Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7684–7688, December 1982
Biochemistry

Collagen structural microheterogeneity and a possible role for glycosylated hydroxylysine in type I collagen

(nonreducible stable crosslinks/hydroxyaldolhistidine/specific cleavage/molecular location)

MITSUO YAMAUCHI*†, CLAUDIA NOYES*‡, YOSHINORI KUBOKI*†, AND GERALD L. MECHANIC*§¶

*Dental Research Center, §Department of Biochemistry and Nutrition, and ‡Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Communicated by Ernest L. Eliel, September 20, 1982

ABSTRACT **A three-chained peptide from type I collagen, crosslinked by hydroxyaldolhistidine, has been isolated from a tryptic digest of 5 M guanidine·HCl-insoluble bovine skin collagen (a small but as yet unknown percentage of the total collagen in whole skin). $OsO_4/NaIO_4$ specifically cleaved the crosslink at its double bond into a two-chained crosslink peptide and a single peptide. The sequence of the two-chained peptide containing the bifunctional crosslink was determined after amino acid analysis of the separated peptides. The crosslink consists of an aldehyde derived from hydroxylysine-87 in the aldehyde-containing cyanogen bromide fragment α1CB5^{ald} and an aldehyde derived from the lysine in the COOH-terminal nonhelical region of the α1CB6^{ald} fragment. The α1CB6^{ald} portion of the peptide exhibited structural microheterogeneity, containing the inverted sequence Ala-Lys-His instead of the normal sequence Lys-Ala-His. This indicates that another structural gene exists for α1(I) chain. The original three-chained peptide did not contain any glycosylated hydroxylysine or glycosylated hydroxyaldolhistidine. The lack of glycosylation of hydroxylysine-87 in α1CB5, which is usually glycosylated, allowed formation of the aldehyde, and this, coupled with the sequence inversion, may have allowed formation of the nonreducible crosslink hydroxyaldolhistidine. We suggest that the role of glycosylation, a posttranslational modification, of specific hydroxylysine residues is to prevent their oxidative deamination to aldehydes, thereby precluding formation of complex stable crosslinks. Complex crosslinks would decrease the rate of collagen turnover. The decrease, with time, would increase the population of stable crosslinked collagen molecules, which would eventually accumulate with age.**

The crosslinking and maturation of collagen is initiated by the action of lysyl oxidase on fibrils. The enzyme converts the ε-carbon atoms of specific peptidyl lysine and hydroxylysine moieties to aldehydes by enzymic oxidative deamination (1, 2). In the first stages, aldehydes can either self-condense to form aldol condensation product (aldol, an α,β-unsaturated aldehyde, the intramolecular crosslink) or condense with ε-NH_2 groups of other specific peptidyl lysine and hydroxylysine residues to form imminium intermolecular crosslinks (3, 4). Recently Fukae and Mechanic (5) have demonstrated that the initial step in the temporal sequence is conversion of the lysine residue at position 17[c] in the α1(I) chain to α-aminoadipoyl-δ-semialdehyde, and this aldehyde forms imminium intermolecular crosslinks before aldol intramolecular crosslinks.

Histidine is an amino acid that is very sparse in collagen (14 of a total of approximately 3,150 amino acids), yet it is a constituent of the relatively abundant $NaBH_4$-reduced imminium crosslink; histidinohydroxymerodesmosine (HisOHMerDes) (6,7). Recently it has been clearly demonstrated by Bernstein and Mechanic (8) that dehydro-HisOHMerDes, which was thought to be artifactual (9, 10) is a true crosslink in collagen fibrils. Bernstein and Mechanic found that one HisOHMerDes crosslink was present per molecule of collagen in freshly reconstituted soluble collagen fibrils.

Histidine was also found to be a constituent of the stable nonreducible trifunctional crosslink hydroxyaldolhistidine (OHAlHis), whose structure was elucidated by PMR and mass spectrometry (11). OHAlHis was isolated from bovine skin collagen. Becker *et al.* (12) isolated a three-chained crosslinked peptide from a tryptic digest of insoluble bovine skin; it contained three NH_2- and three COOH-terminal residues. Housley *et al.* (11) demonstrated that the crosslink in the peptide isolated by Becker *et al.* (12) corresponded to OHAlHis.

Serological studies, end-group analysis, and CNBr cleavage indicated that the three-chained crosslinked peptide contained a portion of the COOH-terminal nonhelical peptide of cleavage fragment α1CB6 and a portion of fragment α1CB5 as two of the three chains of the peptide (12). However, assignments of the location of the contributing amino acid residues or the involvement of their prosthetic groups were not made.

