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Identification of *Clostridium histolyticum* Collagenase Hyperreactive Sites in Type I, II, and III Collagens: Lack of Correlation with Local Triple Helical Stability

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The class I and II *Clostridium histolyticum* collagenases (CHC) have been used to identify hyperreactive sites in rat type I, bovine type II, and human type III collagens. The class I CHC attack both collagens at loci concentrated in the N-terminal half of these collagens starting with the site closest to the N-terminus. The class II CHC initiate collagenolysis by attacking both collagens in the interior to produce a mixture of C-terminal 62,000 and a N-terminal 36,000 fragments. Both fragments are next shortened by removal of a 3000 fragment. These results are very similar to those reported earlier for the hydrolysis of rat type I collagen by these CHC, indicating that the three collagens share many hyperreactive sites. Similar reactions carried out with the respective gelatins show that they are cleaved at many sites at approximately the same rate. Thus, the hyperreactivity of the sites identified must be attributed to their environment in the native collagens. N-terminal sequencing of the fragments produced in these reactions has allowed the identification of 16 cleavage sites in the α1(I), α2(I), α1(II), and α1(III) collagen chains. An analysis of the triple helical stabilities of these cleavage site regions as reflected by their imino acid contents fails to yield a correlation between reactivity and triple helical stability. The existence of these hyperreactive CHC cleavage sites suggests that type I, II, and III collagens contain regions that have specific nontriple helical conformations. The sequence of these sites presented here now makes it possible to investigate these conformations by computational and peptide mimetic techniques.

KEY WORDS: Collagen structure; cleavage site; *Clostridium histolyticum* collagenase; interstitial collagens; sequencing.

1. INTRODUCTION[4]

The collagens are the major protein constituents of the extracellular matrix and the most abundant proteins in all higher organisms (Mayne and Burgeson, 1987). The ability of the fibril-forming interstitial collagens to serve as structural proteins is derived from their tightly coiled triple helical structures that endow them with stiff rod-like conformations (Piez, 1984). While it is clear that the great majority of the collagen molecule adopts a regular triple helical conformation, it is very likely that there are also variations in structure imposed by local sequence. A reasonable analogy is to DNA, which was first assumed to be uniformly double helical, but which is now known to adopt alternate conformations dictated by local nucleotide sequence (Cozzarelli and Wang, 1990). Such regions in collagens may have specific nontriple helical conformations that may be related to important physiological functions such as fibrillogenesis, cell or receptor interactions, etc.

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[4] Abbreviations used: CHC, *Clostridium histolyticum* collagenase; Tricine, N-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; Bis-tris, [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane]; Tes, [tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid]; Caps, 3-(cyclohexylamino)-1-propane sulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polycrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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Unfortunately, almost nothing is known about the local structures of collagens since their fibril-forming propensities have prevented crystallographic structure determinations from being carried out. As a result, more indirect methods must be used to detect and locate regions with unusual properties. Once identified, the structures of such regions could be more expeditiously studied in collagen fragments or in model peptides. One means of locating sites with nontriple helical conformations is by their hypersensitivity to proteinases. Since collagens are almost totally resistant to nonspecific proteinases, only specific collagenases are suited for this purpose. The collagenases can be divided into two categories. The fibroblast-type (Stricklin *et al.*, 1977; Goldberg *et al.*, 1986) and the neutrophil-type (Mookhtiar and Van Wart, 1990; Mallya *et al.*, 1990; Hasty *et al.*, 1990) tissue collagenases (EC 3.4.24.7) are produced by higher organisms for the presumed purpose of catalyzing their own collagen breakdown. These are highly specific enzymes that dissolve fibrils of type I, II, and III interstitial collagens by making a single scission across all three α chains of exposed triple helical collagen monomers (Hasty *et al.*, 1987; Horwitz *et al.*, 1977; Welgus *et al.*, 1981; Mallya *et al.*, 1990) at a specific sensitive locus that has been identified and is currently the only hyperreactive site of known sequence.

A second category of collagenase is that from lower organisms that themselves do not contain collagen. These organisms presumably secrete the enzyme to digest protein for nutritional purposes, or use it to facilitate invasion into a collagen-containing host. The best studied bacterial collagenases are those produced by the pathogenic anaerobe *Clostridium histolyticum* (EC 3.4.24.3) (Seifter and Harper, 1971; Mookhtiar and Van Wart, 1991). The culture filtrate of *Clostridium histolyticum* contains at least seven different collagenases that have been designed as α-, β-, γ-, δ-, ε-, ζ- and η-CHC, according to the order in which they were purified (Bond and Van Wart, 1984a; Van Wart and Steinbrink, 1985). Extensive characterization of these enzymes has shown that all are zinc proteinases (Bond and Van Wart, 1984b), but that they can be assigned to two classes on the basis of their sequence homologies, as revealed by chromatographic analysis of their tryptic digests (Bond and Van Wart, 1984b, c), their peptide substrate specificities (Steinbrink *et al.*, 1985; Van Wart and Steinbrink, 1985; Mookhtiar *et al.*, 1985), and their response to inhibitors (Mookhtiar *et al.*, 1988) and active site metal substitutions (Angleton and Van Wart, 1988a, b). These and other bacterial collagenases have a broad specificity and are distinguished by their ability to hydrolyze interstitial collagens at multiple sites. Thus, the CHC are an ideal tool for locating hyperreactive regions of collagens.

In an earlier study, the class I (α, β, γ, and η) and class II (δ, ε, ζ) CHC were shown to attack native, triple helical type I collagen at a limited number of sites, all distinct from the tissue collagenase cleavage site (French *et al.*, 1987). The class I enzymes initially hydrolyze all three α chains near the N- and C-termini to produce 88,000 and 80,000 fragments, respectively, while the class II CHC make an initial cleavage in the interior of the triple helix to yield 35,000 and 62,000 fragments. In this investigation, we have extended these studies to include type II and III collagens, while refining details of their attack on type I collagen. These studies define the pathway of hydrolysis of these collagens and provide insights into how collagens might be fully catabolized *in vivo*. More importantly, however, the locations of many of the hyperreactive cleavage sites in these three collagens have been identified by N-terminal sequencing. These studies show that many of these sites are conserved in all three collagens, implying that they may serve important functional roles. This makes it possible for the first time to consider how the sequences at these sites might endow them with hyperreactivity by changing local collagen structure.

2. MATERIALS AND METHODS

2.1. Materials

Crude preparations of CHC were purchased from both Sigma Chemical Company and Advanced Biofactures Corporation and were used as starting materials for the isolation of β-, γ-, ε-, and ζ-CHC as described previously (Bond and Van Wart, 1984a). The concentrations of all CHC were determined spectrophotometrically using the extinction coefficients published earlier (Bond and Van Wart, 1984b). Type I collagen was isolated from rat tail tendon, type II collagen from bovine nasal septum, and type III collagen from the amnion of human placenta (Mookhtiar *et al.*, 1986). Collagen concentrations were determined by the biuret method (Gornall *et al.*, 1949), using standard curves prepared from oven-dried samples of the collagens. EDTA and 1,10-phenanthroline were purchased from Sigma Chemical Company. All other reagents and solvents were reagent grade or

higher in quality. Polyvinylidene difluoride membranes were obtained from Millipore.

2.2. Limited Proteolysis of Type I, II, and III Collagens

Limited proteolysis experiments were carried out by adding an aliquot of the CHC to a sample of 0.1–2 mg/ml collagen in 50 mM Tricine, 10 mM $CaCl_2$, 0.2 M NaCl, *p*H 7.5, in a water bath equilibrated at either 15 or 20°C. The final CHC concentrations varied from 40–300 nM, depending on the reaction. Aliquots (20 μl) were withdrawn from the reaction tube as a function of time, quenched by the addition of 1,10-phenanthroline to a final concentration of 1 mM, and mixed with SDS-PAGE denaturing buffer.

