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Covalent Structure of Collagen: Amino Acid Sequence of α2-CB5 of Chick Skin Collagen Containing the Animal Collagenase Cleavage Site†

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ABSTRACT: The amino acid sequence of the 112 residues from the amino terminus of α2-CB5 from chick skin collagen was determined by automated sequential degradation of intact α2-CB5 and several chymotryptic and tryptic peptides. This segment of the peptide includes the site of the action of animal collagenases. As compared to the sequence around the α1 cleavage site, the α2 sequence is notable for the remarkable constancy of the residues to the amino side and the relative abundance of hydrophobic residues to the carboxyl side of the cleavage site, suggesting that these features are important in the recognition by the enzyme. The sequence of this region of the α2 chain is consistent with the Gly-X-Y triplet structure and the preference of certain residues for either the X or Y position in distribution. However, three of the six residues of leucine were found in the Y position rather than the X position. Leucine residues were found only once in the Y position in the α1(I) chain. This preference does not appear to hold in the α2 chain.

Comparisons of the primary structure of collagens from different species can provide useful information relating structure to molecular properties. The interstitial collagen type I, the major protein constituent of skin, bone, and tendon, exists as a triple-stranded helix composed of two α1(I) chains and one α2 chain, each containing over 1000 amino acid residues. The complete amino acid sequence of the α1(I) chain from chick skin collagen has now been established (Kang & Gross, 1970; Kang et al., 1975; Dixit et al., 1975a,b; Highberger et al., 1975), as have the amino acid sequences of substantial portions of rat and calf α1(I) (Hulmes et al., 1973; Gallop & Paz, 1975; Piez, 1976; Fietzek & Kuhn, 1967). Knowledge of the covalent structure of the α2 chain is still incomplete. Cleavage of the α2 chain with CNBr gives rise to three small peptides, α2-CB1 (14 residues), α2-CB0 (3 residues), and α2-CB2 (30 residues), and three large peptides, α2-CB3, α2-CB4, and α2-CB5, containing 338, 321, and 320 amino acid residues respectively (Kang et al., 1969a; Fietzek & Piez, 1969). The order of the CNBr peptides in the α chain has been determined to be CB1-0-4-2-3-5 (Vuust et al., 1970; Igarashi et al., 1970). The covalent structure of α2-CB1 and α2-CB0 of chick, rat, and calf and α2-CB2 of chick, calf, human, rabbit, rat, and guinea pig skin collagens has been reported (Kang & Gross, 1970; Kang et al., 1967; Fietzek et al., 1974a,b; Highberger et al., 1971). In addition, the complete amino acid sequence of α2-CB3 of chick skin (Dixit et al., 1977a,b) and α2-CB4 of calf skin (Fietzek & Rexrodt, 1975) has been published. Also the sequence of 45 amino-terminal residues of the rat skin α2-CB5 has been determined (Fietzek & Kuhn, 1973).

As a continuation of our systematic investigations on the primary structure of type I collagen from chick skin, we now report the amino acid sequence of the amino-terminal region of α2-CB5 of chick skin which contains the cleavage site of animal collagenases (Gross & Nagai, 1965; Gross et al., 1974). To our knowledge the covalent structure of the α2 chain around the cleavage site has not previously been determined.

Materials and Methods

Preparation of α2-CB5. Purified chick skin collagen was prepared from 3-week-old lathyritic white Leghorn chicks by neutral salt and acid extraction procedures described previously (Kang et al., 1969b). The α2 chain was isolated by CM-cellulose[1] chromatography and was subjected to CNBr cleavage in 0.1 N HCl at a concentration of 4 to 5 mg/mL at 37 °C for 4 h (Bornstein & Piez, 1966) with approximately twice the weight of CNBr. At the end of incubation, the digest was diluted 10-fold with cold water and lyophilized. The peptide

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[1] Abbreviations used: CM-cellulose, carboxymethylcellulose; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; >PhNCS, phenylthiohydantoin.

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α2-CB5 was isolated by ion-exchange chromatography on CM-cellulose and molecular sieve chromatography on agarose A1.5M (200–400 mesh, Bio-Rad Laboratories). The procedural details as well as the criteria of the purity of the peptide were previously published (Kang et al., 1969a).