The functional role of the sugar residues linked to hydroxylysine in collagen is not clearly known. However, it has been suggested that they may be involved in directing molecular assembly because their locations in the molecule would place them in the "hole" regions [0.6D spaces of fibril (D = 670 Å)] (13, 14). It has also been suggested that if glycosylation of ε-amino groups occurred, it could be a form by which the availability of such groups could be regulated for crosslink formation (15). The role of sugar in collagen in this type of mechanism and the former functional hypothesis still await confirmation. It has also been suggested that glycosylation of hydroxylysine directs the formation of some crosslinks (16). This hypothesis also still requires verification.

In order to understand the specific structure–function relationships of collagen matrices in different tissues, and to elucidate the fibrillar three-dimensional packing of collagen molecules, it is necessary to identify the molecular loci of the various intermolecular crosslinks.

The postulated structure for OHAlHis contains a doubly bonded carbon atom that is linked to the N^{τ} of the imidazole ring of histidine (11). Previously we have used osmium tetroxide to convert a double bond into a vic-diol in the unequivocal synthesis of dihydroxynorleucine (17). In order to determine the location of OHAlHis, we isolated the crosslinked peptide by the method of Becker *et al.* (12) and converted the double bond to a vic-diol and specifically cleaved the three-chained peptide

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: HisOHMerDes, histidinohydroxymerodesmosine; OHAlHis, hydroxyaldohistidine; *t*-Boc, *t*-butoxycarbonyl.

† Present address: Tokyo Medical and Dental University, Tokyo, Japan.
¶ To whom reprint requests should be addressed.

with metaperiodate into a double-chained peptide and a single peptide.

MATERIALS AND METHODS

Peptide fraction 5, the fraction containing the three-chained peptide linked by OHAlHis, was prepared as described (12) from 78.8 g of the same batch of insoluble bovine skin collagen from which OHAlHis was isolated previously (11).

Amino Acid Analyses. Samples were hydrolyzed for 24 hr at 110°C *in vacuo* with 3 M *p*-toluenesulfonic acid in glass test tubes or with 2 M NaOH in polypropylene tubes enclosed in an evacuated glass test tube. Analyses were performed in duplicate on an LKB model 4400 HPLC amino acid analyzer.

Chromatographic Procedures. See legends of Figs. 1, 2, and 3 for details. Columns of Bio-Gel P-2 were used for desalting.

Amino Acid Sequence Analysis. A Beckman 890 C sequencer using an 0.1 M Quadrol program and 3 mg of Polybrene (18) was utilized. The phenylthiohydantoin derivatives of amino acids were identified by reverse-phase chromatography using a Beckman/Altex 324 HPLC unit with an Ultrasphere octadecylsilyl (ODS) column (4.6 × 250 mm). Gradient elution was carried out at 55°C; the aqueous mobile phase component was 0.01 M sodium acetate (pH 4.9), and the organic component was acetonitrile.

Chemical Procedures. The isolated three-chained crosslink peptide (5 mg) was completely derivatized by using a 10-fold molar excess of di-*tert*-butylcarbonate (calculated on the basis of 10 possible derivatizable nitrogen groups) according to the method described in ref. 19. The blocked peptide was desalted by using a column of Bio-Gel P-2.

The crosslink peptide was cleaved specifically at the double bond in OHAlHis by the use of $OsO_4/NaIO_4$ by the method of Reinecke *et al.* (20). The peptides were deblocked after cleavage, for sequence analysis, by using HCl in formic acid by a described method (21).

RESULTS

Peptide fraction 5 (12) was rechromatographed on phosphocellulose with a concave gradient and was found to be heterogeneous (Fig. 1).

The fraction denoted by the bar in Fig. 1 was chromatographed on DEAE-cellulose and the results are shown in Fig. 2. Only the peaks denoted IV, V, and VI contained OHAlHis. Peak VI was rechromatographed on Sephadex G-50, and it eluted as a single symmetrical peak corresponding to the position of peak I in Fig. 3. The amino acid composition of peak VI (Fig. 2) is shown in Table 1. Peaks IV and V of Fig. 2 had extremely similar compositions. No calculation is presented for OHAlHis, which eluted 6.2 min after phenylalanine and 4.3 min before hydroxylysine. It possessed 3.1 times the color factor of leucine in our system. It eluted at a different position from that of an authentic sample of the pyridinoline crosslink obtained from David Eyre. The composition of the peptide differed somewhat from that previously reported by Becker *et al.* (12) (column 5, Table 1).

