mixture were quenched with 1,10-phenanthroline as a function of time. A portion of each aliquot was treated as a normal assay. Thus, each was diluted with assay buffer to a final concentration of 66 μg/ml and incubated at 30° for rat type I, 34° for bovine type II, and 35°C for human type III collagens. Dioxane was then added and the aliquot chilled, centrifuged, and the extent of hydrolysis determined by scintillation counting. The other portion of each aliquot was added to denaturing buffer and subjected to SDS-PAGE on a 6–9% gradient gel. The collagen chains and the hydrolysis fragments were visualized by silver staining, and the extent of reaction quantitated by scanning the gel with a densitometer.

For the reaction of all three collagens with ζ-CHC, only the native 95,000 α chains and fragments with molecular weights of 62,000 and 35,000 produced by the initial scission were visible on the gels. The percent hydrolysis from the SDS-PAGE experiments was calculated from densitometer scans as the area under the two fragments divided by the sum of the total area under collagen plus fragments. Correlations between the percent hydrolysis obtained from the radioassays vs. the percent conversion of the collagens into the 62,000 and 35,000 fragments are shown in Fig. 3 for the three collagens. In all cases, there is an excellent correlation between these parameters, indicating that the assays are monitoring this single, proteolytic event.

The high degree of correlation between the gel and radioassays implies that the 62,000 and 35,000 fragments for all three collagens fully denature under the incubation conditions employed, whereas the intact collagens do not. To confirm this, melting

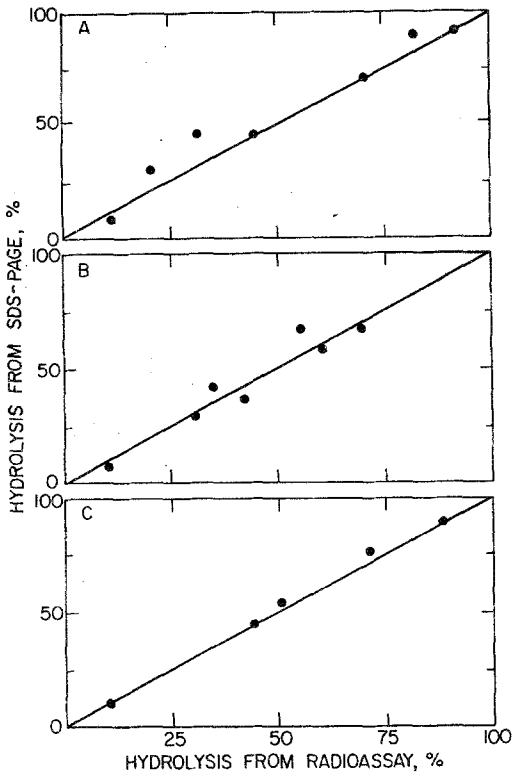

Fig. 3. Correlation between the percent hydrolysis of [^{3}H]acetylated (A) rat type I, (B) bovine type II, and (C) human type III collagens by ζ-CHC as measured with the radioassay and SDS-PAGE. The reactions were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 15°C.

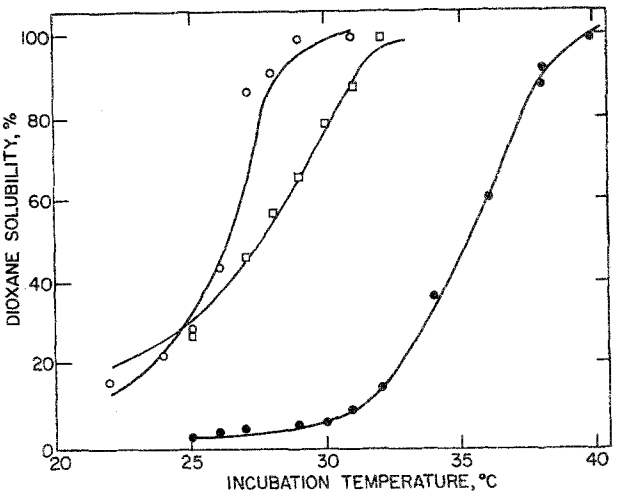

Fig. 4. Melting curves for [^{3}H]acetylated (●) rat type I collagen, (○) the 62,000 and 35,000 fragment mixture initially formed on reaction with ε-CHC, and (□) the 88,000 and 7000 fragment mixture initially formed on reaction with β-CHC. The fragments were formed at 15°C at a collagen concentration of 100 μg/ml. The fragments and collagens were diluted to 66 μg/ml and incubated at the desired temperature for 10 min.

