Cleavage of Type II and III Collagens with Mammalian Collagenase: Site of Cleavage and Primary Structure at the NH_2-Terminal Portion of the Smaller Fragment Released from Both Collagens†

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ABSTRACT: Collagenase cleavage of human Type II and III collagens has been studied using a highly purified preparation of rabbit tumor collagenase. Progress of the reactions in solution was followed by viscometry and the results indicated that under the conditions employed Type III collagen molecules were cleaved at approximately five times the rate of Type II molecules. Cleavage products of the reactions were isolated in denatured form by agarose molecular sieve chromatography. The molecular weights and amino acid compositions of the products demonstrated that Type II and III molecules had been cleaved at the characteristic three-quarter, one-quarter locus, giving rise to a large fragment derived from the NH_2-terminal portion of the molecule and a smaller fragment representing the COOH-terminal region. The amino acid sequence at the NH_2-terminal portion of the smaller fragment derived from Type II collagen was determined to be Ile-Ala-Gly-Gln-Arg, and the corresponding region from Type III collagen was found to have the sequence Leu-Ala-Gly-Leu-Arg. These sequences for α1(II) and α1(III) chains adjacent to the site of collagenase cleavage along with previous data for α1(I) and α2 chains indicate that the minimum specific sequence required for collagenase cleavage is Gly-Ile-Ala or Gly-Leu-Ala. Inspection of the available sequence data for collagen α chains indicates that the latter sequences are found in at least three additional locations at which collagenase cleavage does not occur. Each of the sequences which are apparently not substrates for collagenase, however, are followed by a Gly-X-Hyp sequence. We suggest, then, that a minimum of five residues in collagen α chains COOH-terminal to the cleavage site comprise the substrate recognition site.

The activity of collagenase is recognized as an indispensable initial step in the resorption of collagen during the processes of connective tissue remodelling and turnover in vertebrate organisms. Most animal collagenases exhibit restricted activity with respect to substrate and cleave all three chains of the native collagen molecule at a specific site located approximately one-quarter of the length of the molecule from the COOH-terminus (Harris and Krane, 1974; Gross, 1974). Early studies on the mode of action of collagenase using purified preparations of the tadpole enzyme suggested that fragmentation of the native Type I collagen molecule (chain composition, $\{\alpha1(I)\}_2\alpha2$) was achieved by cleavage at a Gly–Ile bond in the α1(I) chains and at a Gly–Leu bond in the α2 chain (Nagai et al., 1964). These results were confirmed and extended in studies with highly purified preparations of tadpole collagenase as well as a collagenase isolated from rabbit V_2 ascites cell carcinoma growing in muscle (Gross et al., 1974). Using denatured chick α1(I) and α2 chains as well as selected cyanogen bromide peptides from α1(I) as substrates, the latter workers showed that both the amphibian and mammalian enzymes specifically cleaved α1(I) at a Gly–Ile bond corresponding to residues 772–773 in the repetitive triplet sequence of the chain.[1] The data further indicated that α2 was specifically cleaved at a Gly–Leu bond in the homologous region of the latter chain.

The Gly–Ile bond at residues 772–773 is not unique in α1(I) chains. It occurs in different species in at least two other locations, residues 226–227 in rat skin α1(I) (Balian et al., 1972) and residues 925–926 in calf skin α1(I) (Wendt et al., 1972). Thus, in view of the high degree of structural homology known to occur for the α1(I) chains of various species, the results of Gross et al. (1974) using highly purified collagenases to cleave nonhelical chick α1(I) strongly suggest that collagenase specificity is largely independent of substrate conformation and reflects a unique affinity on the part of the enzyme for amino acid sequences surrounding the cleavage site.

The studies presented here have been designed to further evaluate substrate structural features associated with susceptibility to cleavage by mammalian collagenase. For this purpose we have: (1) cleaved native Type II (chain composition, $\{\alpha1(II)\}_3$) and Type III (chain composition, $\{\alpha1(III)\}_3$) collagen molecules in solution with highly purified preparations of rabbit V_2 ascites cell tumor collagenase; (2) isolated and characterized the reaction products following complete cleavage of the substrate molecules; and (3) determined the primary structure of α1(II) and α1(III)

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[1] Residue numbers in the α1(I) chain refer to data on the complete amino acid sequence of α1(I) derived from studies on rat and calf skin α1(I) and compiled by Hulmes et al., 1973.