Preparation of $\alpha 2^B$. The rheumatoid synovial collagenase (Vater et al., 1978, 1979) was purified from the culture media of synovial explants by DEAE-cellulose chromatography, gel filtration on Ultrogel ACA-54 (Vater et al., 1979), and affinity chromatography on collagen–Sepharose (Gellet et al., 1977) after activation with trypsin (TPCK-treated, Worthington). The mammalian collagenase was found to be protease free by lack of proteolytic activity against azocasein and demonstration of the specific reaction products α^A and α^B at 34.5 °C. At this temperature these peptides are denatured and would be susceptible to the action of nonspecific proteases (Sakai & Gross, 1967). The native acid-extracted chick skin collagen (Kang et al., 1969b) was cleaved with the purified collagenase. The reaction was monitored by measuring the specific viscosity of the collagen solution in a constant temperature water bath in viscometers (Cannon Instruments). The reaction was stopped by the addition of EDTA to a final concentration of 0.005 M when the specific viscosity reached 40% of the initial specific viscosity. The presence of the specific reaction products was confirmed by $NaDodSO_4$–polyacrylamide gel electrophoresis in 7.5% acrylamide according to the method of Laemmli (1970). The collagen peptides were precipitated by addition of solid ammonium sulfate to a final concentration of 25%. The precipitate was collected by centrifugation, and the pellet was dissolved in 0.5 M acetic acid, desalted on Bio-Rad P-2, and lyophilized. The $\alpha 2^B$ was purified by a combination of molecular gel filtration and CM-cellulose chromatography as described previously (Kang et al., 1966).

In addition, the 25 000 molecular weight fragment of $\alpha 1$ ($\alpha 1^B$) was isolated from the reaction mixture by gel filtration and CM-cellulose chromatography. NH_2-terminal sequence analysis revealed Ile-Ala-Gly-Gln-Arg-Gly-Val-Val, confirming the established sequence of the collagenase cleavage site in the $\alpha 1$(I) chain (Gross et al., 1974).

Enzymatic Hydrolyses. Digestion with α-chymotrypsin was performed in 0.2 M NH_4HCO_3, pH 8.0. α-Chymotrypsin (3× crystallized, Worthington) was dissolved in 0.1 M HCl (1 mg/mL), and soybean trypsin inhibitor (Worthington) was added in the amount corresponding to 1/100 of chymotrypsin by weight. The peptide α2-CB5 was solubilized in 0.2 M NH_4HCO_3 (5 mg/mL) and heat denatured at 40 °C for 10 min. The appropriate quantity of enzyme solution corresponding to the enzyme/substrate molar ratio of 1:100 was added. Incubation was carried out at 37 °C for 30 min. The reaction was terminated by lyophilization.

Digestion with trypsin (TPCK-treated, Worthington) was performed for 30 min at the enzyme/substrate molar ratio of 1:100 in 0.2 M Tris–0.001 M $CaCl_2$, pH 7.6, at 37 °C. At the end of incubation, the sample was lyophilized.

Column Chromatography. Chymotryptic digests of α2-CB5 were fractionated initially by molecular sieve chromatography on a column (4.5 × 120 cm) of Sephadex G-75S (Pharmacia) equilibrated with 0.04 M sodium acetate, pH 4.8. The samples were applied to the column, dissolved in 10 mL of the same buffer, and eluted at a flow rate of 40 mL/h.

The tryptic digest of α2-CB5 was fractionated on a Sephadex G-50S (Pharmacia) column (2 × 120 cm) equilibrated with 0.04 M sodium acetate, pH 4.8. The samples were applied in 2 mL of the buffer, and the column was eluted with the same buffer at a flow rate of 35 mL/h.

Ion-exchange chromatography of chymotryptic and tryptic peptides was performed on a column (1 × 6 cm) of phosphocellulose (Whatman P-11) equilibrated with 0.001 M sodium acetate, pH 3.8, at 44 °C. After application of the samples, the column was eluted with a linear gradient of NaCl from 0 to 0.3 M over a total volume of 500 mL at a flow rate of 60 mL/h. The effluents were continuously monitored at 230 nm in a Gilford spectrophotometer equipped with a flow cell. Peptides were desalted on a column (2 × 120 cm) of Bio-Gel P-2 (200–400 mesh) using 0.1 M acetic acid as eluent and lyophilized.

Amino Acid Analysis. Samples for amino acid analysis were sealed under an atmosphere of nitrogen in doubly distilled constant-boiling HCl and were hydrolyzed for 24 h at 108 °C. Analyses were performed on an automatic analyzer (Beckman 121) using a single-column method (Kang, 1972). No correction factors were used for losses of labile amino acids (threonine and serine) or the incomplete release of valine.

Amino Acid Sequence Analysis. Automated sequential degradation of peptides was performed according to established principles (Edman & Begg, 1967) on a Beckman sequencer (890C). Either the fast protein Quadrol (072172C) or the slow peptide DMAA (071472) program with only minor modification was used to run the sequenator. In all cases, except α-CB5, peptides were modified at carboxyl groups by treatment with 2-amino-1,5-naphthalenedisulfonic acid in the presence of *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide by the procedure of Foster et al. (1973) with minor modification (Dixit et al., 1975a). The phenylthiohydantoin amino acids were identified by high-pressure liquid chromatography (Zimmerman et al., 1973). The COOH-terminal residues were inferred from the amino acid composition of peptides, from the known specificities of enzymes, or by subsequent overlapping amino acid sequence analysis.