Amino acid analysis after alkaline hydrolysis of the peptide indicated that hydroxylysine and OHAlHis were not glycosylated, because their elution positions were unchanged.

Derivatization of the crosslink peptide with *t*-Boc was complete, because there was no retardation of the peptide when a solution of the peptide in 0.001 M lithium formate at pH 3.8 was applied to the phosphocellulose column. This was confirmed by amino acid analysis after metaperiodate (see below) treatment of the blocked peptide. No hydroxylysine was de-

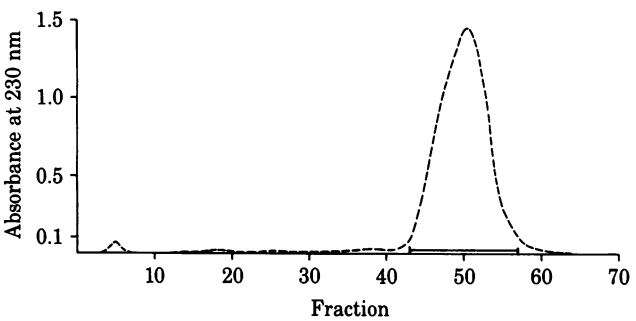

FIG. 1. Rechromatography of peptide fraction 5 (ref. 12) on a phosphocellulose column (1.5 × 24 cm) jacketed at 42°C and equilibrated with 0.001 M lithium formate buffer, pH 3.8, containing 0.5% 2-propanol. After application of the sample in equilibration buffer, the column was eluted with a simple concave gradient generated by a three-chambered Varigrad (Mark Instrument, Villanova, PA) containing 180 ml of 0.001 M lithium formate, pH 3.8/0.5% 2-propanol in the first two chambers and 180 ml of 0.001 M lithium formate, pH 3.8/0.5% 2-propanol/0.3 M LiCl in the third chamber. Flow rate was 150 ml/hr; 5-ml fractions were collected. The bar under the peak denotes the fractions that were pooled for chromatography on DEAE-cellulose (see Fig. 2).

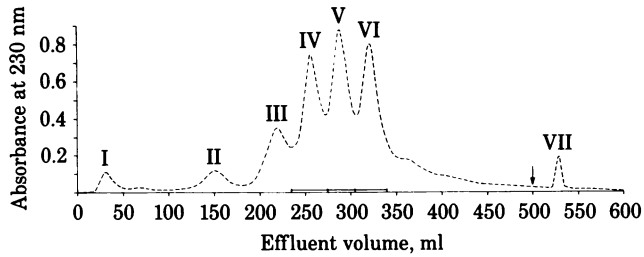

FIG. 2. Chromatography of the pooled peak from Fig. 1 on a DEAE-cellulose column (2.5 × 8 cm) jacketed at 42°C and equilibrated with 0.01 M Tris·HCl (pH 8.2) as the starting buffer. After application of the sample, the column was eluted with 260 ml of starting buffer and 240 ml of a linear gradient generated by two equal chambers. The first contained 200 ml of starting buffer and the second contained 200 ml of starting buffer and 0.2 M NaCl. The column was then eluted with 100 ml of starting buffer containing 0.5 M NaCl (arrow). Flow rate was 150 ml/hr; 5-ml fractions were collected. The fractions that were pooled for further work are represented by the bars under the three peaks IV, V, and VI. The fractions in each peak were pooled separately.

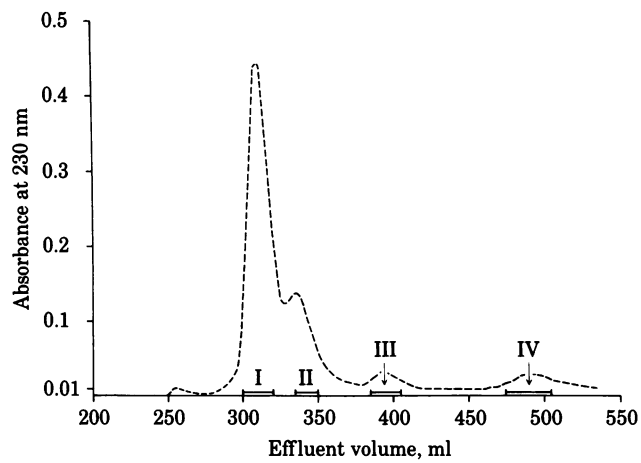

FIG. 3. Chromatography of peak VI from Fig. 2 after complete derivatization with *t*-butoxycarbonyl (*t*-Boc) and reaction with $OsO_4/NaIO_4$ on a Sephadex G-50 superfine column (1.8 × 230 cm) equilibrated with 2 M guanidine·HCl buffered with 0.05 M Tris·HCl (pH 7.5). Flow rate was 25 ml/hr; 5-ml fractions were collected. The fractions under each peak denoted by the bars were pooled.