curves for intact rat type I collagen and a mixture of the 62,000 and 35,000 fragments produced on hydrolysis by ε-CHC has been measured. Enzyme was added to the collagen at 15°C until SDS-PAGE showed that the 62,000 and 35,000 fragments had been fully formed. Next, the fragments were diluted to 66 μg/ml, incubated for 10 min at various temperatures between 22 and 30°C, and the percentage of denatured fragments quantitated by scintillation counting as described above. Melting curves obtained in this way for both the intact collagen and fragment mixture confirm that a 10 min incubation at 30°C for rat type I collagen denatures all of the 62,000 and 35,000 fragments, while leaving the intact collagen triple helical (Fig. 4). Thus, these radioassays directly monitor the rate of the initial proteolytic event for the class II CHC.

It is considerably more difficult to quantitate the progress of the initial events in the hydrolysis of these collagens by the class I CHC using SDS-PAGE, since the native 95,000 chains and 88,000–93,000 fragments that are initially formed (French *et al.*, 1990) are not well enough resolved on the gels to be integrated by the densitometer for these collagens. However, a comparison of the SDS-PAGE results with the radioassays for the hydrolysis of rat type I collagen by β-CHC at 30°C shows that *only* the initial 88,000 fragment is formed up to a point in the reaction where the radioassay indicates 70% of full hydrolysis. This suggests that the 88,000 fragment is substantially denatured by the 10 min incubation at 30°C. To investigate this further, rate type I collagen was treated with β-CHC until it was fully converted to the 88,000 fragment at 15°C and its melting curve measured as described above. A comparison of this curve with that for intact type I collagen shows somewhat surprisingly that it is approximately 75% denatured at 30°C, while the native collagen remains triple helical (Fig. 4). Thus, assays for the reaction of rat type I collagen with the class I CHC which utilize a 10 min incubation of the quenched time points at 30°C give a slightly low estimate of the rate of production of the 88,000 fragment. A comparison of SDS-PAGE and radioassay data for types II and III collagens gives similar results, indicating that the radioassays reflect the formation of the initial 88,000–93,000 fragments.

The experiments described above demonstrate that these radioassays provide good estimates of the initial rates of the primary proteolytic events in the

hydrolysis of all three collagens by both classes of CHC. The assays can be carried out under a variety of conditions, including variable enzyme and substrate concentrations, any temperature up to the incubation temperatures, and a range of *p*H values. Assays for the hydrolysis of all three collagens by both classes of CHC have been carried out as a function of enzyme concentration at 30°C. Plots of v vs. CHC concentration (not shown) increase linearly for all three collagen types over the entire enzyme concentration range of 0.1–6 nM that was examined. Thus, these assays give initial rates that are first order in enzyme concentration. In order to determine the kinetic parameters for these reactions, initial rate measurements have been carried out over a wide range of collagen concentrations. The values of v have been converted to units of $\mu M\ hr^{-1}$ by assuming a molecular weight for collagen of 300,000. In all cases, the substrate concentration range examined was both well below and well above the K_M value for the reaction. Lineweaver–Burk plots for the hydrolysis of all three collagen types by both β- and ε-CHC at 30°C are shown in Fig. 5. All six plots are linear and show no kinetic anomalies. Thus, these reactions all obey Michaelis–Menten kinetics.

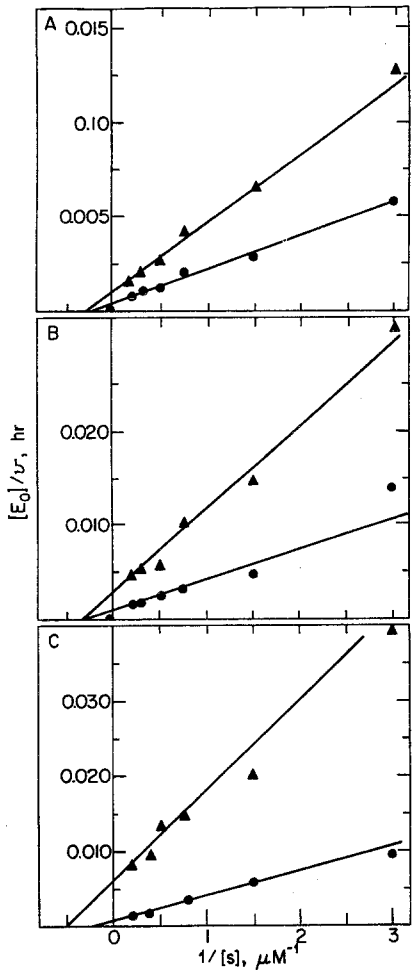

Fig. 5. Lineweaver–Burk plots for the hydrolysis of [^{3}H]acetylated (A) rat type I, (B) bovine type II, and (C) human type III collagens by ε-(▲) and β-(●) CHC. Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 30°C.