Results

Isolation of Chymotryptic Peptides. The chymotryptic digest of α2-CB5 was fractionated initially on Sephadex G-75S. The elution profile of the chymotryptic peptides is presented Figure 1. The fractions corresponding to peaks 4 and 5 were pooled separately, desalted, and lyophilized. Further fractionation of peptides was performed on phosphocellulose. The material in peak 4 (Figure 1) on phosphocellulose column chromatography separated into two distinct major peaks, C2 and C3, as shown in Figure 2. Under similar conditions, the material in peak 5 (Figure 1) was resolved into peptide C1 and C4 (Figure 3). Only chymotryptic peptides C1, C2, and C3 were needed to establish the amino acid sequence of the portion of α2-CB5 presented in this report. The amino acid compositions of C1, C2, C3, and C4 are presented in Table I. The separation of C1 on Sephadex G-75S from C2 and C3 may have been effected by the presence of the single phenylalanine in the peptide, which possibly retarded its elution from the column.

Isolation of Intact Tryptic Peptide from Residues 46–97 (Figure 6). The initial fractionation of tryptic digest was performed on Sephadex G-50S. The typical elution profile of tryptic peptides obtained under the conditions described under Materials and Methods above is presented in Figure 4. The peptide material in peak 1 represented by the solid bar (Figure 4) was pooled, desalted, and further fractionated on phosphocellulose. Two peaks, T1 and T2 (Figure 5), were consistently obtained. The amino acid compositions of tryptic peptides T1 and T2 are summarized in Table II.

Amino Acid Sequence Analysis. Intact α2-CB5, the chymotryptic peptides C1, C2, and C3, the tryptic peptide T2,

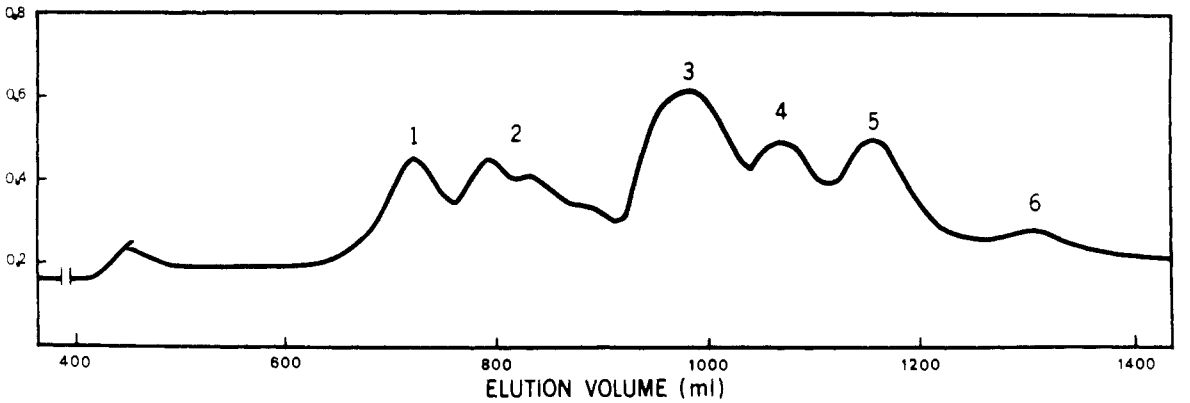

FIGURE 1: Sephadex G-75S chromatography of a chymotryptic digest of α2-CB5. The column (4.5 × 120 cm) was packed with the resin equilibrated with 0.04 M sodium acetate, pH 4.8, and after application of the sample was eluted with the same buffer at a rate of 40 mL/h. For the present study, fractions in peaks 3 and 4 were pooled, desalted, and lyophilized for further fractionation.

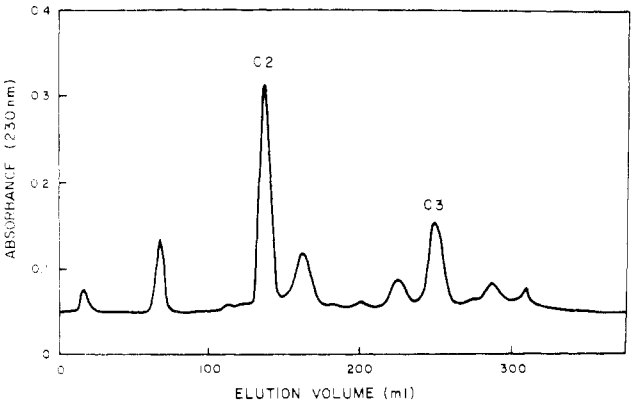

FIGURE 2: Phosphocellulose chromatography of the chymotryptic peptide material collected in peak 3 (Figure 1). Chromatography was performed on a 1 × 6 cm jacketed column of phosphocellulose equilibrated with 0.001 M sodium acetate, pH 3.8, at 44 °C. Peptides were eluted with a linear gradient of NaCl from 0 to 0.3 M over a total volume of 500 mL.