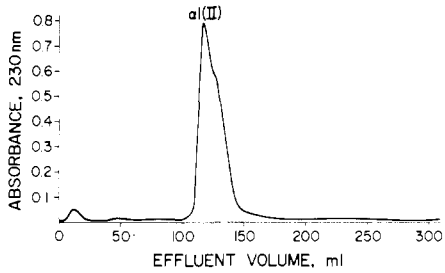

FIGURE 1: Carboxymethyl-cellulose elution pattern of 50 mg of denatured Type II collagen derived from articular cartilage. Chromatography was performed as described in the text.

chains immediately adjacent (COOH-terminal) to the site of collagenase cleavage. The results confirm earlier studies indicating that collagenase cleaves native Type II collagen molecules at the characteristic three-quarter, one-quarter locus (Woolley et al., 1973, 1975) and demonstrate that Type III molecules are likewise cleaved at this site. The data further indicate that the primary structure of $\alpha 1$(II) and $\alpha 1$(III) chains immediately adjacent to the site of collagenase cleavage are remarkably similar to known sequences in homologous regions of $\alpha 1$(I) and $\alpha 2$ chains.

Materials and Methods

Preparation of Collagens. Human Type II collagen was prepared from slices of infant articular cartilage dissected from the surface of several major joints. The fresh tissue slices were initially extracted at room temperature for 2 days in several volumes of 4.0 M guanidine hydrochloride (pH 7.5, 0.05 M Tris) in order to remove proteoglycan molecules (Sajdera and Hascall, 1969). Following extraction with the guanidine solution, the tissues were thoroughly rinsed with distilled water and equilibrated with 0.5 M acetic acid. The cartilage collagen was then solubilized by incubation in 0.5 M acetic acid containing pepsin at 4 °C for 24 h as previously described (Miller, 1972). Collagen was precipitated from the incubation solution by the addition of crystalline NaCl to a concentration of 0.9 M. The precipitate was redissolved in 1.0 M NaCl (pH 7.5, 0.05 M Tris) and reprecipitated by the addition of crystalline NaCl to a final concentration of 3.5 M. This precipitate was redissolved in 0.5 M acetic acid and the collagen was again precipitated at 0.9 M NaCl. The latter precipitate was redissolved in 0.5 M acetic acid, dialyzed against several changes of the solvent, and lyophilized.

Human Type III collagen was prepared by selective precipitation at 1.5 M NaCl from neutral salt solutions of pepsin-solubilized infant dermal collagen as described previously (Chung and Miller, 1974). The collagen thus obtained was twice redissolved in 1.0 M NaCl (pH 7.5, 0.05 M Tris) and reprecipitated each time by the addition of sufficient crystalline NaCl to achieve a final concentration of 1.5 M. The final precipitate was redissolved in 0.5 M acetic acid, dialyzed against the solvent, and lyophilized.

Carboxymethyl-(CM-)cellulose Chromatography. Aliquots of the collagens prepared as described above were dissolved in 0.04 M (Na^+) sodium acetate (pH 4.8), containing 1.0 M urea, and chromatographed after denaturation on a 1.8 × 10 cm column of CM-cellulose as previously described (Miller, 1971). Elution was achieved by means of a linear gradient from 0 to 0.1 M NaCl over a total volume of 500 ml.

Amino Acid Analyses. Amino acid analyses were performed as described previously (Miller, 1972) on an automatic amino acid analyzer (Beckman, Model 119).