2.3. Electrophoresis

SDS-PAGE experiments were carried out using either a Tris-glycine, *p*H 8.3 (Laemmli, 1970), or a Bis-Tris Tes, *p*H 7.2, buffer system (Moos *et al.*, 1988). The concentration of acrylamide in the running gel was varied in different experiments from 6–15%. Samples were denatured by heating in boiling water for 5 min. For experiments with type III collagen, 2-mercaptoethanol was added to a final concentration of 2%. Proteins were visualized either by Coomassie Brilliant Blue R-250 staining or by silver staining (Merril *et al.*, 1981). Quantitation of protein bands was carried out by densitometry using a Hoefer Scientific densitometer equipped with a Hewlett-Packard model 3390A integrator.

2.4. Electrophoretic Transfers

SDS-PAGE experiments that preceded electroblotting were carried out under several conditions designed to minimize N-terminal blocking reactions (Moos *et al.*, 1988). These included the use of the lower *p*H Bis-Tris Tes running buffer, pre-electrophoresis of the gel in the presence of thioglycolate, and running the gels at 4°C. Collagen fragments were subsequently transferred onto polyvinylidene difluoride membranes using a Bio Rad Mini Trans-Blot apparatus. After the initial electrophoresis, the gels were soaked in 10 mM Caps, *p*H 11.0, for 5 min. The polyvinylidene difluoride membranes were activated by treatment with HPLC-grade methanol for 5 min, followed by a 5 min wash in 10 mM Caps, *p*H 11.0. The gel-membrane sandwich was assembled and placed in the electroblot apparatus containing 10 mM Caps, *p*H 11.0, and transfer was carried out at 300 mA for 1 hr at 4°C. After transfer was complete, the membranes were stained in Coomassie Blue R-250 dissolved in 50% methanol/water for 5 min, destained with a methanol:water solution (5:2:1), washed with several changes of water, and dried on Whatman filter paper. The desired bands were cut out and subjected to sequencing.

2.5. High-Performance Liquid Chromatography

HPLC experiments were performed to isolate certain collagen fragments with a system that consisted of a NEC PC8300 controller, an Altex 210 injector, a Beckman System Gold 126 solvent module, and a Varian UV-50 detector. Reversed-phase separations were carried out using a Vydac 218 TP546 C18 (4.6 × 250 mm) column at 45°C, using either gradient elution with acetonitrile and 0.1% trifluoroacetic acid. Gel filtration experiments were carried out using a Bio Rad Bio-Sil TSK-250 (7.5 × 250 mm) column at 40°C in 0.2 M sodium acetate, *p*H 6. The fractions containing the desired products were dialyzed vs. 0.10 M acetic acid and the collagen fragments recovered by lyophilization.

2.6. N-Terminal Sequencing

N-terminal sequence analysis of either HPLC purified or electroblotted proteins was carried out using an Applied Biosystems Model 477-A pulsed liquid-phase protein sequencer. PTH-amino acids were analyzed using an on-line Applied Biosystems Model 120A PTH-amino acid analyzer that contained a PTH-C18 cartridge (2.1 × 200 mm). Quantitation of amino acids was carried out using the 900A data analysis system. The reactions leading to formation of fragments shown to arise from hydrolysis of Arg–Gly bonds were repeated in the presence of 0.1 mM p-chloromercuribenzoate to inhibit any traces of clostripain present. However, the same results were obtained, indicating that the CHC were indeed responsible for the cleavage.

3. RESULTS

In an earlier study, the initial proteolytic events in the hydrolysis of rat tendon type I collagen by two class I (β and γ) and two class II (ε and ζ) CHC were investigated by monitoring the products of the reaction by SDS-PAGE (French *et al.*, 1987). In this study, these limited proteolysis experiments are first

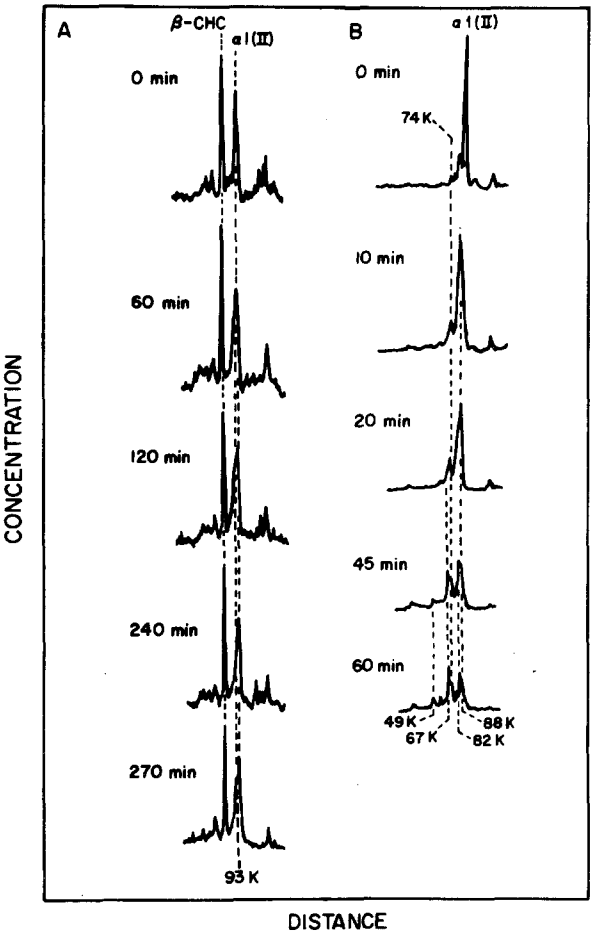

Fig. 1. Densitometer tracings of an SDS gel showing the reaction of bovine nasal septum type II collagen with β-CHC where: (A) the collagen and β-CHC concentrations were 0.1 mg/ml and 220 nM, respectively, and the temperature 15°C; and (B) the collagen and β-CHC concentrations were 2 mg/ml and 220 nM, respectively, and the temperature 20°C.

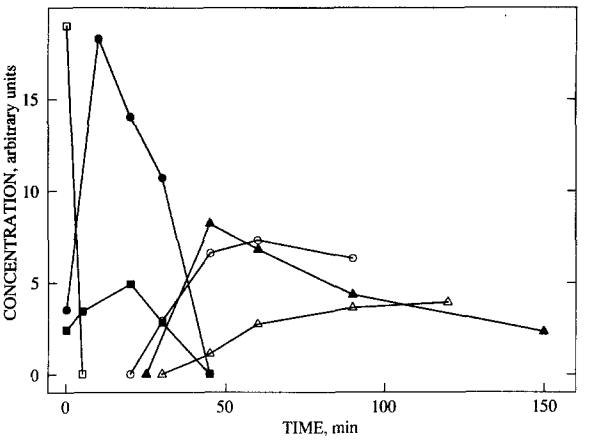

Fig. 2. Variation in the concentration of the (□) α1(II) chain and (●) 88,000, (▲) 82,000, (■) 74,000, (○) 67,000, and (△) 49,000 fragments as a function of time during the reaction of bovine nasal septum type II collagen with β-CHC at 20°C, as shown in Fig. 1B.

extended to the hydrolysis of bovine type II and human type III collagens. After this, the hydrolysis of rat type I collagen is reexamined and a pathway for the degradation of all three collagens by both classes of CHC is proposed. Last, N-terminal sequencing has been used to identify as many sites as possible so that the basis for their hyperreactivity could be considered in the light of their local sequences.

The reactions of two class I (β and γ) CHC with type II collagen will be considered first. In order to observe all of the initial proteolytic events in these reactions, it was necessary to carry them out under two sets of conditions. Both reactions were carried out in 50 mM Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, *p*H 7.5, at CHC concentrations of 220 nM. However, the collagen concentration and temperature were varied to focus on different stages of the reaction. Densitometer tracings from a gel showing the products of the reactions of 0.1 mg/ml type II collagen with β-CHC at 15°C are shown in Fig. 1A. At the start of the reaction, only the α1(II) chain and the band due to β-CHC are prominent. After 60 min, the first evidence of a proteolytic fragment starts to appear as a shoulder on the low molecular weight side of the intact α1(II) chain. The estimated molecular weight of this fragment obtained using a calibration curve made from the α1(II) chain, the $TC^A[\alpha 1(II)]$ chain and the $TC^B[\alpha 1(II)]$ chain is 93,000. The appearance of the fragment continues at longer reaction times and correlates with the decay of the α1(II) chain. After 120 min, the concentration of the 93,000 fragment levels off and no other fragments are detected.