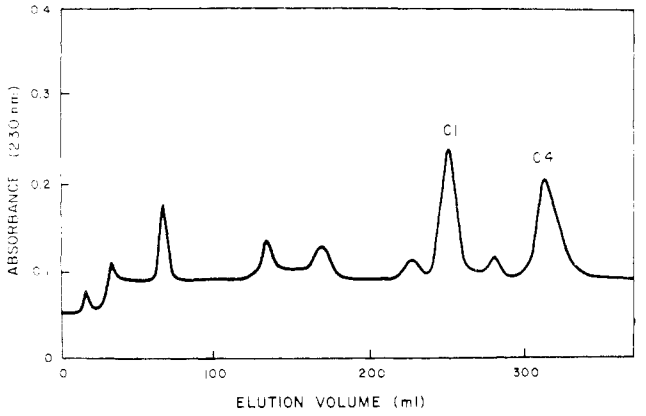

FIGURE 3: Phosphocellulose chromatography of the chymotryptic peptide material collected in peak 4 (Figure 1).

and the animal collagenase peptide $\alpha 2^B$ were subjected to automated Edman degradation to establish the amino acid sequence of the 112 residues of the amino-terminal region of α2-CB5. The peptide α2-CB5 was degraded through 49 residues without modification. The peptides C1, C2, C3, and T2 were modified at carboxyl groups by condensing with 2-amino-1,5-naphthalenedisulfonic acid in the presence of *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide. The peptides C1, C2, and C3 were degraded through the penultimate amino acid residues. The tryptic peptide T2 was degraded through 28 residues. The peptide $\alpha 2^B$ was subjected to Edman

Table I: Amino Acid Composition of Chymotryptic Peptides of α2-CB5 of Chick Skin Collagen[a]

	C1	C2	C3	C4
4-Hyp	1 (1.1)	2 (2.2)	3 (3.1)	1 (1.3)
Asp	1 (1.0)	-	-	1 (1.0)
Thr	1 (0.8)	1 (0.9)	1 (0.8)	-
Ser	-	1 (1.0)	1 (1.0)	1 (0.8)
Glu	2 (2.1)	3 (3.1)	2 (2.1)	1 (1.0)
Pro	2 (2.2)	3 (3.3)	1 (1.2)	2 (1.7)
Gly	7 (7.2)	8 (8.1)	8 (8.3)	5 (5.0)
Ala	1 (1.0)	4 (4.0)	2 (2.0)	2 (2.0)
Val	2 (1.7)	-	-	-
Ile	1 (0.9)	1 (1.0)	1 (0.8)	-
Leu	-	1 (1.0)	3 (3.1)	1 (1.0)
Phe	1 (1.1)	-	-	-
Hyl	-	-	-	0.3
Lys	-	1 (1.0)	-	0.7
Arg	2 (2.1)	-	2 (2.0)	1 (1.0)
total	21	25	24	16

[a] Expressed as assumed residues per peptide. The numbers in parentheses are the observed values. A dash indicates 0.1 residue or less.

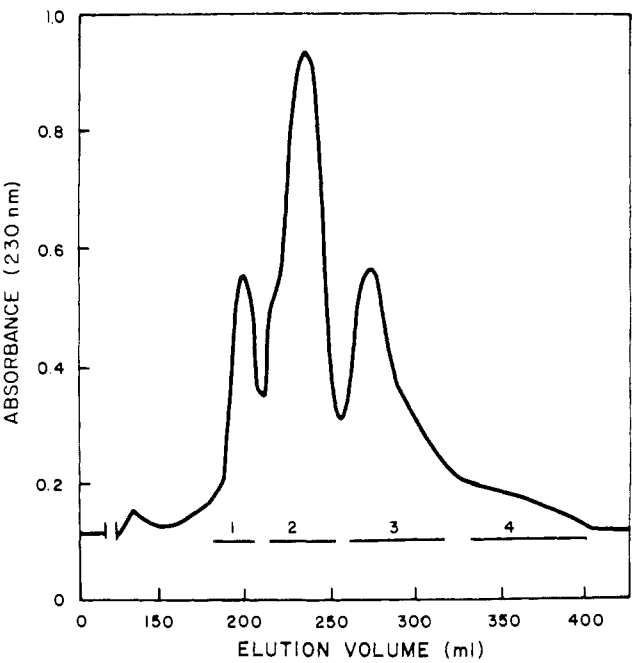

FIGURE 4: Fractionation of a tryptic digest of α2-CB5 on a column (2 × 120 cm) of Sephadex G-50S. Conditions were similar to those in Figure 1, except that the flow rate was 35 mL/h. For the present study, the peptide material eluting in the peak labeled 1 was further investigated.