Table 1. Amino acid compositions

Residue	VI*	II†	III†	II + III	Peptide 5‡
Hyp	8 (8.1)	6 (5.8)	2 (1.8)	8	10
Asp	8 (8.2)	6 (5.6)	2 (1.8)	8	6
Thr§	4 (3.9)	3 (2.6)	1 (1.1)	4	4
Ser§	2 (1.9)	1 (1.1)	1 (1.0)	2	3
Glu	9 (9.2)	6 (6.2)	4 (3.8)	10	8
Pro	8 (7.9)	5 (5.3)	3 (3.2)	8	13
Gly	27 (27.4)	18 (18.3)	7 (7.4)	25	31
Ala	8 (7.5)	5 (4.9)	2 (2.1)	7	8
Val	1 (1.4)	1 (1.0)	—	1	1
Met¶	1 (0.8)	0 (0.3)	1 (1.0)	1	1
Ile	1 (0.7)	0 (0.4)	—	—	1
Leu	8 (7.7)	5 (5.1)	4 (3.7)	9	7
Tyr	0 (0.3)	0 (0.3)	—	—	1
Phe	2 (1.6)	1 (0.8)	1 (1.0)	2	2
Hyl	3 (3.3)	3 (2.8)	—	3	2
His	2 (1.7)	1 (1.0)	2 (1.8)	3	2
Lys	0 (0.4)	0 (0.2)	—	—	—
Arg	4 (4.0)	2 (2.4)	2 (2.3)	4	4
Total	96	63	32	95	104

Values are expressed as the integral number of residues per peptide, with experimental values in parentheses. Unreported values indicate less than 0.2 residue found.
* Fig. 2.
† Fig. 3.
‡ Composition from ref. 12.
§ Uncorrected for destruction on hydrolysis.
¶ Methionine and methionine sulfoxide.

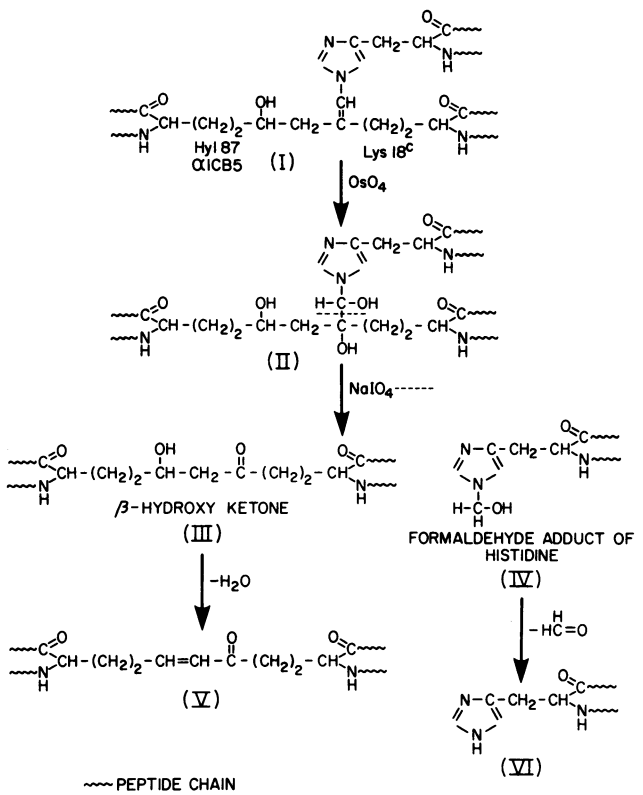

FIG. 4. Specific cleavage of OHAlHis three-chained peptide. The completely *t*-Boc-derivatized peptide (I) reacted with OsO_4 to yield the vic-diol (II). The vic-diol reacted with $NaIO_4$ to yield the β-hydroxyketone crosslinked two-chained peptide (III) and the single peptide containing the formaldehyde adduct of histidine (IV). Elimination of H_2O from the unstable β-hydroxyketone to yield the conjugated α,β-unsaturated ketone (V) occurs in the same manner as aldol condensation in collagen, to yield the α,β-unsaturated aldehyde intramolecular crosslink. Loss of formaldehyde from histidine to give VI is analogous to metaperiodate cleavage of hydroxylysinonorleucine to yield lysine (see ref. 22).