Table I. Kinetic Parameters for the Hydrolysis of Type I, II, and III Collagens by Class I CHC[a]

Collagen	Enzyme	k_{cat} (hr^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}\ hr^{-1}$)
Rat type I	β	2100	4.0	530
	γ	1000	3.5	290
Bovine type II	β	1100	4.0	280
	γ	660	3.3	200
Human type III	β	1900	5.0	380
	γ	840	5.0	170

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 30°C.

The values of the kinematic parameters k_{cat} and K_M obtained from the y- and x-intercepts, respectively, of the Lineweaver–Burke plots are listed in Tables I and II for two class I (β and γ) and two class II (ε and ζ) CHC, respectively. The K_M values for β- and γ-CHC are similar and lie between 3.3 and 5.0 μM. Surprisingly, the k_{cat} values only vary from 660–2100 hr^{-1}, with the result that the k_{cat}/K_M values are also very similar. Thus, β- and γ-CHC exhibit very little collagen specificity. This is in marked contrast to the tissue collagenases that have very distinct preferences toward the different collagen types (Hasty *et al.*, 1987; Horwitz *et al.*, 1977; Welgus *et al.*, 1981; Mallya *et al.*, 1990). The kinetic parameters for the hydrolysis of these collagens by ε- and ζ-CHC show the same pattern (Table II). The K_M values for both enzymes are slightly lower than those for the class I

Table II. Kinetic Parameters for the Hydrolysis of Type I, II, and III Collagens by Class II CHC[a]

Collagen	Enzyme	k_{cat} (hr^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}\ hr^{-1}$)
Rat type I	ε	900	3.1	290
	ζ	1100	5.5	200
Bovine type II	ε	340	3.3	100
	ζ	300	2.8	110
Human type III	ε	190	2.0	95
	ζ	410	3.3	120

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 30°C.

CHC, but are all between 2.0 and 5.5 μM. The k_{cat} values show more variability than those for the class I enzymes and are in the 190–1100 hr^{-1} range. For type I collagen, the k_{cat} values are two- to threefold higher than the values for type II and III collagens for both ε- and ζ-CHC. The k_{cat}/K_M values, however, show very little variation between the different types of collagens. Thus, the CHC discriminate very little between these collagens. A comparison of the k_{cat}/K_M values for the three collagens shows that the class I CHC hydrolyze these collagens somewhat more efficiently than the class II CHC.

The temperature dependence of the k_{cat} and K_M values for the hydrolysis of rat type I collagen by β- and ζ-CHC has been investigated in order to monitor the changes in binding and catalysis as the reaction temperature was reduced away from the collagen denaturation temperature. Assays were carried out at 15, 20, and 25°C and the quenched time points were diluted and incubated at 30°C as described above. The kinetic parameters obtained from the Lineweaver–Burk plots at these temperatures are compared with the values obtained at 30°C in Table III. For both enzymes, the k_{cat} values increase markedly with temperature. For β-CHC, k_{cat} increases by approximately a factor of two for every 5° increase in temperature from 15°–30°C. The same is true for ζ-CHC up to 25°, except that k_{cat} levels off between 25 and 30°C. For both enzymes, K_M is invariant from 15–25°C, after which there is a significant drop for β-CHC and slight increase for ζ-CHC. The k_{cat}/K_M values rise steeply for β-CHC over the whole temperature range and for ζ-CHC up to 25°C. Apparently, subtle changes in collagen structure in the region of the cleavage sites by these two CHC occur near 30° that influence the reactions differently.

Table III. Kinetic Parameters for the Hydrolysis of Rat Type I Collagen by β- and ζ-CHC at Various Temperatures

Temperature (°C)	Enzyme	K_M (μM)	k_{cat} (hr^{-1})	k_{cat}/K_M (μM^{-1} hr^{-1})
15	β	12	250	21
20		12	500	42
25		12	1000	83
30		4.0	2100	530
15	ζ	4.2	270	64
20		4.2	400	95
25		4.2	1100	260
30		5.5	1100	200

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at the indicated temperature.

Table IV. Specific Activities (A_{sp}) of β- and ε-CHC Toward the Dissolution of Fibrillar Rat Type I and Human Type III Collagens[a]

Enzyme	A_{sp} (μg/min/mg)		A_{sp} (type I)/ A_{sp} (type III)
	Type I	Type III	
β	1600	1800	0.89
ε	6800	5900	1.2

[a] Assays were carried out in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, at 37°C.