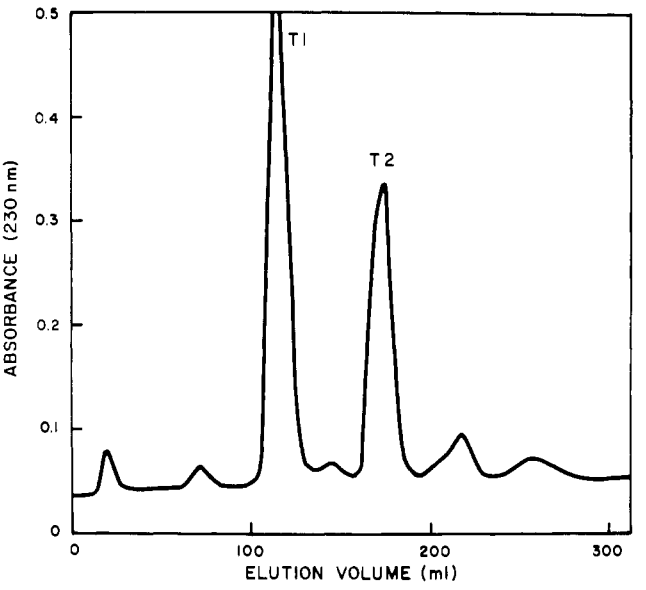

FIGURE 5: Phosphocellulose chromatography of the tryptic peptide material collected in peak 1 (Figure 4). Conditions of chromatography were the same as those described in Figure 2.

Table II: Amino Acid Composition of Tryptic Peptides T1 and T2 of α2-CB5 of Chick Skin Collagen[a]

	T1	T2
4-Hyp	4 (3.7)	5 (4.8)
Asp	1 (1.0)	-
Thr	2 (1.8)	2 (1.8)
Ser	3 (2.8)	2 (1.7)
Glu	3 (3.2)	6 (5.0)
Pro	3 (3.2)	4 (4.2)
Gly	16	17
Ala	6 (6.0)	6 (6.0)
Val	1 (0.8)	-
Ile	2 (1.8)	3 (2.7)
Leu	3 (3.0)	3 (3.0)
Phe	1 (1.0)	1 (1.0)
Hyl	0 (0.4)	0 (0.1)
Lys	1 (0.6)	1 (0.9)
Arg	1 (1.0)	2 (2.0)
total	47	52

[a] Expressed as assumed residues per peptide. The numbers in parentheses are the observed values. A dash indicates 0.1 residue or less.

Table III: Amount of >PhNCS Derivative Recovered from Peptides T2 and C2 of α2-CB5 of Chick Skin Collagen after Automatic Edman Degradation[a]

residue no.	amino acid identified	T2[b]	C2[b]
1	Thr	386	
2	Gly	460	
3	Glu	421	
4	Gln	394	
5	Gly	444	
6	Ile	421	
7	Ala	411	
8	Gly	409	
9	Pro	383	
10	Hyp	365	
11	Gly	384	
12	Phe	362	
13	Ala	348	410[c]
14	Gly	343	464
15	Glu	329	392
16	Lys	301	321
17	Gly	312	376
18	Pro	296	344
19	Ser	68	80
20	Gly	288	342
21	Glu	273	328
22	Ala	270	312
23	Gly	264	321
24	Ala	243	324
25	Ala	261	342
26	Gly	250	298
27	Pro	204	264
29	Hyp	192	215
29	Gly	208	228
30	Thr	102	160
31	Hyp	186	184
32	Gly	220	188
33	Pro	164	121
34	Gln	118	66
35	Gly	160	104
36	Ile	120	42
37	Leu	102	
38	Gly	126	
39	Ala	86	
40	Hyp	64	
41	Gly	108	
42	Ile	36	

[a] Values are expressed as total nanomoles of the phenylthiohydantoin derivative after each cycle. [b] Amount of peptide used was 500 nmol in each case. [c] C2 began at residue 13 from the Phe-Ala cleavage site of chymotrypsin.

degradation without modification through 25 residues. Each peptide was analyzed at least twice, except α2-CB5 which was degraded 3 times. Figure 6 shows each peptide with the number of residues degraded. The repetitive yields were determined by calculating the yield of Gly>PhNCS and were generally 95%. Actual values obtained with T2 and C2 are presented in Table III.

Alignment of Peptides. The position of chymotryptic peptide C1 was determined from the amino acid sequence of intact α2-CB5 which was degraded through 49 residues (Figure 6). The peptide α2-CB5 has an overlap sequence of Arg-Gly-Asp-Val-Gly-Pro-Val-Gly-Arg-Thr-Gly-Glu-Gln common with the amino acid sequence of C1 from residues 1–13 (Figure 6, residues 37–49). The chymotryptic peptide C2 follows C1, as deduced from the amino acid sequence determination of the overlap peptide T2 which was degraded through 28 residues (Figure 6, residues 46–73). The peptide C2 consisted of residues 58–82, which was established by its sequence determination through the penultimate residue 81 and by the presence of a residue of Leu in the amino acid composition (Table I). The amino acid sequence was extended to residue 105 by sequential degradation of $\alpha 2^{B}$ (Figure 6). The fact that the chymotryptic peptide C3 began with residue 89 (Figure 6) is also established by the amino acid sequence analysis of $\alpha 2^{B}$. Further, the amino acid sequence was extended to residue 112 by the degradation of chymotryptic peptide C3 through the penultimate residue. Leu is the carboxyl end of C3 as suggested by the amino acid composition of C3 (Table I).