Preparation of Collagenase. Collagenase free of nonspecific proteolytic activity was purified from homogenates of tumors resulting from implantation of V_2 ascites cell carcinoma in muscle of rabbits (Harris et al., 1972; McCroskery et al., 1973, 1975). Purification steps, outlined previously in detail (McCroskery et al., 1975), included precipitation of enzyme activity at 20–50% saturation with ammonium sulfate, elution from a column of diethylaminoethyl-Sephadex A-50, and passage through a column of Bio-Gel A-1.5m. These steps achieved a purification of 55 000-fold and resulted in a 10-fold increase in recovery of enzyme activity due to the elimination of natural inhibitors of collagenase found in the tissue homogenates (McCroskery et al., 1975).

Incubation of Substrates with Collagenase. All operations preparatory to incubation with collagenase were performed at 4 °C. The collagens were dissolved in 0.5 M acetic acid at a concentration of 3 mg/ml and dialyzed extensively against 0.15 M NaCl (pH 7.4, 0.01 M Tris). Following dialysis, an equal volume of 0.2 M NaCl containing 6 mM sodium azide and collagenase (1:500, w/w, with respect to collagen) was added to each collagen solution. Flasks containing the reaction mixtures were incubated in a large water bath at 27 °C. The progress of each reaction was followed by viscometry (see below) and disc gel electrophoresis (McCroskery et al., 1975) on aliquots of the reaction mixtures taken periodically throughout the incubations.

On completion of the reactions, the incubation mixtures were immediately freeze-dried and stored at −20 °C.

Viscometry. The progress of the reactions as well as the relative rates of cleavage for Type II and III collagens were determined viscometrically. For this purpose, 1.0-ml aliquots of the reaction mixtures described above were taken at various times during the incubations and placed in semimicro viscometers (Cannon Instruments) with a flow time for water at 27 °C of approximately 1 min The results were calculated as percent of original specific viscosity, and initial velocity of each reaction was calculated by extrapolation to zero time.

Isolation of Cleavage Products. The lyophilized reaction mixtures were redissolved in approximately 2 ml of 2.0 M guanidine hydrochloride (pH 7.5, 0.05 M Tris) warmed to 45 °C for 30 min to ensure denaturation of the collagen cleavage products, and chromatographed at a flow rate of 12.8 ml/h on a calibrated 1.5 × 155 cm column of agarose beads (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories). The column was equilibrated and eluted with the same solvent. Portions of the column effluent containing collagenase cleavage products were desalted and lyophilized. The cleavage products derived from Type III collagen preparations were redissolved in 5.0 M urea containing 0.1 M 2-mercaptoethanol, pH 8.0 (Chung and Miller, 1974). Following a 4-h interval for reduction, the reaction mixture was applied directly to and eluted from the same agarose column.

Amino Acid Sequences. Amino acid sequences were determined by automated Edman degradations (Edman and Begg, 1967) performed in a Beckman Sequencer (Model 890 C) and employing the Slow Protein-Quadrol (042772) Program supplied by Beckman Instruments. The phenylthiohydantoin (Pth)-amino acids were identified by gas chromatography on a Hewlett-Packard High Efficiency Gas Chromatograph (Model 7610 A) according to the pro-

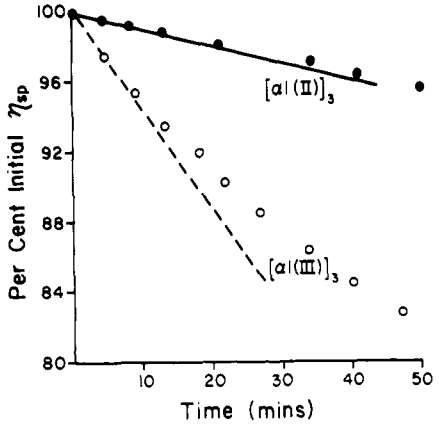

FIGURE 2: Comparison of the fall in viscosity of Type II and III collagens in solution on incubation with purified rabbit tumor collagenase. Incubation was performed at 27 °C. The contents of each reaction mixture is described in the text.