It is doubtful that collagenases come in contact with soluble, monomeric collagens in physiological environments, since these collagens exist in insoluble fibrillar forms *in vivo*. Thus, the action of the CHC on fibrillar rat type I and human type III collagens has been investigated. The specific activities for the dissolution of both types of collagen fibrils and also the ratio of activities toward type I and III collagens are listed in Table IV for β- and ε-CHC. Both collagenases cleave the two collagens at similar rates. However, while the class I enzyme β-CHC hydrolyzes the soluble collagens faster than the class II CHC (Tables I and II), the class II enzyme ε-CHC solubilizes these fibrillar collagens better than β-CHC.

4. DISCUSSION

It has long been thought that the committed and rate-determining step in the breakdown of interstitial collagens *in vivo* is their conversion into TC^A, TC^B fragments by tissue collagenases (Woolley, 1984; Harris and Cartwright, 1977). These fragments denature at body temperature and their degradation is completed rapidly by specific gelatinases or other proteinases (Harris and Cartwright, 1977; Seltzer *et al.*, 1981). While the CHC are capable of degrading these collagens to small dialyzable peptides without the action of synergistic proteinases (Seifter and Harper, 1971), it has been established that they *initiate* the breakdown of these collagens by making a single cleavage within their central triple helical domains at locations that differ from that made by the the tissue collagenases (French *et al.*, 1990; French *et al.*, 1987). Thus, although the location of the initial cleavage differs, the initial event in breakdown of collagen by these two types of collagenases is similar. Since the rates of the initial cleavages made by bacterial collagenases have not been studied previously, the kinetic parameters that describe these events for the CHC have been investigated here.

Since the initial proteolytic events for the CHC are similar to those for the tissue collagenases, we

have adapted the radioassays that were developed for the latter (Mallya *et al.*, 1986; Mookhtiar *et al.*, 1986) to facilitate these studies. The lowering of the melting temperature of the collagens by approximately 7° and 9°C on initial cleavage by the class I and class II CHC, respectively, resembles the lowering in the melting temperature on cleavage of these collagens by tissue collagenases (Sakai and Gross, 1967; Birkedal-Hansen, 1987; Mookhtiar *et al.*, 1986). It is this feature that makes adaptation of the tissue collagenase assay possible for the CHC. It is quite interesting that the melting temperature of the 88,000 fragment produced by the class I CHC is lowered so much compared to the intact collagen. Since only a small C-terminal piece is removed, a smaller change in melting temperature was expected. It is possible that the C-terminal end of the triple helix and/or the C-terminal telopeptide play a prominent role in stabilizing the triple helix. This suggestion is consistent with the observation that the C-terminal end of type I collagen is rich in amino acids (Fasman, 1977). These assays provide an accurate estimate of the rate of formation of 62,000 and 35,000 fragments for the class II enzymes, but a slight (~25%) underestimate of the rate of formation of the initial 80,000–93,000 fragments produced by the class I CHC.

The kinetic parameters for the reactions of the class I and II CHC with type I, II, and III collagens in solution obtained using these assays allow us to directly compare them with the parameters for the tissue collagenase reactions reported earlier (Mallya *et al.*, 1990; Welgus *et al.*, 1981; Hasty *et al.*, 1987). The first comparison of interest is that of the relative collagen specificities of the two types of collagenases, as reflected by the k_{cat}/K_M values. Using similar radioassays on these identical collagen samples in the same buffer at 30°C, the k_{cat}/K_M values for the hydrolysis of rat type I collagen by PCMB-activated human neutrophil collagenase are 12- and 8.6-fold higher than that for bovine type II and human type III collagens, respectively (Mallya *et al.*, 1990). In contrast, human fibroblast collagenase prefers type II collagen 11- and 160-fold over type I and II collagens, respectively. The marked preference of human neutrophil and fibroblast collagenases for type I and III collagens, respectively, is not found for either class of CHC. The largest preference observed is the 3.1-fold higher k_{cat}/K_M value for the hydrolysis of type I over type III collagen by β-CHC. It was shown earlier for the two human collagenases that their marked collagen specificities cannot be explained by the specificities of the enzymes for the sequences found at the cleavage sites (Mallya *et al.*, 1990). Conversely, the rates of hydrolysis of type I, II, and II collagens by the class II CHC are similar, in spite of the fact that the cleavage site sequences differ (French *et al.*, 1990). The Arg_{396}–Gly_{397} bond is hydrolyzed in both the α1(I) and α2(I) chains of type I collagen, the Hyp_{405}–Gly_{406} bond in the α1(II) chains of type II collagen, and the Arg_{399}–Gly_{400} bond in the α1(III) chains of type III collagen. Thus, the relative rates of cleavage of sites in interstitial collagens seems to depend more on some presently unappreciated conformational feature than the actual sequence.