Discussion

The primary structure of the amino-terminal region of α2-CB5 containing the animal collagenase cleavage site is summarized in Figure 6. The presented sequence data are consistent with the characteristics of the helical portion of all known collagen sequences. Glycine is present at every third residue in the triplet Gly-X-Y sequence. The presence of two residues of phenylalanine (residues 3 and 57) in the X position of the triplet is consistent with previous findings (Balian et al., 1971; Butler et al., 1976, 1977a,b; Seyer & Kang, 1977, 1978). The segment of amino acid sequence from residues 18–112 contains a total of six residues of leucine and five

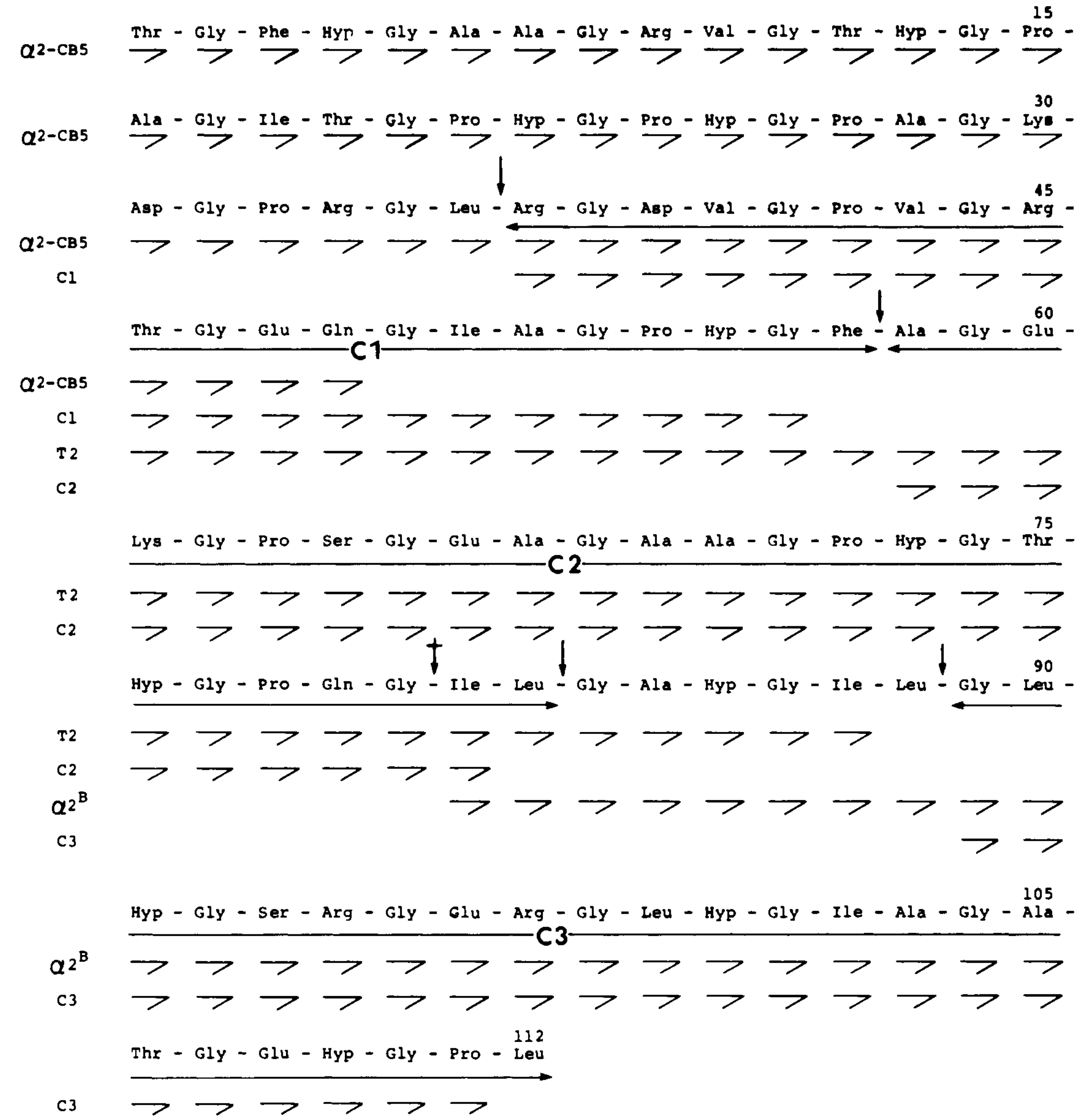

FIGURE 6: Amino acid sequence of 112 residues of α2-CB5 from the amino terminus. The short horizontal half arrows (⇀) indicate the residues degraded in the sequencer. The vertical arrows (↓) indicate the sites of cleavage by chymotrypsin. The symbol ↨ indicates the site of synovial collagenase cleavage.