stroyed. Fig. 4 represents the series of reactions used to specifically cleave the *t*-Boc-derivatized three-chained peptide. Amino acid analyses indicated quantitative conversion of compound I (Fig. 4) to compound II. OHAlHis had completely disappeared from the chromatogram. After metaperiodate cleavage (Fig. 4) the reaction mixture was filtered on the Sephadex G-50 superfine column (Fig. 3). Peak II was refiltered and was separated from the small amount of contaminating peak I peptide. Amino acid analyses of peaks II and III are shown in Table 1. Note that the histidine in column II + III represents the predicted amount after metaperiodate cleavage of compound II (Fig. 4). Incomplete cleavage of the peptide was obtained (Fig. 3). It is probable that the reaction conditions (time, temperature, and pH) were not optimal for more complete cleavage. Conditions must be sought to attain quantitative splitting of the crosslink.

The summary of the sequence data for peak III (Fig. 3) is presented in Table 2 and Fig. 5. The data indicates a two-chained crosslinked peptide. No peak representing any phenylthiohydantoin derivative was observed in the organic layer of cycle 14. These results indicated that the hydroxylysine at residue 87 and the lysine in the COOH-terminal nonhelical peptide of α1(I) were involved in the crosslink in the two-chain peptide sequenced.

Our data suggest that an inversion in the sequence occurs at residue 17^c, which corresponds to sequence cycle 13 (Table 2), at which one residue of alanine is clearly identified. In cycle 14, the crosslinked lysine residue is not seen, but it is cleaved because we find a residue of histidine in cycle 15. It must be emphasized that equal amounts of histidine phenylthiohydantoin were recovered in cycles 14 and 15. The same sequence results were obtained five times with the same and other preparations of peak III (Fig. 3).

Attempts to determine the sequence of peak II Fig. 3 as well as the complete three-chained peptide to arrive at the identity of the third chain, which contributes its histidine to OHAlHis, were unsuccessful; the sequence results of the complete three-chained peptide yielded only the expected two sequences seen with peak III. This was believed due to a blocked NH_2-terminal residue on this third chain. It is necessary to prepare more material so that it will be possible to enzymically cleave the isolated third chain, separate the pieces, and determine their sequences to arrive at its structure.

DISCUSSION

Our laboratory has been interested in the structure–function relationships of collagenous tissues containing type I collagen. Although all type I collagen matrices have the same packing arrangements and identical amino acid sequences (24, 25), recent data have indicated that differences in the molecular distribution of the same intermolecular crosslinks might explain the varied physiological functions these tissues display. In order to relate crosslink distribution with structure and function of collagen matrices it is necessary to demonstrate the molecular loci of those prosthetic groups that contribute to the formation of the crosslinks.

It has been suggested that the crosslink OHAlHis forms by condensation, by an as yet unknown mechanism, from one residue each of peptidyl δ-hydroxy-α-aminoadipoyl-δ-semialdehyde, α-aminoadipoyl-δ-semialdehyde, and histidine, with the

Table 2. Amino acid sequence and composition of peak III (Fig. 3) derived after cleavage of peak VI (Fig. 2)

Cycle*	Residue	No. of residues: By sequence	By composition†	Difference‡	Residues 76–90 + 5^c–23^c§
3, 9	Hyp	2	2 (1.8)	0	2
1	Asp	1	2 (1.8)	1	2
5	Thr	1	1 (1.1)¶	0	1
3	Ser	1	1 (1.0)¶	0	1
7, 10	Gln	2			
	Glx		4 (3.8)		
11, 12	Glu	2		0	2
6, 8, 9	Pro	3	3 (3.2)	0	3
1, 4, 7, 10, 13	Gly	5	7 (7.4)	2	7
6, 13	Ala	2	2 (2.1)	0	2
11	Met	1	1 (1.0)‖	0	1
2, 5, 8	Leu	4	4 (3.7)	0	4
4	Phe	1	1 (1.0)	0	1
14, 15	His	2	2 (1.8)	0	2
15	Arg	1	2 (2.3)	1	2
	Lys		—	—	1
12, 14	X**	2			
	Hyl		(0.2)	—	1
	Total	30	32	4	34

* Only 15 cycles on the sequenator were completed. The HPLC chromatogram exhibited single peaks in cycles 12 and 14 that corresponded to one residue of glutamic acid and histidine in each cycle, respectively. Cycle 2 exhibited one peak, which corresponded to two residues of leucine.