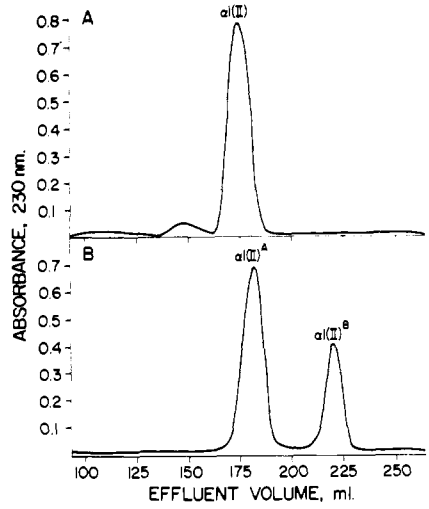

FIGURE 3: Bio-Gel A-5m elution pattern of: (A) denatured Type II collagen; and (B) the denatured cleavage products following exposure of Type II collagen to rabbit tumor collagenase. Chromatography was performed as described in the text.

cedure of Pisano et al. (1972). When necessary, the Pth derivatives were chromatographed following trimethylsilylation according to the method outlined in the Beckman Sequencer Manual. Identities of the Pth-amino acids were further investigated by thin-layer chromatography on plastic plates coated with silica gel (Inagami and Murakami, 1972). Pth-arginine, remaining in the aqueous layer of various degradation cycles, was identified by the phenanthroquinone spot test (Yamada and Itano, 1966).

Results

Collagen Preparations. In order to unequivocally establish the identity of the collagens used in these studies, an aliquot of each preparation was denatured and chromatographed on CM-cellulose. As illustrated in Figure 1, the Type II collagen preparation chromatographed as a single somewhat heterogeneous peak with no detectable protein appearing in the effluent where $\alpha 2$ elutes (at approximately 250 ml). Thus, the CM-cellulose elution pattern for pepsin-solubilized human cartilage collagen closely resembles the CM-cellulose elution pattern previously observed for chick (Miller, 1971, 1972) and bovine (Strawich and Nimni, 1971) Type II collagen. In addition, amino acid analyses of the protein eluted from CM-cellulose demonstrated that it possessed the characteristic compositional features of a collagen $\alpha 1$(II) chain, i.e., a relatively high content of glutamic acid, leucine, and hydroxylysine, as well as a relatively low content of alanine. Indeed, the amino acid composition of the human $\alpha 1$(II) chain isolated in the present study was virtually identical with that previously calculated as the sum of amino acid residues in $\alpha 1$(II) cyanogen bromide peptides derived from slices of human articular cartilage (Miller and Lunde, 1973).

The Type III collagen preparation was examined in a similar fashion and found to exhibit the chromatographic properties and compositional features recently described as characteristic for Type III collagen and its component $\alpha 1$(III) chains in human tissues (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974; Trelstad, 1974).

Collagenase Cleavage of Type II and III Collagens. At 27 °C, with equal amounts of each substrate per unit of enzyme, it was observed that the rate of cleavage of Type II collagen was approximately one-fifth that of Type III collagen (Figure 2). Continued incubation resulted in a *final* specific viscosity of 47% of the original for each collagen which was attained at 42 h for Type II collagen and at 9 h for Type III collagen (data not shown). At these times, it was assumed that complete cleavage of the respective collagens had been attained and this was confirmed by polyacrylamide disc gel electrophoresis on aliquots of the reaction mixtures.

Characterization of Collagenase Cleavage Products. Figure 3A illustrates the agarose molecular sieve elution pattern of approximately 20 mg of Type II collagen which was dissolved in 2.0 M guanidine hydrochloride and chromatographed without prior exposure to collagenase. Virtually all of the protein was recovered as $\alpha 1$(II) chains eluting at approximately 175 ml, corresponding to a molecular weight of 95 000 daltons. In contrast, Figure 3B shows the agarose elution pattern of an approximately equal aliquot of Type II collagen following incubation with rabbit tumor collagenase. The protein is recovered as two components, one eluting at approximately 180 ml (molecular weight, 71 000 daltons) and the second eluting at approximately 220 ml (molecular weight, 23 000 daltons). Since these components represent the expected denaturation products of segments obtained by cleavage of the native molecule at the characteristic three-quarter, one-quarter locus, they have been designated $\alpha 1(II)^A$ and $\alpha 1(II)^B$ following the nomenclature introduced by Kang et al. (1966).