									9	10		12			
Chick α2-CB5	Thr -	Gly -	Phe -	Hyp -	Gly -	Ala -	Ala -	Gly -	Arg -	Val -	Gly -	Thr -	Hyp -	Gly -	Pro -
Rat α2-CB5										Thr		Pro			
	16		18									27			
Chick α2-CB5	Ala -	Gly -	Ile -	Thr -	Gly -	Pro -	Hyp -	Gly -	Pro -	Hyp -	Gly -	Pro -	Ala -	Gly -	Lys -
Rat α2-CB5	Ser		Asn									Ala			
	31		33	34		36				40					45
Chick α2-CB5	Asp -	Gly -	Pro -	Arg -	Gly -	Leu -	Arg -	Gly -	Asp -	Val -	Gly -	Pro -	Val -	Gly -	Arg -
Rat α2-CB5	Glu		Ile	Lys		Pro				Gln					

FIGURE 7: Comparison of amino acid sequence of the first 45 residues of α2-CB5 from chick and rat skin. Only the substitutions are indicated. The rat data are from Fietzek & Kuhn (1973).

residues of isoleucine. Three of the leucine residues (residues 82, 88, and 112) are in the Y position of the triplet. This is unusual since only one leucine occurs in the Y position of the entire α1(I) chain of rat and calf skin collagen (Butler et al., 1974; Fietzek et al., 1972).

The present data permit an interspecies comparison with the reported amino-terminal 45 residues of α2-CB5 of rat skin collagen (Fietzek & Kuhn, 1973). There are 10 substitutions (Figure 7) with a sequence identity of 78%, which is in close agreement with the previously reported sequence identity of ~80% among known homologous regions of α2-CB3 of chick, calf, and rat skin collagen (Dixit et al., 1977a). The present work also allows a comparison with the homologous region of chick α1-CB7 (Highberger et al., 1975). There are 38 substitutions (Figure 8) within a stretch of 112 residues. If the glycine residues are omitted from the calculation, over 50%

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                                                                    10
α2-CB5   Thr - Gly - Phe - Hyp - Gly - Ala - Ala - Gly - Arg - Val - Gly - Thr - Hyp - Gly - Pro -
α1-CB7                                                                      Pro

                                        20
α2-CB5   Ala - Gly - Ile - Thr - Gly - Pro - Hyp - Gly - Pro - Hyp - Gly - Pro - Ala - Gly - Lys -
α1-CB    Ser         Asn   Ile         Leu

                                                                    40
α2-CB5   Asp - Gly - Pro - Arg - Gly - Leu - Arg - Gly - Asp - Val - Gly - Pro - Val - Gly - Arg -
α1-CB7   Glx         Ser   Lys         Pro               Glu   Thr               Ala

                                49                                  54
α2-CB5   Thr - Gly - Glu - Gln - Gly - Ile - Ala - Gly - Pro - Hyp - Gly - Phe - Ala - Gly - Glu -
α1-CB7   Hyp               Hyp         Pro                                 Pro   Hyp

                          63                                                72
α2-CB5   Lys - Gly - Pro - Ser - Gly - Glu - Ala - Gly - Ala - Ala - Gly - Pro - Hyp - Gly - Thr -
α1-CB7               (Ala) Hyp         (Ala) Asp         Pro   Ile         (Ala)

                                        81                                                  90
α2-CB5   Hyp - Gly - Pro - Gln - Gly - Ile - Ala - Gly - Ala - Hyp - Gly - Ile - Leu - Gly - Leu -
α1-CB7   Pro                                         Gln   Arg         Val   Val

                                                                    99
α2-CB5   Hyp - Gly - Ser - Arg - Gly - Glu - Arg - Gly - Leu - Hyp - Gly - Ile - Ala - Gly - Ala -
α1-CB7               Gly                         Phe               Leu   Hyp         Pro

                          108                           112
α2-CB5   Thr - Gly - Gln - Hyp - Gly - Pro - Leu
α1-CB7   Ser                         Lys   Gln
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FIGURE 8: Comparison of amino acid sequence of the first 112 residues of α2-CB5 with the homologous segment of α1-CB7 from chick skin. The data on α1-CB7 are from Highberger et al. (1975).