In order to observe later proteolytic events, a second digestion was carried out using a higher (2 mg/ml) collagen concentration and a higher (20°C) temperature. Densitometer tracings of a gel showing the products of this reaction are pictured in Fig. 1B. Over the first 60 min of the reaction, fragments with α-chain molecular weights of 88,000, 82,000, 74,000, 67,000, and 49,000 are formed. Under these reaction conditions, the 93,000 fragment is never observed. After 60 min, no new fragments are detectable and the existing fragments are degraded to nondetectable products (not shown). A similar digestion pattern is observed with γ-CHC. The variation in the concentrations of the α1(II) chain and these five fragments with time is displayed in Fig. 2. After 10 min,

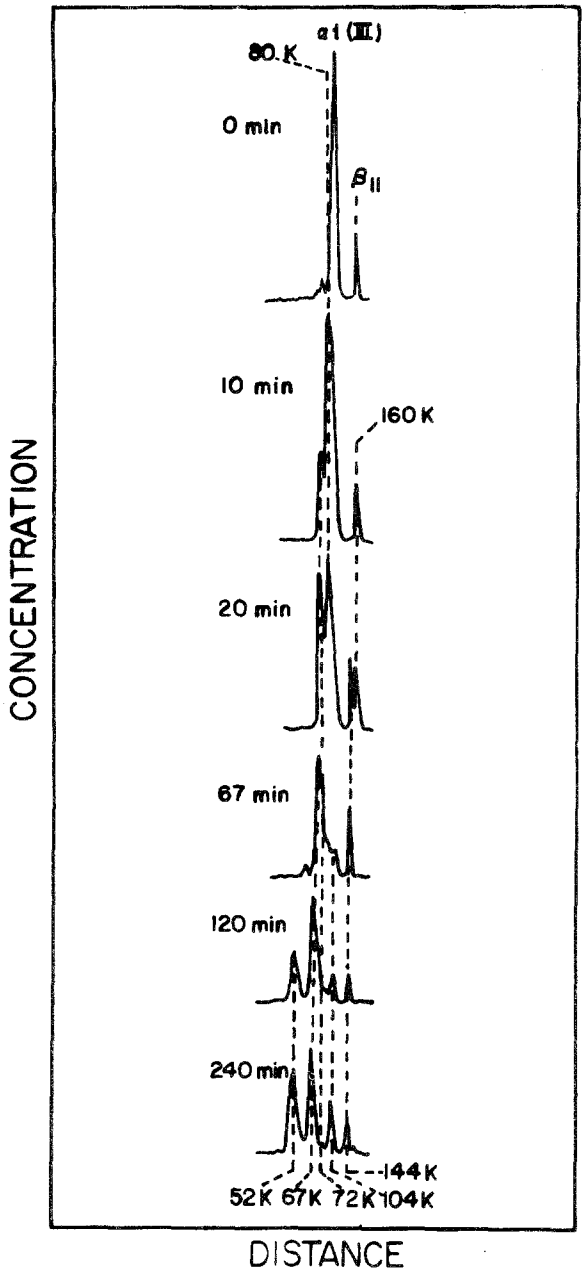

Fig. 3. Densitometer tracings of an SDS gel showing the reaction of human placental type III collagen with β-CHC at 20°C. The collagen and β-CHC concentrations were 1 mg/ml and 220 nM, respectively.

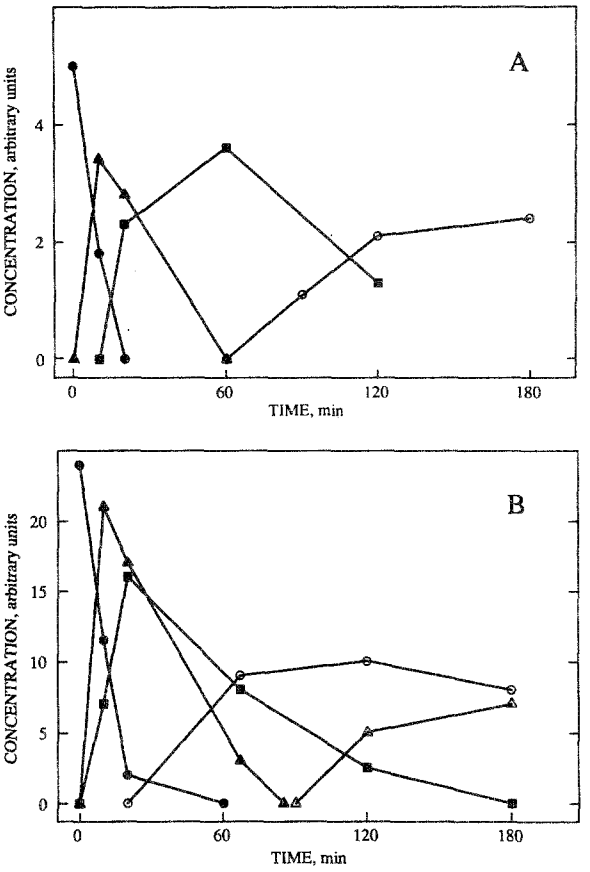

Fig. 4. Variation in the concentration of the (A) (●) β_{11} chain and (▲) 160,000 (■) 144,000+135,000 and (○) 104,000 fragments, and (B) (●) α1(III) chains, (▲) 80,000, (■) 72,000, (○) 67,000, and (△) 52,000 fragments, as a function of time during the reaction of human placental type III collagen with β-CHC at 20°C as shown in Fig. 3.

the α1(II) chain is completely converted to major and minor products with molecular weights of 88,000 and 74,000, respectively. At this time, the 88,000 fragment has already reached its maximum concentration. The 74,000 fragment is produced in parallel with the 88,000 species, but continues to increase in concentration after 10 min, even though all of the α1(II) chains have been hydrolyzed. This suggests that both fragments arise by simultaneous cleavage of the 93,000 fragment and that some of the 88,000 fragment is converted into 74,000 fragment. Between 20 and 45 min, the 82,000 and 67,000 fragments are produced in parallel and their appearance correlates with the decay of the 88,000 and 74,000 fragments. The 82,000 fragment can only arise from the 88,000 fragment. However, the 67,000 fragment could arise from hydrolysis of either the 88,000 or 74,000 fragments, or both. The fact that its intensity surpasses that of the 74,000 fragment argues that at least some of it is derived from the 88,000 fragment. Finally, the rise in concentration of the 49,000 fragment correlates best with the decay of the 82,000 and 67,000 fragments.

Similar limited proteolysis experiments have been carried out between both β- and γ-CHC and human

placental type III collagen and analyzed by SDS-PAGE experiments carried out in the presence of 2-mercaptoethanol to reduce all disulfide cross-links. By manipulation of the reaction conditions as described above for type II collagen, the initial event observable is the rapid formation of an 88,000 fragment derived from the α1(III) chains and a 176,000 dimer fragment from the β_{11} chains (not shown). The β_{11} bands arise from an internal covalent cross-link within the triple helical domain (Fujii and Tanzer, 1976). Subsequent events are pictured in Fig. 3, which shows the reaction of type III collagen (1 mg/ml) with β-CHC at 20°C. A similar digestion pattern is observed with γ-CHC. At the start of the reaction, only the monomeric α1(III) and dimeric β_{11} bands are present. Over the first 240 min of the reaction, fragments with molecular weights of 160,000, 144,000, 104,000, 80,000, 72,000, 67,000, and 52,000 are detected. The variation in the concentrations of the β_{11} chain and 160,000, 144,000, and 104,000 fragments as a function of time is shown in Fig. 4A, while that of the α1(III) chain and 80,000, 72,000, 67,000, and 52,000 fragments is shown in Fig. 4B.