The derivation of $\alpha 1(II)^B$ from the COOH-terminal quarter of the Type II collagen molecule was independently established by amino acid analyses which showed that $\alpha 1(II)^B$ contained two residues of 3-hydroxyproline. The human $\alpha 1$(II) chain contains only two of these amino acid residues, both of which occur in the sequence represented by the cyanogen bromide peptide, $\alpha 1$(II)-CB9,7 (Miller and Lunde, 1973) and the latter sequence is located in the COOH-terminal portion of $\alpha 1$(II) (Miller et al., 1973). Further, amino acid analyses of $\alpha 1(II)^A$ revealed that it contained no detectable 3-hydroxyproline confirming that under the conditions of incubation employed in this study collagenase cleavage of the Type II molecule was essentially complete.

The collagenase cleavage products of the Type III collagen molecule were recovered and examined in much the same fashion. As indicated in Figure 4A, a 20-mg aliquot of

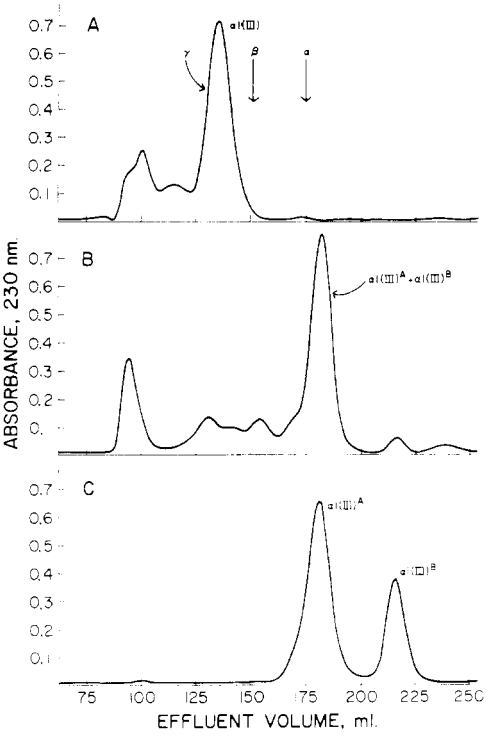

FIGURE 4: Bio-Gel A-5m elution pattern of: (A) denatured Type III collagen; (B) the denatured cleavage products following exposure of Type III collagen to rabbit tumor collagenase; and (C) the denatured cleavage products following reduction with 2-mercaptoethanol. Chromatography was performed as described in the text.

Type III collagen chromatographed on agarose largely as γ components (molecular weight, 300 000 daltons) accompanied by a moderate amount of higher molecular weight material as previously described for similar preparations of this collagen (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974). Following incubation with the rabbit tumor collagenase, however, there was a pronounced shift in molecular weight distribution and virtually all the protein was recovered as material eluting in a position indicating a molecular weight of approximately 70 000 daltons (Figure 4B). The apparent absence of a lower molecular weight collagenase cleavage product, $\alpha 1(III)^B$, in the latter chromatogram was attributed to interchain disulfide bonding through the two cysteinyl residues which are known to occur in the COOH-terminal portion of the $\alpha 1(III)$ chain (Chung et al., 1974). Accordingly, the protein eluted at 180 ml (Figure 4B) was reduced with 2-mercaptoethanol and rechromatographed on agarose. As shown in Figure 4C, these procedures resulted in conversion of the disulfide-bonded $\alpha 1(III)^B$ trimer (molecular weight, 70 000 daltons) to $\alpha 1(III)^B$ monomer (molecular weight, 23 000 daltons) and recovery of the latter in the column effluent at approximately 220 ml.

These results confirmed that, under the conditions employed in these experiments, collagenase cleavage of the Type III collagen was essentially complete. In addition, the data indicate that animal collagenase specifically cleaves the native Type III molecule at the characteristic three-quarter, one-quarter locus producing a large segment derived from the NH_2-terminal portion of the molecule and a smaller segment representing the COOH-terminal region.