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Chick                                  5                  ↓                10
α2-CB5   Gly - Thr - Hyp - Gly - Pro - Gln - Gly - Ile - Leu - Gly - Ala - Hyp - Gly - Ile - Leu - Gly - Leu - Hyp
Chick
α1-CB7   Gly - Thr - Pro - Gly - Pro - Gln - Gly - Ile - Ala - Gly - Gln - Arg - Gly - Val - Val - Gly - Leu - Hyp
Calf
α1-CB7   Gly - Thr - Pro - Gly - Pro - Gln - Gly - Ile - Ala - Gly - Gln - Arg - Gly - Val - Val - Gly - Leu - Hyp
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FIGURE 9: Amino acid sequence around the animal collagenase cleavage site in chick α2-CB5 and α1-CB7 and calf α1-CB7. The data on chick α1-CB7 are from Highberger et al. (1975), and those for calf α1-CB7 are from Wendt et al. (1972). The arrow indicates the synovial collagenase cleavage site.

of the residues are different. This high degree of variability between the α1(I) and α2 chains of chick skin collagen has been observed earlier in the case of α2-CB3 and the homologous region of the α1(I) chain (Dixit et al., 1977a,b).

The purified rheumatoid synovial collagenase (Vater et al., 1978, 1979) cleaves the native collagen at a specific site one-quarter the length of the collagen molecule from the carboxyl end in each of the three chains. In α2-CB5, the Gly–Ile bond between residues 80 and 81 (Figure 6) is cleaved by the collagenase. The amino acid sequence around the collagenase cleavage site in α2-CB5 is depicted in Figure 9. For comparison, the amino acid sequences around the cleavage site in α1-CB7 in chick (Gross et al., 1974; Highberger et al., 1975) and in calf (Wendt et al., 1972) are also presented. The amino acid sequences around Gly-Ile (Figure 9) in both α1-CB7 and α2-CB5 have unique structural identities. Residues 1–7 are identical except for hydroxylation of Pro in α2-CB5. On the other hand, the amino acid sequences on the carboxyl side (residues 9–18 in Figure 9) of Gly-Ile in α2-CB5 contain several substitutions (Ala → Leu; Gln → Ala; Arg → Hyp; Val → Ile; Val → Leu). It is probable that certain identical sequences are necessary on the carboxyl side of Gly-Ile with a minimum requirement for several hydrophobic residues for the specific affinity of animal collagenases. It is interesting to note that another Gly–Ile bond between residues 13 and 14 (Figure 9) in close vicinity of the cleavage site is not cleaved by collagenase. In addition, there are two more Gly–Ile bonds in α2-CB5 between residues 17 and 18 and 101 and 102 (Figure 6) which were not cleaved by collagenase, thereby ruling out Gly-Ile as sufficient for the action of animal collagenase and demonstrating that a much more complex recognition site may be necessary for enzymatic activity.

The amino-terminal sequence of $\alpha 2^{B}$ obtained in this study, Ile-Leu-Gly-, is not in agreement with preliminary results reported in a previous study, Leu-Ala-Gly- (Gross et al., 1974). The reason for the discrepancy is not clear at present. It might be the result of a possible microheterogeneity in α2, as has been documented in the α1(II) chain of cartilage by Butler et al. (1977a,b). Further investigations are in progess to clarify this issue.

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Interaction between Cytochrome *c* and Cytochrome b_5[†]

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ABSTRACT: The reduction of cytochrome *c* by cytochrome b_5 was studied over a wide range of ionic strengths in four different buffer systems. The reaction rate decreased linearly as the $I^{1/2}$ was increased, suggesting that electrostatic interactions are important in the interaction. The ionic strength dependence of the reaction rate was in quantitative agreement with the theory of Wherland & Gray [Wherland, S., & Gray, H. B. (1976) *Proc. Natl. Acad. Sci U.S.A. 73*, 2950] only if the effective radius of the interaction was 2 Å. This indicates that the interaction between the two proteins is best described as the sum of *n* complementary charge interactions, each involving a specific lysine on cytochrome *c* and a specific carboxyl group on cytochrome b_5. The number of complementary charge interactions, *n*, was calculated to be five to seven, in agreement with the results of our specific modification studies. Ultracentrifugation and gel permeation techniques were used to demonstrate that cytochrome b_5 and cytochrome *c* formed a stable complex at low ionic strength.

The development of a comprehensive theory of electron transport in biological systems has been hampered by the lack of detailed structural information on interacting components of these systems. The in vitro reaction between cytochrome b_5 and cytochrome *c* is a unique model system because the X-ray crystal structures of both proteins have recently been determined to high resolution (Argos & Mathews, 1975; Swanson et al., 1977), and the reaction rate is as rapid as the reactions of either protein with its physiological oxidants and reductants (Strittmatter, 1964). Although cytochrome b_5 was first discovered in the membrane of the endoplasmic reticulum, a very similar form has been found in other organelles, including the outer membrane of the mitochondrion (Borgese & Meldolesi, 1976). Matlib & O'Brien (1976) have suggested that the reaction between cytochrome b_5 and cytochrome *c* might have some limited physiological significance because under conditions of high intermembrane ionic strength cytochrome *c* is released from the inner membrane and can transport electrons from cytochrome b_5 located on the inner

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