† Amino acid analysis indicated only 32 residues. A residue each of lysine and hydroxylysine, which are coupled in a crosslink, bring the total to 34, equal to the theoretical amount from the literature. Values are residues per peptide, experimental values in parentheses. Unreported values indicates less than 0.2 residue found.

‡ The difference of four residues corresponds to positions 20^c to 23^c of the COOH-terminal nonhelical peptide of the α1(I) chain, which has the sequence of Asp-Gly-Gly-Arg.

§ Data from ref. 23.

¶ Uncorrected for destruction on hydrolysis.

‖ Methionine and methionine sulfoxide.

** From sequence considerations the crosslink in this two-chain peptide consists of a residue of hydroxylysine linked to a residue of lysine.

latter contributing the N$^\tau$ of the imidazole ring (11).

The proposed structure containing an ethylene group on the N$^\tau$ of His (I in Fig. 4) allowed one of us (G.L.M.) to propose from previous experiences (17) a series of reactions that would specifically cleave the crosslink into two moieties (Fig. 4). We

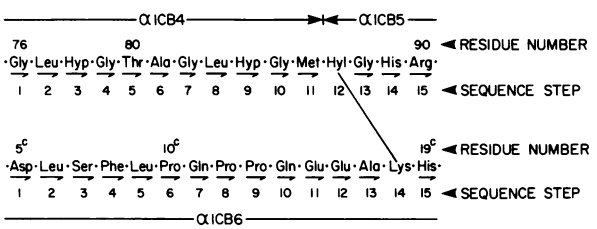

FIG. 5. Sequence of two-chained crosslinked peptide obtained by specific cleavage of three-chained OHAlHis crosslink peptide. The residue numbers in the collagen sequence are above the amino acid residues. The half-headed arrows with the numbers of the sequence step below are results obtained from the sequenator. The arrows denote that the residue was identified in the sequence step assigned. See Table 2 for other details.

therefore prepared peptide 5, the three-chained peptide containing OHAlHis, by the methods described previously (12). We further purified this heterogeneous fraction (Fig. 2) and found three peaks (IV, V, and VI) that contained OHAlHis, with each peak possessing very similar amino acid compositions (typical example, peak VI in Table 1). Chromatographic separation of the three on the DEAE-cellulose column could possibly be due to variances in amidation of COOH groups.

The amino acid compositions and the sequence results obtained after cleavage seemed to confirm the proposed structure of OHAlHis (11).

Sequence analyses and amino acid composition of peak III (Fig. 3) unequivocally demonstrated that it was a two-chained crosslink peptide (Table 2 and Fig. 5). The residues that participate in the crosslink are Hyl-87 (in α1CB5^{ald}; ald indicating the aldehyde form) and the lysine residue in the COOH-terminal nonhelical peptide portion of α1CB6^{ald}. The amino acid composition and sequence indicate that the portion of α1CB6^{ald} isolated as part of the two-chained peptide described in this report consists of 19 residues starting with Asp-5^c and ending with Arg-23^c. In the earlier work describing the sequence of the COOH-terminal portion of α1(I), a 44-residue peptide isolated from a tryptic digest of the α1(I) chain of bovine collagen started with Thr-993 and terminated with Arg-23^c (26, 27). The peptide isolated in this report undoubtedly resulted from a chymotryptic cleavage that occurred between Tyr-4^c and Asp-5^c. It is known that even tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, which was used to digest the collagen, contains significant chymotryptic activity (28). The other branch of the crosslinked two-chained peptide, which started with Gly-76 and terminated with Arg-90, resulted from a typical tryptic cleavage at Arg-75 and Arg-90.

In this paper we report apparent structural microheterogeneity surrounding the crosslinking moiety (α-aminoadipoyl-δ-semialdehyde) in the COOH-terminal nonhelical region of α1CB6^{ald}. Starting with 15^c and ending at 19^c, the previously published sequence (23, 26, 27) is Glx-Glx-Lys(ald)-Ala-His, whereas we have found the sequence in the crosslinked peptide for the same residue numbers to be Glu-Glu-Ala-Lys(ald)-His. The evidence for this sequence inversion relies on the finding that one residue of glycine and one residue of alanine were recovered in cycle 13 and one residue of histidine and no residue for the crosslinked lysine was detected in cycle 14, a finding that departs from the previously published sequence Lys-Ala-His. In order for this sequence of recoveries to occur for the sequence Lys-Ala-His, a completely unknown chemical reaction would have to be proposed whereby the modified lysine-derived crosslinking residue cleaves from the alanine in the sequencing cycle before it is supposed to be degraded. The latter is highly unlikely because our data from multiple sequencer runs demonstrate alanine in good yield on cycle 13. For its true location to be cycle 14, the previous residue (crosslinked lysine) would have to undergo complete cleavage during the coupling step (basic pH). For example, histidine, which has a tendency to undergo premature cleavage, does so only to the extent of 10% in our sequencing system when it does occur. However, because we found equal amounts of histidine in cycles 14 and 15 we can presume that no premature cleavage is occurring. In addition, the other chain containing the other member of the crosslink has the sequence Hyl-Gly-His. This cleaved normally and reads as the normal sequence for α1CB5. It is these facts that permit us to propose the sequence microheterogeneity found in α1CB6.