After 10 min, most of the α1(III) chains have been completely degraded to major and minor products with molecular weights of 80,000 and 72,000, respectively, while the β_{11} chains have been simultaneously converted to a major 160,000 fragment with an unresolved shoulder due to some 144,000 fragment. The 160,000 and 144,000 fragments are attributed to dimers of the 80,000 and 72,000 fragments because of their molecular weight relationship and the fact that their appearance and decay are correlated in time. The concentrations of the 80,000 and 160,000 fragments decrease between 10 and 20 min, while the 72,000 and 144,000 fragments continue to rise in concentration. This suggests that the 80,000/160,000 pair decays to the 72,000/144,000 pair. Between 20 and 90 min, the 67,000 fragment is produced by cleavage of one or both of the 80,000 and 72,000 fragments. A corresponding 135,00 fragment is probably produced, but is not resolved from the 144,000 band. Last, the 52,000 fragment appears at a rate that suggests that it is produced primarily from the 67,000 fragment, while a clearly resolved 104,000 dimer fragment increases in parallel via breakdown of the 135,000 and/or 144,000 fragments.

In order to locate the cleavage sites that lead to the production of the fragments discussed above and those detected earlier for type I collagen (French *et al.*, 1987), N-terminal sequencing experiments have been carried out on all fragments that could be successfully isolated by HPLC or electroblotting procedures. Although repeated attempts were made to sequence every fragment discussed, this could not be accomplished either because the isolated fragment gave a mixture of several N-terminal amino acids at every cycle (e.g., the 80,000 type I collagen fragment), or the fragment could not be isolated due to its small size (e.g., the 2000 and 7000 fragments from the N-terminal cleavage of type II and III collagens) or overlaps with other fragments (e.g., many of the type I fragments). The results of these N-terminal analyses for the fragments produced by the class I CHC are shown in Table I.

For each fragment, the sequence detected is presented on the top line. Occasionally, more than a single amino acid was found to be present during a particular Edman cycle. In these cases, the less abundant residue is listed above the more abundant residue detected. The cleavage sites shown on the bottom line were located by comparison of the fragment N-terminal sequence with the most closely related collagen chain of known sequence. To perform this comparison, the combined rat/calf skin sequence data were used for the α1(I) and α2(I) chains (Fasman, 1977; Chapman *et al.*, 1981), bovine cartilage data for the α1(II) chains (Fasman, 1977; Seyer *et al.*, 1989) and human liver data for the α1(III) chains (Seyer and Kang, 1981). In all cases, the cleavage site identified was consistent with the molecular weight of the fragment. The numbering of the residues starts with the Gly of the first Gly–X–Y triplet of the triple helical domain of these collagens. Thus, the first triplets are assumed to be Gly–Pro–Met for rat α1(I) and α2(I) chains (Fasman, 1977; Chapman *et al.*, 1981), Gly–Val–Met for bovine α1(II) (Fasman, 1977), and Gly–Tyr–Hyp for human liver α1(III) (Seyer and Kang, 1981). There are several mismatches with the published sequences (shown in bold typeface in Table I) that arise either from errors in sequencing or from true differences in sequence due to comparison with collagens of different species or tissue type.

By combining all of the data discussed above and that obtained earlier (French *et al.*, 1987), a pathway of hydrolysis of type I, II, and III collagens by the class I CHC can be constructed. The locations of the sites of hydrolysis in these collagens by the class I CHC are shown pictorially in Fig. 5. Here, each collagen chain is denoted by a horizontal line with the N-terminus on the left. Each cleavage site is denoted by a vertical arrow placed at its approximate location in the collagen molecule, and each is numbered

Table I. Location of Class I CHC Hyperreactive Cleavage Sites in Type II and III Collagens[a]

Collagen chain	Molecular weight	Sequence	Reference
		↓	
		Glu	
α1(II)	93,000	Gly -Asn-Pro-Gly-Glu-Hyp-Gly-Val-Hyp-Gly-Val-Ser	
α1(II)	95,000	Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln_{24} / Gly_{25}-Asn-Pro-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Val-Ser	—[b]
α1(II)	88,000	Gly -Gln-Arg-Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp	
α1(II)	95,000	Gly-Lys-Ser-Gly-Glu-Arg-Gly-Pro-Hyp-Gly-Pro-Gln_{72} / Gly_{73}-Ala-Arg-Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp	—[b]
α1(II)	82,000	Gly -Pro-Arg-Gly-Gln-Hyp	
α1(II)	95,000	Gly-Pro-Gln-Gly-Ala-Arg-Gly-Pro-Glu-Gly-Ala-Gln_{189} / Gly_{170}-Pro-Arg-Gly-Gln-Hyp-Gly-Thr-Hyp-Gly-Ala-Hyp	—[c]
		Arg Ala Leu	
α1(II)	67,000	Gly -Pro-Hyp-Gly-Glu-Hyp-Gly-Glu-Hyp	
α1(II)	95,000	Gly-Ala-Val-Gly-Pro-Hyl-Gly-Ser-Hyp-Gly-Glu-Ala_{348} / Gly_{349}-Arg-Hyp-Gly-Glu-Ala-Gly-Leu-Hyp-Gly-Ala-Hyl	—[c]
α1(II)	49,000	Gly -Pro-Hyp-Gly-Glu-Gly-Gly-Lys-Hyp-Gly-Ala-Gln	
α1(II)	95,000	Gly-Pro-Ser-Gly-Phe-Gln-Gly-Leu-Hyp-Gly-Pro-Hyp_{471} / Gly_{472}-Pro-Hyp-Gly-Glu-Gly-Gly-Lys-Hyp-Gly-Asp-Gln	—[b]
α1(III)	88,000	Gly -Leu-Hyp-Gly-Thr-Hyp	
α1(III)	95,000	Gly-Glu-Ser-Gly-Arg-Hyp-Gly-Arg-Hyp-Gly-Glu-Arg_{69} / Gly_{70}-Leu-Hyp-Gly-Pro-Hyp-Gly-Ile-Hyl-Gly-Pro-Ala	—[d]
		Leu Pro	
α1(III)	176,000	Gly -Gln-Hyp-Gly-Thr-Hyp	
α1(III)	95,000	Gly-Glu-Ser-Gly-Arg-Hyp-Gly-Arg-Hyp-Gly-Glu-Arg_{69} / Gly_{70}-Leu-Hyp-Gly-Pro-Hyp-Gly-Ile-Hyl-Gly-Pro-Ala	—[d]
α1(III)	72,000	Gly -Leu-Arg-Gly-Gly-Lys-Gly-Glu-Hyp	
α1(III)	95,000	Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Ala-Asn-Gly-Ala-Hyp_{252} / Gly_{253}-Leu-Arg-Gly-Gly-Ala-Gly-Glu-Hyp-Gly-Lys-Asn	—[d]
α1(III)	144,000	Gly -Leu-Arg-Gly-Gly-Ala-Gly-Glu-Hyp-Gly-Lys-Asn	
α1(III)	95,000	Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Ala-Asn-Gly-Ala-Hyp_{252} / Gly_{253}-Leu-Arg-Gly-Gly-Ala-Gly-Glu-Hyp-Gly-Lys-Asn	—[d]
		Gly Asn	
α1(III)	52,000	Gly -Leu-Hyp-Gly-Thr-Hyp-Gly-Pro-**Pro**-Gly-Glu-Asn	
α1(III)	95,000	Gly-Asp-Thr-Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Leu-Gln_{468} / Gly_{469}-Leu-Hyp-Gly-Thr-Gly-Gly-Pro-Hyp-Gly-Glu-Asn	—[d]
		Hyp	
α1(III)	52,000	Gly -Thr-Gly-Gly-Pro-Gly-Gly-Glu-Asn-Gly-Lys-Hyp	
α1(III)	95,000	Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Leu-Gln-Gly-Leu-Hyp_{471} / Gly_{472}-Thr-Gly-Gly-Pro-Hyp-Gly-Glu-Asn-Gly-Lys-Hyp	—[d]

[a] For each entry, the experimentally determined sequence is shown on top (in some cases, more than one amino acid was detected, with the minor one shown above). The most consistent cleavage site from the most closely related collagen chain of known sequence is shown on bottom.