With regard to the recognition of the collagen cleavage sites by collagenases, the K_M values for the reaction of all three collagens with both classes of CHC are very similar and fall in the 2.0–5.5 μM range. These K_M values are also very similar to those for the tissue collagenases which fall in the 0.82–2.6 μM range (Mallya *et al.*, 1990). This suggests that the clostridial and human collagenases share some common characteristics in recognition and binding of their respective cleavage sites in the collagen molecules. The tight binding of collagenases to collagens is not simply a direct result of recognition of the specific sequence at the cleavage site. It has been shown both for tissue collagenases (Fields *et al.*, 1987) and the CHC (Van Wart and Steinbrink, 1985) that the K_M values for hydrolysis of peptides with collagen-like sequences are much higher than those for collagens. Thus, the triple helical structure and/or local conformation around the cleavage site must play an important role in the recognition and binding of all collagenases. Like the tissue collagenases, the k_{cat} values for the CHC show more variability than the K_M values. The k_{cat} values for the hydrolysis of different collagen types by the CHC range from 190–2100 hr^{-1}, while they range from 3.2–350 hr^{-1} for human fibroblast collagenase and 130–690 for human neutrophil collagenase. Interestingly, these values are much smaller than the k_{cat} values obtained for the hydrolysis of small peptides by the same enzymes (Van Wart and Steinbrink, 1985; Mallya *et al.*, 1990). This large difference may be due to the inaccessibility of the scissile bond in the collagen triple helix. Since access to the scissile bond is critical for hydrolysis, the actual value of k_{cat} may be determined by the flexibility of the triple helix at the cleavage site, as determined by the surrounding sequence.

The temperature dependence of the kinetic parameters for both classes of CHC shows that the factors that influence k_{cat} and K_M are likely to be complex. Over the 15–25°C range, the K_M values

remain constant, while the k_{cat} values increase monotonically with temperature for both class of CHC. This implies that there is no change in the binding step and that the rate of the rate-determining step is greatly increased at higher temperatures. On raising the temperature to 30°C, the k_{cat} and K_M values for the two enzymes exhibit opposite behavior that would require a more detailed study to fully understand. This different behavior suggests that the factors that are responsible for binding and catalysis in these reactions might be quite subtle and involve small changes in local collagen structure.

Another interesting comparison between the CHC and tissue collagenases relates to their collagenolytic potencies. It has been widely believed that the CHC are more potent collagenases, a view derived in part from the extensive use of crude CHC preparations for harvesting cells from human tissue. The kinetic parameters presented here for the CHC show that this belief is only partially valid. The k_{cat}/K_M values of 690 μM^{-1} hr^{-1} for the hydrolysis of rate type I collagen by human neutrophil collagenase and of 210 μM^{-1} hr^{-1} for the hydrolysis of type II collagen by human fibroblast collagenase are of the same general magnitude as the 95–530 μM^{-1} hr^{-1} values for the CHC reactions listed in Tables I and II. The same conclusion is obtained by comparing the hydrolysis rates of type I and III collagen fibrils (Table IV). The specific activities for human neutrophil and fibroblast collagenases are in the 1000–7000 μg/min/mg range, while the values for the CHC are in the 1800–6800 μg/min/mg range. What is notable about the CHC is that they hydrolyze *all three* soluble collagen types efficiently and that they do not stop after the initial cleavage, but go on to degrade these collagens to a mixture of small peptides.

In conclusion, the kinetic parameters that describe the primary events in the hydrolysis of type I, II, and III collagens by the CHC and human collagenases highlight both the similarities and differences between these enzymes. Both the k_{cat} and K_M values for the hydrolysis of soluble collagens are of comparable magnitude for the two types of collagenases. Moreover, their potencies toward dissolving fibrillar collagens are also very similar. In contrast to the tissue collagenases, however, the CHC have markedly reduced specificities toward the interstitial collagen types in solution. All of these reactions share the property that they are markedly influenced by local collagen structure in ways that are not currently understood. More detailed studies are required to elucidate the features of tissue and bacterial cleavage sites in these collagenases that play such a dominant role in influencing their reactions.

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