Amino Acid Sequences. Approximately 0.4 μmol of $\alpha 1(II)^B$ or $\alpha 1(III)^B$ was used in the determination of primary structure, and 20 cycles of automatic Edman degradation were performed with each peptide. Repetitive yields of the Pth-amino acids were approximately 95%.

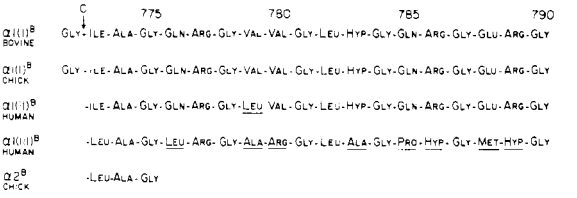

FIGURE 5: The primary structure of $\alpha 1(I)$, $\alpha 1(II)$, $\alpha 1(III)$, and $\alpha 2$ in homologous portions of the chains immediately COOH-terminal to the site of collagenase cleavage. The site of collagenase cleavage at residues 772–773 is depicted by the arrow. The data for bovine $\alpha 1(I)$, chick $\alpha 1(I)$, and chick $\alpha 2$ have been reported by Fietzek et al. (1973), Highberger et al. (1975), and Gross et al. (1974), respectively. The underlined residue in the $\alpha 1(II)^B$ sequence denotes an amino acid substitution relative to the $\alpha 1(I)^B$ sequence. The underlined residues in the $\alpha 1(III)^B$ sequence denote amino acid substitutions relative to both $\alpha 1(I)^B$ and $\alpha 1(II)^B$ sequences.

The results of these studies on human $\alpha 1(II)^B$ and $\alpha 1(III)^B$ are presented in Figure 5 along with the previously determined primary structure for homologous portions of $\alpha 1(I)$ and $\alpha 2$. The data for $\alpha 1(II)$ indicate that, similar to $\alpha 1(I)$, a Gly–Ile bond is cleaved in $\alpha 1(II)$ as the result of collagenase activity. The data further indicate that the $\alpha 1(II)$ sequence adjacent (COOH-terminal) to the site of collagenase cleavage is very similar to that of $\alpha 1(I)$. In this regard, there is only one difference in 18 amino acid residues COOH-terminal to the site of collagenase cleavage. This occurs as a leucine–valine interchange at residue number 779, seven amino acid residues from the site of collagenase cleavage. Since comparable data are not currently available for human $\alpha 1(I)$, this substitution could reflect a species difference rather than a chain difference. In any event, the results indicate that although $\alpha 1(I)$ and $\alpha 1(II)$ chains exhibit numerous differences in primary structure at certain other homologous regions (Butler et al., 1974), the primary structure of these chains at the site of collagenase cleavage is highly conserved.

The data for $\alpha 1(III)^B$ indicate that, similar to $\alpha 2$, collagenase activity results in cleavage of a Gly–Leu bond in $\alpha 1(III)$. The data further demonstrate that, within the 18 amino acid residues adjacent (COOH-terminal) to the site of collagenase cleavage, the $\alpha 1(III)$ sequence exhibits a total of eight amino acid substitutions relative to $\alpha 1(I)$ or $\alpha 1(II)$ chains. However, only one of these occurs within the six amino acids adjacent to the cleavage site. The latter is a leucine–glutamine interchange at residue number 776, four amino acids from the cleavage site.

Discussion

The distinctive feature of most animal collagenases is their capacity to cleave all three chains of the native collagen molecule at a single specific locus producing two fragments, a three-quarter fragment derived from the NH_2-terminal portion of the original molecule and a one-quarter fragment representing the COOH-terminal region of the original molecule. These general characteristics of collagenolytic activity have heretofore been determined largely in studies using Type I collagen from various species as substrate. The present studies have established that mammalian collagenase cleaves the Type II and III collagen molecules at precisely the same locus as previously observed for the Type I molecule. In view of the apparent close structur-

al homology between the various chains at the site of collagenase cleavage, the data also indicate that fragmentation of the Type II and III molecules is achieved by cleavage at the same types of bond (Gly–Ile and Gly–Leu, respectively) as previously shown to be cleaved during collagenolysis of the Type I molecule. In addition, the amino acid sequence data for $\alpha 1(II)^B$ and $\alpha 1(III)^B$, in conjunction with previous data on homologous regions of $\alpha 1(I)$ and $\alpha 2$, support the concept that collagenase specificity is dependent on a unique primary structure surrounding the cleavage site in native substrate molecules or their component chains (Gross et al., 1974).