[b] Fasman (1977).

[c] Seyer *et al.* (1989).

[d] Seyer and Kang (1981).

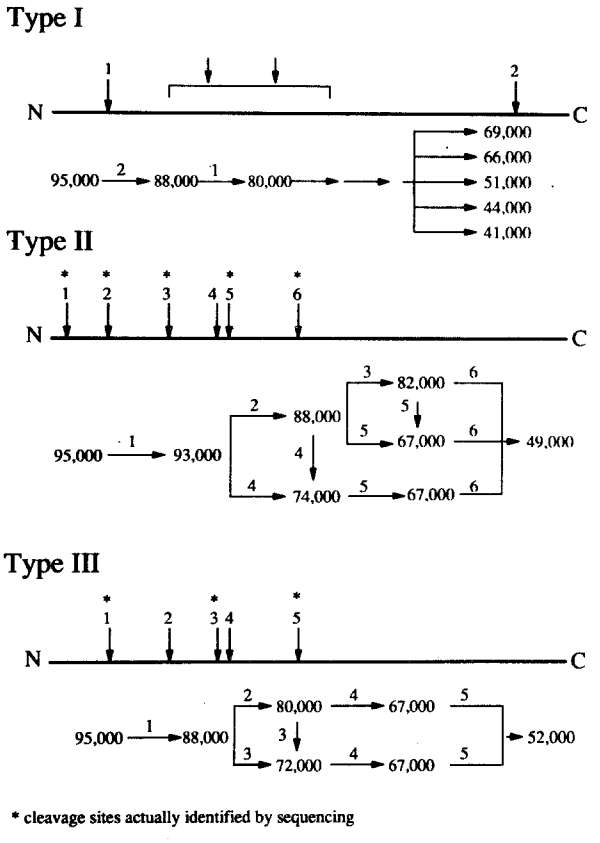

Fig. 5. Schematic showing the sites of hydrolysis (vertical arrows) of type I, II, and III collagens (solid horizontal lines) by the class I CHC, and a degradation scheme for each. Cleavage sites identified by N-terminal sequencing are indicated by an asterisk.

consecutively from then N-terminus. Those sites identified by sequencing (Tables I and II) are indicated by an asterisk. Starting with type II collagen for which the most sequence data are available, the first hydrolytic event is the hydrolysis of all three α1(II) chains at the Gln_{24}–Gly_{25} bond at site 1 to produce the 93,000 fragment. This fragment is then hydrolyzed at sites 2 and 4 to give fragments with molecular weights of 88,000 and 74,000, respectively. While both cleavages occur over the same time scale, hydrolysis of Gln_{72}–Gly_{73} bond at site 2 is faster, as evidenced by the faster rise and greater concentration of the 88,000 fragment (Fig. 2). As noted above, some of the 74,000 fragment arises from hydrolysis of the 88,000 fragment at site 4. Next, the 88,000 fragment is simultaneously hydrolyzed at the Gln_{189}–Gly_{190} bond at site 3 to produce the 82,000 fragment and at the Ala_{348}–Gly_{349} bond of site 5 to produce the 67,000 fragment. Over the same time scale, the 82,000 and 74,000 fragments also undergo hydrolysis at site 5 to produce the 67,000 fragment. Finally, the 49,000 fragment arises from hydrolysis of both the 82,000 and 67,000 fragments at the Hyp_{471}–Gly_{472} bond at site 6.

The hyperreactive cleavage sites for type III collagen are similar to those in type II collagen, except that there is no evidence for the most N-terminal site that would have produced a 93,000 fragment. The first event detected is hydrolysis of the Arg_{69}–Gly_{70} bonds of the α1(III) and β_{11} chains at site 1 to produce the 88,000 and 176,000 fragments, respectively. The sequence data confirm the monomer–dimer relationship between the 88,000 and 176,000 fragments (Table I). The next event is the parallel hydrolysis of the 88,000 fragment at sites 2 and 3 to form the 80,000 and 72,000 fragments, respectively. N-terminal sequence analysis of the 72,000 fragment shows that cleavage has occurred at the Hyp_{252}–Gly_{253} bond. The 144,000 fragment has the same N-terminal sequence (Table I), confirming that it is a dimer of the 72,000 fragment. Some of the 72,000 fragment also arises from hydrolysis of the 80,000 fragment at site 3. Next, the 67,000 fragment is formed by hydrolysis of the 80,000 and 72,000 fragments at site 4. Finally, the 52,000 fragment is formed by hydrolysis of the 67,000 fragment at site 5. N-terminal sequence analysis of the 52,000 fragment shows that it is actually a mixture of two fragments whose N-termini differ by three amino acids due to simultaneous cleavage at both the Gln_{468}–Gly_{469} and Hyp_{471}–Gly_{472} bonds at site 5. A comparison of the pathways of hydrolysis of type II and III collagens (Fig. 5) shows that they are similar except for the faster rate of formation of the 74,000 fragment in type II collagen.

The results for the digestion of type I collagen by the class I CHC reported earlier (French *et al.*, 1987) are intrinsically more difficult to decipher because the molecule is a heterotrimer composed of two α1(I) chains and one α2(I) chain. Thus, there are twice as many bands to consider, making the SDS gels more difficult to analyze. This problem has also prevented any successful N-terminal sequence analysis of the type I collagen fragments. However, the early events have been deciphered. The initial proteolytic event is hydrolysis at site 2 to produce a fragment with a molecular weight of 88,000. The evidence that site 2 is at the C-terminus of the collagen molecule is derived from the observation of a dimer fragment from the β_{11} and β_{12} chains that has a molecular weight of 175,000. The next event is the hydrolysis of the 88,000 fragment at site 1 to produce the 80,000 fragment. Hydrolysis must occur at the N-terminus to remove the cross-link present in the N-terminal telopeptide

Table II. Location of Class II CHC Hyperreactive Cleavage Sites in Type I, II, and III Collagens[a]

Collagen chain	Molecular weight	Sequence	Reference
		↓	
α1(I)	62,000	Gly -Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp-Gly-Pro-Lys	
α1(I)	95,000	Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Ala-Arg$_{396}$ Gly$_{397}$-Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp-Gly-Pro-Lys	—[b,c]
α2(I)	62,000	Gly -Glu-Ala-Gly-Ala-Ile	
α2(I)	95,000	Gly-Arg-Hyp-Gly-Pro-Ile-Gly-Pro-Ala-Gly-Pro-Arg$_{396}$ Gly$_{397}$-Glu-Ala-Gly-Ala-Ile-Gly-Phe-Hyp-Gly-Pro-Lys	—[b,c]
α1(I)	58,000	Gly -Pro-Lys-Gly-**Thr**-Ala-Gly-Glu-Hyp-Gly	
α1(I)	95,000	Gly-Ala-Arg-Gly-Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp$_{405}$ Gly$_{406}$-Pro-Lys-Gly-Ala-Ala-Gly-Glu-Hyp-Gly-Lys-Ala	—[b,c]
α2(I)	58,000	Gly -Pro-**Ala**-Gly-Pro-**Thr**-Gly-Glu	
α2(I)	95,000	Gly-Pro-Arg-Gly-Glu-Ala-Gly-Ala-Ile-Gly-Phe-Hyp$_{405}$ Gly$_{406}$-Pro-Lys-Gly-Pro-Thr-Gly-Glu-Hyp-Gly-Lys-Hyp	—[b,c]
α1(II)	62,000	Gly -Pro-**Hyp**-Gly-Ala-Asn-Gly-Glu-Hyp-Gly-Lys-Ala	
α1(II)	95,000	Gly-Ala-Arg-Gly-Gln-Hyp-Gly-Val-Met-Gly-Phe-Hyp$_{405}$ Gly$_{406}$-Pro-Hyl-Gly Ala-Asn-Gly-Glu-Hyp-Gly-Lys-Ala	—[b]
α1(III)	59,000	Gly -Gln-Hyp-Gly-Val-Met-Gly-Phe-Hyp	
α1(III)	95,000	Gly-Arg-Hyp-Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Pro-Arg$_{399}$ Gly$_{400}$-Gln-Hyp-Gly-Val-Met-Gly-Phe-Hyp-Gly-Pro-Lys	—[d]
α1(III)	56,000	Gly -Asn-**Lys**-Gly-Ala-Hyp-Gly-Lys-Asn-Gly	
α1(III)	95,000	Gly-Gln-Hyp-Gly-Val-Met-Gly-Phe-Hyp-Gly-Pro-Lys$_{411}$ Gly$_{412}$-Asn-Asp-Gly-Ala-Hyp-Gly-Lys-Asn-Gly-Glu-Arg	—[d]
		Glu Arg	
α1(III)	44,000	Gly -Leu-Gln-Gly-Met-Hyp-Gly-Leu-Hyp	
α1(III)	95,000	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Ala-Ala-Gly-Thr-Hyp$_{549}$ Gly$_{550}$-Leu-Gln-Gly-Met-Hyp-Gly-Glu-Arg-Gly-Gly-Lys	—[d]