The molar amount of three-chained peptide in the insoluble bovine skin collagen represents a small percentage of the total amount of the three same peptides uncrosslinked in the type

I collagen present in bovine skin. Recently the sequence of the bases in a portion of the more abundant cDNA that codes for α1(I) in chicken collagen has been determined (29). The peptide sequence represented by the base triplets for the same peptide residues as above are Glu-Glu-Lys-Ala-His, the sequence reported in the literature that was described above. It therefore seems likely that there may exist small amounts of another structural gene that codes for an α1(I) whose expression is the altered sequence found here. The newly found sequence Ala-Lys, coupled to the formation of δ-hydroxy-α-aminoadipoyl-δ-semialdehyde from Hyl-87, allows for the "anomalous" production of the complex nonreducible stable crosslink OHAlHis in bovine skin collagen. It is well known that, the more crosslinked a collagen is, the less susceptible it is to enzymic digestion. With age OHAlHis crosslinked collagen may build up in a collagenous tissue because its degradation rate is slower.

Another odd fact concerns the residue δ-hydroxy-α-aminoadipoyl-δ-semialdehyde (derived from Hyl-87), whose prosthetic group is part of the crosslink. It has been reported that Hyl-87 in α1CB5 is about 80% glycosylated in rat skin (30) whereas it is only 60% glycosylated in bovine skin collagen (15). We believe that glycosylation of hydroxylysine prevents it from forming in aldehyde. This is substantiated by the fact that glycosylated dihydroxynorleucine has never been found in any NaB^3H_4-reduced collagen in any genetic type (unpublished data), whereas nonglycosylated dihydroxynorleucine is abundant in reduced collagens (17, 31). Glycosylated Hyl-87 in α1CB5 has been found in crosslinked peptide form with Lys-17^c in α1CB6^{ald} previously as glycosylated dihydroxylysinonorleucine and hydroxylysinonorleucine (16, 32–34). It is remarkable that the small portion of nonglycosylated hydroxylysine at residue 87 in bovine skin collagen forms an aldehyde and, coupled with the sequence inversion, allows the formation of the crosslink OHAlHis. This suggests that the role of glycosylation of certain specific hydroxylysine residues in collagen is to ensure the formation of simple bimolecular crosslinks and prevent the formation of δ-hydroxy-α-aminoadipoyl-δ-semialdehyde in order to control complex stable crosslink formation. The lack of complete glycosylation of Hyl-87 and the presence of a small amount of another α1(I) structural gene that codes the sequence inversion in collagen are possibly biologic errors of evolution.

Recently it has been demonstrated that the COOH-terminal nonhelical portion of the collagen molecule contains the Lys-17^c that is converted to a residue of α-aminoadipoyl-δ-semialdehyde before any NH_2-terminal nonhelical peptidyl lysine is converted to aldehyde (5). In this report we have concluded that Hyl-87 in α1CB5^{ald} participates as an aldehyde function in the formation of the crosslink OHAlHis. In the fibril packing of collagen molecules, as derived from crosslinking studies (8), residues 87–89 in α1CB5 are juxtaposed to Lys(ald)-17^c, which Fukae and Mechanic (5) propose as the binding site for lysyl oxidase. The conversion of nonglycosylated Hyl-87 in α1CB5 to an aldehyde supports the proposal that the conversion of other ε-NH_2 groups to aldehydes in collagen is due to the juxtaposition of the lysyl oxidase binding site to other sites because of the specific "quarter-stagger" packing of collagen molecules in the fibril.

We thank Dr. David Eyre for an authentic sample of the pyridinoline crosslink. This work was supported by National Institutes of Health Grants DE-02668, HL 22040, and AM 19969.