These data further allow some initial generalizations concerning the specific sequence required for collagenase activity in the region immediately COOH-terminal to the cleavage site. In all four chains, $\alpha 1(I)$, $\alpha 1(II)$, $\alpha 1(III)$, and $\alpha 2$, the collagenase-susceptible Gly–Ile or Gly–Leu bond is followed by an alanyl residue suggesting that the minimum specific sequence compatible with cleavage by collagenase is either Gly-Ile-Ala or Gly-Leu-Ala. It would appear, however, that neither of these minimum sequences can fully account for the observed specificity in collagenase cleavage. One additional Gly-Ile-Ala sequence occurs at residues 226–228 in the repetitive triplet structure of rat skin $\alpha 1(I)$ (Balian et al., 1972) and collagenase cleavage apparently does not occur at this site. Similar considerations apply to the Gly-Leu-Ala sequence which is known to occur in at least two other sites at which collagenase cleavage does not occur. These are residues 823–825 in calf skin $\alpha 1(I)$ (Wendt et al., 1972) and residues 781–783 in human skin $\alpha 1(III)$ (Figure 5). It is noteworthy, however, that each of the sequences at which collagenase cleavage apparently does not occur is followed by a Gly-X-Hyp triplet: Gly-Ala-Hyp in rat skin $\alpha 1(I)$ (Balian et al., 1972); Gly-Pro-Hyp in calf skin $\alpha 1(I)$ (Wendt et al., 1972); and Gly-Pro-Hyp in human skin $\alpha 1(III)$ (Figure 5). On the other hand, the sequences susceptible to collagenase cleavage in $\alpha 1(I)$, $\alpha 1(II)$, and $\alpha 1(III)$ are followed by a Gly-X-Arg triplet (Figure 5). These data strongly suggest that the collagenase recognition site in substrate molecules or their component chains involves at least five amino acid residues on the COOH-terminal side of the cleavage site. Additional information on the sequences surrounding the cleavage site in $\alpha 2$ as well as the sequences preceding the cleavage site in $\alpha 1(II)$ and $\alpha 1(III)$ will be required to test this hypothesis and further delineate the nature and extent of the substrate recognition site.

Aside from questions relating to primary structure at the collagenase cleavage site, there remain several considerations relevant to higher orders of structure at this locus. Since the helical portion of the native collagen molecule is generally resistant to proteolysis, it has been suggested that the collagenase cleavage site might exhibit some special structural characteristic such as lack of perfection in, or even total absence of, the usual collagen helicity. Current information, however, does not support this suggestion. Data on the amino acid sequence surrounding the collagenase cleavage site in $\alpha 1(I)$ (Fietzek et al., 1973; Highberger et al., 1975) indicate that the sequence is characterized by repeating Gly-X-Y triplets and thus fulfills the minimum structural requirements for participation in the typical collagen fold. As yet, comparable data are not available for the homologous portion of $\alpha 2$, $\alpha 1(II)$, or $\alpha 1(III)$ chains. Nevertheless, the data presented here on the sequence of $\alpha 1(II)$ and $\alpha 1(III)$ immediately COOH-terminal to the collagenase cleavage site certainly suggest that the triplet structure occurs throughout the cleavage site in these chains as well. In addition, it has been shown that highly purified preparations of collagenase exhibit diminished capacity to cleave denatured collagen chains relative to native molecules (McCroskery et al., 1973; Harris and McCroskery, 1974). Thus, it would appear that, although the helical conformation of native collagen inhibits degradation of the molecule by most proteases, some degree of helicity at the cleavage site facilitates recognition and/or cleavage on the part of mammalian collagenases.