[a] For each entry, the experimentally determined sequence is shown on top (in some cases, more than one amino acid was detected, with the minor one shown above). The most consistent cleavage site from the most closely related collagen chain of known sequence is shown on bottom.

[b] Fasman (1977).

[c] Chapman *et al.* (1981).

[d] Seyer and Kang (1981).

because no accompanying dimer band with a molecular weight of 160,000 is observed. This site appears to be conserved in type II and III collagens (Fig. 5).

After these first two cleavages, many new fragments with molecular weights in the 30,000–75,000 range are produced (see Fig. 1 in French *et al.*, 1987). Reexamination of our earlier data shows that five sets of fragments are formed whose equal spacing suggests that they arise from α1(I) and α2(I) chains, respectively. Based on this assumption, they are predicted to have molecular weights of 69,000, 66,000, 51,000, 44,000, and 41,000. No direct evidence for the locations of the hyperreactive sites that produce these fragments can be given. However, the conservation of hyperreactive sites 3–6 in type II collagen and of sites 2–5 in type III collagen suggests that these sites may be present in type I collagen as well. In type II and III collagens, hydrolysis at these sites produces fragments with molecular weights of approximately 81,000, 73,000, 67,000, and 51,000, respectively. If cleavage of the 80,000 type I collagen fragment occurred at these same sites, fragments with molecular weights of 74,000, 66,000, 60,000, and 44,000 should be formed. Since fragments with molecular weights of 66,000 and 44,000 are found in the digest, this suggests that at least two of these sites are present in type I collagen.

Next, attention was turned to the action of the class II CHC on these collagens. The gel patterns that are observed on reaction of type II collagen with ε-CHC at 20°C are shown in Fig. 6. This reaction produces fragments whose mobilities correspond to molecular weights of 62,000, 58,000, 35,000, 33,000, and 28,000. The variation in the concentrations of these fragments and the α1(II) chain as a function of reaction time is shown in Fig. 7. The appearance of the 62,000 and 35,000 fragments correlates with the decay of the α1(II) chain. The sum of the fragment molecular weights of 97,000 is in good agreement with α1(II) chain molecular weight of 95,000, indicating that both arise from a single cleavage. Next, the 62,000 fragment decays to form the 58,000 fragment, a process that cannot be shown on the plot in Fig. 7, since these bands are not well resolved on the gels; only the sum of their intensities has been plotted. Since the 58,000 fragment does not form until most of the α1(II) chains have been degraded, it must arise by degradation of the 62,000 fragment. The 35,000 fragment decays at a rate that matches the appearance of the 33,000 fragment over a time interval (60–90 min) when no other fragments change concentration appreciably, indicating that the latter is derived from the former. Likewise, the 28,000 fragment arises via proteolysis of the 33,000 fragment. Similar results are obtained on reaction of type II collagen with ζ-CHC.

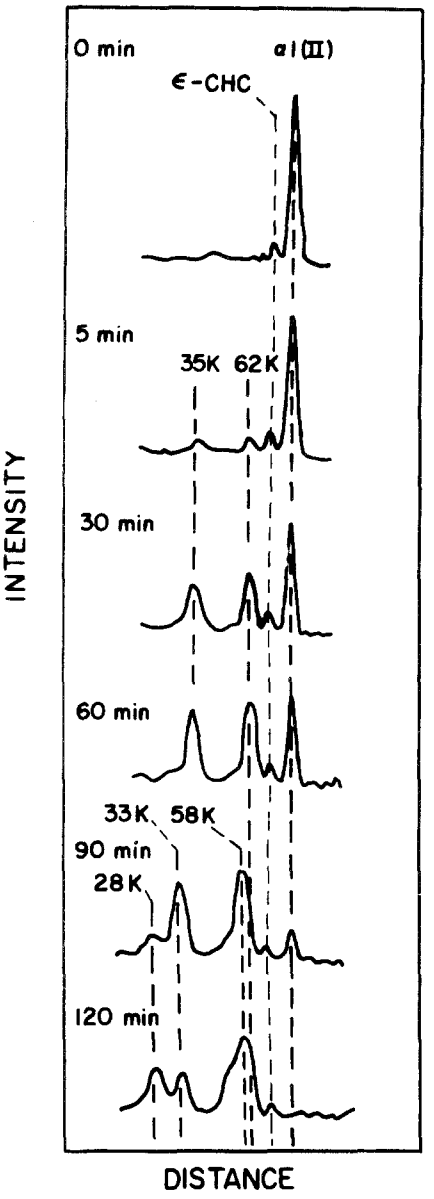

Fig. 6. Densitometer tracings of a gel showing the reaction of bovine nasal septum type II collagen with ε-CHC at 20°C. The concentrations of ε-CHC and collagen were 40 nM and 2 mg/ml, respectively.

The products of reaction of type III collagen with ζ-CHC at 15°C are shown in Fig. 8. Over the 210 min reaction, fragments with molecular weights of 117,000, 112,000, 59,000, 56,000, 37,000, 34,000, and 31,000 are formed. On extended incubation (4–5 hr), a 44,000 fragment is also formed (not shown). The variation in the concentrations of all (except the

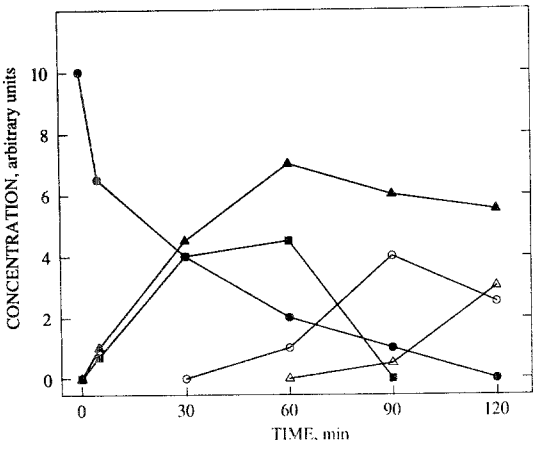

Fig. 7. Variation in the concentration of the (●) α1(II) chain and (▲) 62,000 + 58,000, (■) 35,000, (○) 33,000, and (△) 28,000 fragments as a function of time during the reaction of bovine nasal septum type II collagen with ε-CHC at 20°C, as shown in Fig. 6.

44,000) fragments with time is plotted in Fig. 9. The decay of the β_{11} chains correlates with the appearance of the 117,000 fragment (Fig. 9A), while the decay of the α1(III) chains correlates with the appearance of the 59,000 and 37,000 fragment (Fig. 9B). The sum of these fragments molecular weights is 96,000, indicating that these two bands are products of a single cleavage of the α1(III) chain. The 117,000 fragment has approximately twice the molecular weight of the 59,000, band, suggesting that it is a dimer of the 59,000 fragment. This conclusion is supported by the observation that the 59,000 and 117,000 fragments each reach their maximal concentration at the same time. Between 30 and 90 min, the 117,000, 59,000, and 37,000 fragments disappear and are replaced by new products with molecular weights of 112,000, 56,000, and 34,000. The 5000 drop in the molecular weight of the 117,000 fragment and the 3000 drop in the molecular weight of the 59,000 fragment are consistent with the assumption that the 117,000 and 112,000 fragments are dimers of the 59,000 and 56,000 fragments, respectively. The 34,000 fragment appears with no significant reduction in the concentration of the 59,000 or 56,000 fragments. Therefore, it must be derived from the 37,000 fragment. By 210 min, a 31,000 fragment is clearly evident that appears to be derived from the 34,000 fragment. At longer times, the 56,000 fragment is degraded to a 44,000 fragment (not shown). The same reaction pathway is observed for ε-CHC.