1. Gallop, P. M., Blumenfeld, O. O. & Seifter, S. (1972) *Annu. Rev. Biochem.* **41**, 617–672.
2. Siegel, R. C. (1976) *J. Biol. Chem.* **251**, 5786–5792.
3. Bornstein, P., Kang, A. H. & Piez, K. A. (1966) *Biochemistry* **5**, 3803–3812.
4. Tanzer, M. L. (1968) *J. Biol. Chem.* **243**, 4045–4054.
5. Fukae, M. & Mechanic, G. L. (1980) *J. Biol. Chem.* **255**, 6511–6518.
6. Franzblau, C., Kang, A. H. & Faris, B. (1970) *Biochem. Biophys. Res. Commun.* **40**, 437–444.
7. Tanzer, M. L., Housley, T., Berube, L., Fairweather, R., Franzblau, C. & Gallop, P. M. (1973) *J. Biol. Chem.* **248**, 393–402.
8. Bernstein, P. H. & Mechanic, G. L. (1980) *J. Biol. Chem.* **255**, 10414–10422.
9. Robins, S. P. & Bailey, A. J. (1973) *Biochem. J.* **135**, 657–665.
10. Robins, S. P. & Bailey, A. J. (1977) *Biochem. J.* **163**, 339–346.
11. Housley, T., Tanzer, M. L., Henson, E. & Gallop, P. M. (1975) *Biochem. Biophys. Res. Commun.* **67**, 824–830.
12. Becker, U., Furthmayr, H. & Timpl, R. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 21–32.
13. Morgan, P. H., Jacobs, H. G., Segiest, J. P. & Cunningham, L. W. (1970) *J. Biol. Chem.* **245**, 5042–5048.
14. Piez, K. A., Miller, E. J., Lane, J. M. & Butler, W. T. (1970) *Chemistry and Molecular Biology of the Intercellular Matrix*, ed. Balazs, E. A. (Academic, New York), Vol. 1, pp. 117–125.
15. Bornstein, P. & Traub, W. (1979) in *The Proteins*, eds. Neurath, H. & Hill, R. L. (Academic, New York), 3rd Ed., Vol. 4, pp. 457 & 501.
16. Eyre, D. R. & Glimcher, M. J. (1973) *Biochem. J.* **135**, 393–403.
17. Mechanic, G. L. & Tanzer, M. L. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1597–1604.
18. Tarr, G. E., Beecher, J. F., Bell, M. & McKean, D. J. (1978) *Anal. Biochem.* **84**, 622–627.
19. Moroder, L., Hallett, A., Wunsch, E., Keller, O. & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1651–1653.
20. Reinecke, M. G., Kray, L. R. & Francis, R. F. (1972) *J. Org. Chem.* **37**, 3489–3495.
21. Okno, M., Tsukamoto, S., Makisumi, S. & Izumiya, N. (1972) *Bull. Chem. Soc. Jpn.* **45**, 2852–2855.
22. Tanzer, M. L. & Mechanic, G. (1970) *Biochem. Biophys. Res. Commun.* **39**, 183–189.
23. Fietzek, P. P. & Kuhn, K. (1976) *Int. Rev. Connect. Tissue Res.* **7**, 1–60.
24. Kuboki, Y. & Mechanic, G. L. (1974) *Connect. Tissue Res.* **2**, 223–230.
25. Kuboki, Y. & Mechanic, G. L. (1982) *Calcif. Tissue Int.* **34**, 306–308.
26. Becker, U., Timpl, R. & Kuhn, K. (1972) *Eur. J. Biochem.* **28**, 221–231.
27. Rauterberg, J., Fietzek, P., Rexrodt, F., Becker, U., Stark, M. & Kuhn, K. (1972) *FEBS Lett.* **21**, 75–79.
28. Jany, K. D., Keil, W., Meyer, H. & Kiltz, H. H. (1976) *Biochim. Biophys. Acta* **453**, 62–66.
29. Fuller, F. & Boedtker, H. (1981) *Biochemistry* **20**, 996–1006.
30. Butler, W. T. (1969) *Biochemistry* **9**, 44–50.
31. Mechanic, G. L., Gallop, P. M. & Tanzer, M. L. (1971) *Biochem. Biophys. Res. Commun.* **45**, 644–653.
32. Eyre, D. R. & Glimcher, M. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 663–670.
33. Kuboki, Y., Takagi, T., Shimokawa, H., Oguchi, H., Sasaki, S. & Mechanic, G. L. (1982) *Connect. Tissue Res.* **9**, 107–114.
34. Kuboki, Y., Tsuzaki, M., Sasaki, S., Fang Liu, C. & Mechanic, G. L. (1981) *Biochem. Biophys. Res. Commun.* **102**, 119–126.