A number of studies have indicated that Type II collagen in solution is cleaved by animal collagenases at a slower rate than Type I collagen (Harris and Krane, 1973; Nagai, 1973; Woolley et al., 1975; McCroskery et al., 1975). Type I and III collagens, however, appear to be cleaved at a similar rate (McCroskery et al., 1975). Our results indicating that cleavage of Type III collagen occurs at approximately five times the rate for Type II collagen are consistent with the previous observations. At the moment, no definitive explanation can be offered to account for the relatively slow rate of cleavage observed for the Type II molecule.

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Photoreceptor Protein from the Purple Membrane of *Halobacterium halobium.* Molecular Weight and Retinal Binding Site†

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ABSTRACT: The apparent molecular weight of the purple membrane protein of *Halobacterium halobium* was found to be 20 000 by sodium dodecyl sulfate gel electrophoresis and by gel filtration in sodium dodecyl sulfate. However, the molecular weight value determined by gel filtration in 6 M guanidine was 28 000. To resolve this discrepancy, methods insensitive to or independent of the conformation of the protein were used to estimate the molecular weight. Analytical ultracentrifugation of the sodium dodecyl sulfate–protein complex, peptide mapping, and amino acid analysis all gave values of 25 000 ± 1000, a figure in agreement with a recent x-ray study. Borohydride reduction was used to attach the retinal cofactor covalently to a lysine residue. After digestion with thermolysin, peptide maps were prepared of the protein labeled at lysine residues with [^{14}C] succinic anhydride both before and after reduction. Comparison of the maps showed one radioactive peptide with changed mobility. This peptide was isolated and shown to have the sequence Val-Ser-Asp-Pro-Asp-Lys-Lys with only one of the two lysine residues alkylated. Solid-phase sequencing showed the succinyl group to be at position 6 and hence the retinal group to be at position 7. It was possible that a small amount of retinal was also bound to Lys-6. There was no apparent homology with the corresponding peptide of vertebrate rhodopsin. No evidence of chain heterogeneity was found by radiochemical peptide mapping and sequence analysis of peptides containing lysine residues indicating that all protein chains of purple membrane are very similar or identical.

The halophilic bacterium, *Halobacterium halobium,* exhibits optimum growth in solutions containing high concentrations of sodium chloride. On exposure to water or low salt concentration, the cells lyse and the disintegrated membranes may be separated by centrifugation into two fractions, the "red membrane" and the "purple membrane" (Stoeckenius and Kunau, 1968). The purple fraction apparently contains only one protein (Oesterhelt and Stoeckenius, 1971) which, together with membrane lipids, constitutes a system capable of carrying out the transport of protons through the membrane against a pH gradient (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Hess, 1973). The purple color of the membrane has been shown to be due to the covalent attachment of 1 mol of retinal per mol of protein (Oesterhelt and Stoeckenius, 1971) and, by analogy with rhodopsin, these authors concluded that the mode of attachment was probably via a Schiff's base linkage to the ϵ-NH_2 side chain of a lysine residue. This has been confirmed in a brief report (Oesterhelt, 1971). Addition of borohydride to the native membrane normally has no effect but, after bleaching with 0.01 M cetyltrimethylammonium bromide, Oesterhelt and Stoeckenius (1971) found that borohydride treatment could be used to form a stable covalent bond between the retinal and protein. More recently, Oesterhelt and Schuhmann (1974) have briefly reported that illumination of purple membrane suspensions at 570 nm in the presence of borohydride causes simultaneous reduction of the Schiff's base linkage and bleaching of the membrane. These authors were unable to reconstitute membranes bleached under these conditions with retinal, indicating that reduction occurred at the original retinal binding site.

The molecular weight of the bacterial protein has been reported (Oesterhelt and Stoeckenius, 1971) to be 26 000 on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, retinal content, and the finding of 1 mol of histidine per 26 000 mol of protein. Our preliminary observations indicated that the mobility of this protein on sodium dodecyl sulfate gels was incompatible with this molecular weight and we therefore decided to reinvestigate the molec-

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