The results of N-terminal sequence analyses of fragments isolated from digestion of type I, II, and

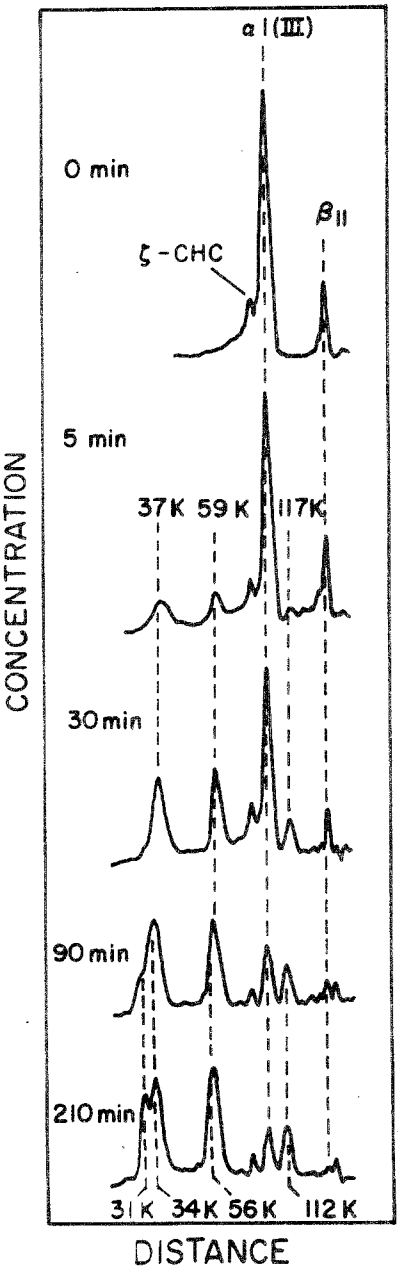

Fig. 8. Densitometer tracings of a gel showing the reaction of human placental type III collagen with ζ-CHC at 15°C. The concentrations of ζ-CHC and collagen were 79 nM and 1 mg/ml, respectively.

III collagens by the class II CHC are shown in Table II. By considering the data described above for type II and III collagens, and that obtained earlier for type I collagen (French *et al.*, 1987), a pathway of hydrolysis can be constructed for each collagen type by the class II CHC (Fig. 10). The initial event in the hydrolysis of type II collagen is hydrolysis of all three α1(II) chains at the Hyp_{405}–Gly_{406} bond at site 1 to

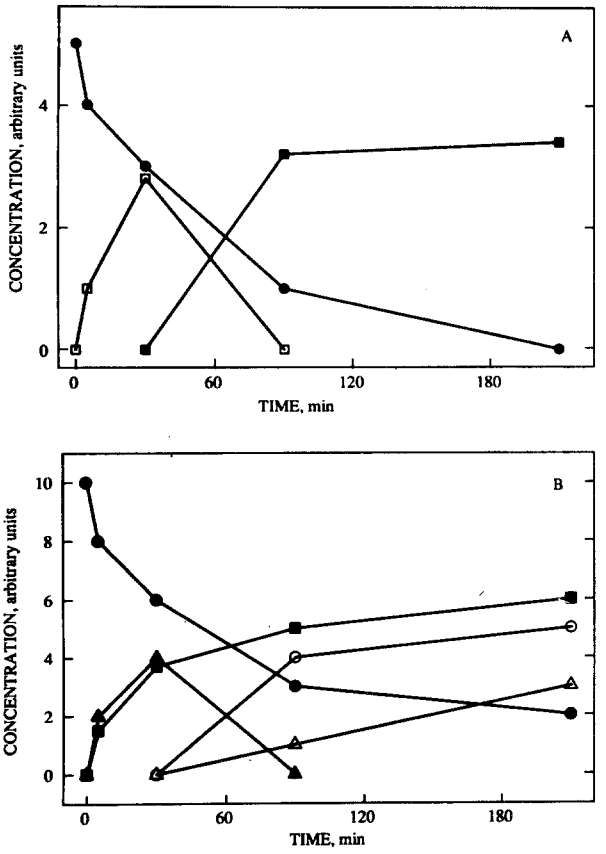

Fig. 9. Variation in the concentration of the (A) (●) β_{11} chains and (□) 117,000 and (■) 112,000 fragments; and (B) (●) α1(III) chains and (■) 56,000+59,000, (▲) 37,000, (○) 34,000, and (△) 31,000 fragments as a functionof time during the reaction of human placental type III collagen with ζ-CHC at 15°C, as shown in Fig. 8.

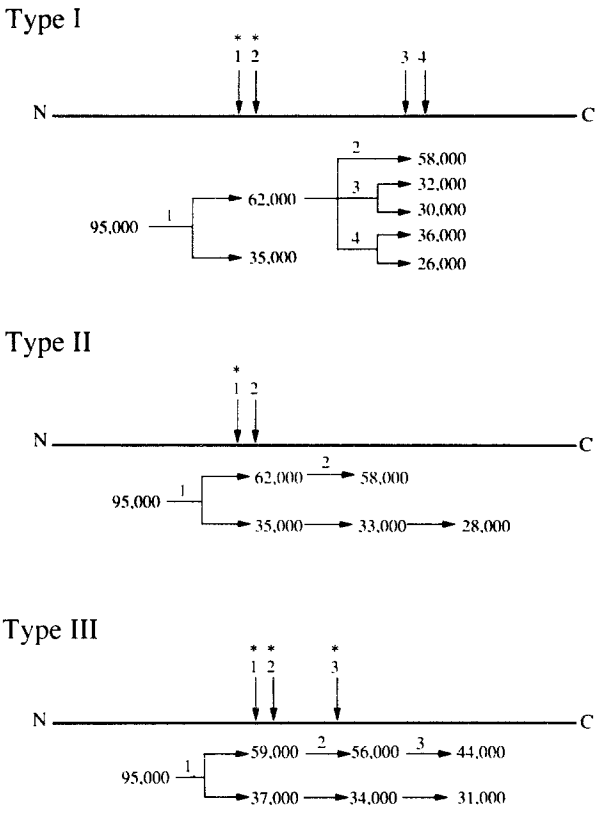

Fig. 10. Schematic showing the sites of hydrolysis (vertical arrows) of type I, II, and III collagens (solid horizontal lines) by the class II CHC, and a degradation scheme for each. Cleavage sites identified by N-terminal sequencing are indicated by an asterisk.

produce an N-terminal 35,000 fragment and a C-terminal 62,000 fragment. Next, the 62,000 and 35,000 fragments are hydrolyzed to 58,000 and 33,000 fragments, respectively, over approximately the same time scale. The 4000 peptide is probably removed from the N-terminus of the 62,000 fragment, as for type I and III collagens (see below). However, there is no information to indicate which end of the 35,000 peptide is shortened. Finally, the 33,000 fragment is converted to a 28,000 fragment by hydrolysis at an unknown site.

The first proteolytic event in the hydrolysis of type III collagen by the class II CHC is cleavage of all three α1(III) chains at the Arg_{399}–Gly_{400} bond at site 1 to produce the 37,000 and 59,000 fragments. Next, these fragments are simultaneously hydrolyzed to 56,000 and 34,000 fragments, respectively. The 56,000 fragment is produced by a cleavage of the Lys_{411}–Gly_{412} bond at site 2, but the site of hydrolysis of the 34,000 fragment is not known. The 34,000 fragment undergoes further hydrolysis at another unknown site to give a 31,000 fragment. On prolonged digestion, a fragment having a molecular weight of 44,000 has been observed due to hydrolysis of the Hyp_{549}–Gly_{550} bond at site 3.

In our earlier study, it was shown that the class II CHC initially convert type I collagen into a mixture of N-terminal 35,000 and C-terminal 62,000 fragments (French *et al.*, 1987). The sequence data in Table II now establish that this is due to hydrolysis of the Arg_{396}–Gly_{397} bonds at site 1 in both the α1(I) and α2(I) chains. A 70,000 fragment is also observed that is a dimer of the 35,000 fragment that arises from a cross-link in the N-terminal telopeptide. Next, the 62,000 fragment decays by what appears to be simultaneous cleavage at three sites. A major portion is converted to a 58,000 fragment by hydrolysis at the Hyp_{405}–Gly_{406} bonds at site 2 in both the α1(I) and α2(I) chains. However, over this same time interval, three prominent sets of bands are observed whose

equal spacing suggests that they are derived from parallel cleavages of $\alpha 1$(I) and $\alpha 2$(I) chains (see Fig. 2 of French *et al.*, 1987). Based on this assumption, these three sets of fragments have molecular weights of 36,000, 32,000, and 26,000, respectively. This suggests that the 62,000 fragment is also hydrolyzed at site 3 to give 32,000 and 30,000 fragments, and at site 4 to give 36,000 and 26,000 fragments.

Similar limited proteolysis experiments have been carried out with type II and III gelatins in order to compare the fragment patterns formed with those of the native collagens discussed above. Since both the class I and II CHC are more potent gelatinases than collagenases, it was necessary to lower the enzyme concentration by at least 10-fold in order to observe high molecular weight fragments. For the reaction of both collagens with both classes of CHC, the digestion patterns were very complex under all conditions studied. The SDS gels clearly showed the simultaneous formation of a very large number of light bands, many of almost equal intensity. Because of the complexity of these mixtures, it was not possible to analyze the pathway of hydrolysis of the gelatins or to determine whether any of their hydrolysis fragments were the same as those observed for the native collagens.

4. DISCUSSION

As found earlier for rat type I collagen (French *et al.*, 1987), the results presented here demonstrate that bovine type II and human type III collagens are initially hydrolyzed at a limited number of hyperreactive sites by the CHC. Many of the sites in these three collagens appear to be conserved, although incomplete sequence data prevents us from establishing the degree of identity definitively. The class I CHC initiate attack on all three collagens at several sites concentrated in the N-terminal half of these molecules. With the exception of type I collagen, whose most hyperreactive site is located close to the C-terminus, the first cleavages are close to the N-terminus. In contrast, the class II CHC initiate their attack on all three collagens at a site well in the interior of these collagens, but still on the N-terminal half. This is followed by additional cleavages near the ends of the fragments, none of which duplicate those of the class I CHC. While one or more hyperreactive sites may be absent in some collagens, the pathways of hydrolysis of the three collagens by a given class of CHC differs primarily in the rate at which hydrolysis at the different hyperreactive sites occurs. Thus, it appears that the CHC recognize some feature characteristic of these sites, while the rate of cleavage depends upon the specific amino acid sequence around the scissile bond. It should be noted that some of the fragment N-termini were heterogeneous, making it impossible to identify a single sequence. Site 5 for the hydrolysis of type III collagen by the class I CHC is an example where two distinct N-termini could be identified. This indicates that the hyperreactivity of these sites may be the result of a conformational feature that covers several triplets, leading in some cases to hydrolysis of more than one bond.

In contrast to the finding that native triple helical type I, II, and III collagens are hydrolyzed at distinct hyperreactive sites by the CHC, experiments carried out on the corresponding gelatins show that many sites are hydrolyzed at similar rates. Thus, the reactivity of potential cleavage sites in the various α chains is modulated by their environments in these native collagens. Since the CHC have very broad specificities and can digest these collagens down to a mixture of small peptides, these collagenases are acting as "probes" of collagen structural microheterogeneity. Accepting the view that the hyperreactivity of the CHC cleavage sites is the consequence of some local conformational feature, the question becomes one of identifying this feature. In this regard, our ability to pinpoint many of these sites by N-terminal sequencing is quite important. While a detailed multiparameter analysis of the different features of these sites will not be presented here, one important factor that could modulate the reactivity of sections of these collagen chains will be considered. This is the local imino acid content of these sites.

It has been pointed out that the single tissue collagenase cleavage site in these collagens is located in a region that is locally deficient in imino acids (Gross *et al.*, 1980). It is known that the triple helical stability of $(Gly\text{-}X\text{-}Y)_n$ polymers are enhanced when X = Pro and Y = Hyp (Piez, 1984). Since the prototypic collagen triple helix is a tightly coiled structure, it is reasonable to conclude that regions deficient in triplets with Pro and Hyp in the X and Y positions, respectively, would be less tightly wound with the result that they would be more mobile and more easily recognized by specific collagenases. While this is probably not the only distinctive feature of the tissue collagenase cleavage site, it is probably one of the most important.

With this in mind, it is of interest to consider whether the CHC hyperreactive cleavage sites also occur in regions of low triple helical stability. Tables I and II show the sequences surrounding the 14 distinct

Table III. Average Imino Acid Content of Triplets Surrounding Hyperreactive CHC Cleavage Sites in Type I, II, and III Collagens

Collagen type	Site	Average imino acid content over n triplets $N=$ 2	4	6
		Class I CHC		
II	1	17	25	28
	2	17	25	22
	3	17	25	22
	5	17	17	22
	6	67	42	33
III	1	17	33	28
	3	17	8.3	17
	5	17	21	28
		Class II CHC		
I	1	17	22	22
	2	22	14	15
II	1	33	17	22
III	1	33	25	33
	2	0	17	11
	3	17	17	22

hyperreactive sites identified in these collagens. Since two of these sites are in type I collagen, which consists of two different types of α chains, there are actually 16 different sequences to consider. The average percentage of imino acids in the two, four, and six triplets centered around each cleavage site has been calculated (Table III). All of the Pro and Hyp residues in these sites are found at the X and Y positions, respectively, except for Pro_{27} at class I CHC site 1 for type II collagen (Table I). Thus, average imino acid content should reflect triple helical stability. For type I collagen, the weighted average of the values for two $\alpha 1$(I) and one $\alpha 2$(I) chains is given, while, for class I CHC site 5 in type III collagen, the average values obtained for the two scissile bonds is reported. These numbers should be viewed in the light of the average imino acid contents of 22, 22 and 23% for type I, II, and III collagens, respectively (Miller and Gay, 1982).

The results for the two classes of CHC are slightly different and will be discussed separately. The first two triplets of seven of the eight class I CHC cleavage sites are slightly deficient in imino acids compared to the average for these collagen chains (17 vs. 22–23%). This deficiency disappears in five of these seven if the calculation is extended to cover the adjoining four or six triplets. The eighth cleavage site (site 6 for type II collagen) is highly unusual in that it corresponds to a very stable triple helical segment. In fact, hydrolysis at this site occurs between two Gly–Pro–Hyp triplets. Thus, most of the class I CHC hyperreactive sites lie in regions where there is a slight deficiency of imino acids over the adjoining two triplets, but not over the adjoining four or six triplets. Also, there are many other regions more deficient than these which contain the potential Gln–Gly, Ala–Gly, and Arg–Gly scissile bonds (Table I) that are not cleaved. The class II CHC hyperreactive cleavage sites do not show any trend with local imino acid content. If the calculation is limited to the adjoining two triplets, three of these sites are deficient in imino acids, while one is average and three are enriched. Extending the calculation to include more adjoining triplets provides no further insight into this question. Thus, no clear correlation between the location of class II CHC cleavage sites and imino acid content can be established. This suggests that the hyperreactivity of these sites is not simply the result of local disorder, but rather that they can adopt specific nontriple helical conformations. The determination of the sequences of these sites presented here now makes it possible to design computational or peptide mimetic approaches capable of studying the structures of these sites.

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