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The Identity of a Cyanogen Bromide Fragment of Bovine Dentin Collagen Containing the Site of an Intermolecular Cross-Link†

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ABSTRACT: A peptide fraction isolated from a cyanogen bromide digest of bovine dentin collagen had a molecular weight of 46 000. Its size and amino acid composition indicated that it could not consist of peptides derived from the cleavage of a single α chain. On reduction with tritiated sodium borohydride, radioactivity was incorporated primarily into 5,5′-dihydroxylysinonorleucine without degradation at the peptide backbone. Periodate cleavage of the reduced or nonreduced peptide fraction generated one fragment of molecular weight 28 000 and one of 18 000 completely accounting for the size of the parent peptide. On amino acid analysis the constituent single-chain peptides were determined to be α2CB4 and α1CB6. Both peptides isolated after periodate oxidation of the tritiated borohydride reduced cross-link peptide were found to contain [^{3}H]hydroxynorvaline. These data show that some hydroxylysine of α2CB4, a helical region peptide, was present in aldehyde form and could act as the aldehyde donor in cross-link, Schiff's base formation. The only cross-linkage of this α2CB4 acting as an aldehyde donor peptide to α1CB6 would be a helical region to helical region bond, perhaps accounting for the unusual stability and low solubility of dentin collagen.

Bone and dentin collagens are stabilized by interchain cross-links of the Schiff base type, involving lysine or hydroxylysine and hydroxyallysine (Mechanic et al., 1971; Davis and Bailey, 1971), the aldehyde formed by oxidative deamination at the ϵ-amino group of hydroxylysine. The Schiff base may then be stabilized by reduction in vivo (Mechanic et al., 1971), by rearrangement to the corresponding ketimine (Miller and Robertson, 1973; Eyre and Glimcher, 1973a; Mechanic et al., 1974), or possibly by condensation with sterically available lysine or hydroxylysine residues on adjacent α chains (Davis et al., 1975).

Little is presently known about the number or location of such cross-links in insoluble collagens, although several authors have stressed the importance of lysine and hydroxylysine residues which may be oxidized to the corresponding aldehydes (Kang et al., 1967; Rauterberg et al., 1972), and which are present in the nonhelical terminal extensions of the α chains. Kang (1972) demonstrated the presence, in sodium borohydride reduced rat tail tendon collagen, of a cross-link peptide which involved the amino terminus of one α1 chain and the carboxy terminus of an adjacent α1 chain. This was purified and determined to be the cross-linked CNBr[1] peptide—(α1CB1 × α1CB6). Dixit and Bensusan (1973) isolated the analogous peptide from a CNBr digest of reduced insoluble bovine corium collagen, together with two others, both involving the carboxy-terminal CNBr peptide of the α1 (I) chain—α1CB6. Volpin and Veis (1973) and Zimmerman et al. (1973) found the yield of this latter peptide to be signifi-

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[1] Abbreviations used: CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine.

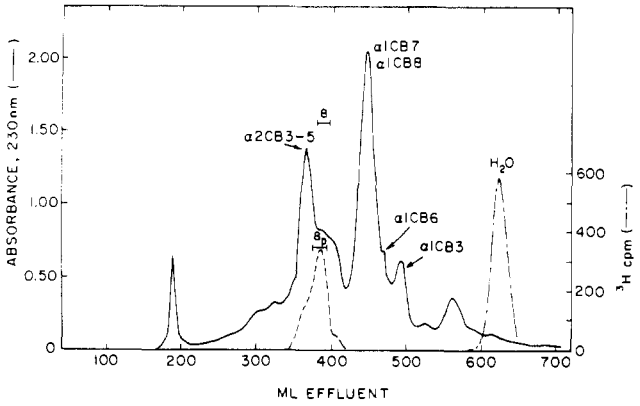

FIGURE 1: Chromatography of bovine dentin collagen CNBr peptides on Agarose A-5m. Material from between the bars labeled "8" was pooled, desalted on Bio-Gel P2, lyophilized, and re-chromatographed on the same system to give the inset elution profile (broken line). Material from this peak, "8p", was desalted, lyophilized, and used for subsequent experiments.

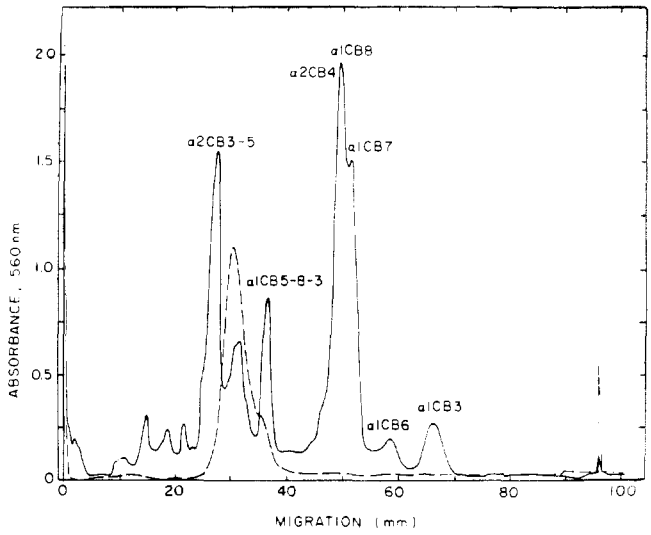

FIGURE 2: Optical density scan of sodium dodecyl sulfate gel electrophoresis of CNBr peptides of bovine dentin insoluble collagen. Peak defined by broken lines between α2CB3-5 and α1CB5-8-3 is that given by agarose fraction 8p (see Figure 1).

cantly reduced in CNBr digests of insoluble bovine skin collagen. Recovery was increased by brief pepsin treatment of the insoluble collagen prior to digestion. This treatment is known to remove the nonhelical carboxy-terminal portion of the α1 chain which contains a lysine or hydroxylysine residue which may be converted to the aldehyde (Zimmerman et al., 1973) and incorporated into a cross-link.

Eyre and Glimcher (1973a) recently described the probable location of a cross-link in calf bone collagen as between this nonhelical region of the α1 chain and the helical region of an adjacent α chain. Kuboki et al. (1973) isolated three tryptic peptides containing 5,5′-dihydroxylysinonorleucine from bovine dentin collagen, but insufficient information was available at that time to permit assignment of the cross-links to specific loci on the donor α chains. However, it was demonstrated that the cross-link peptides did not involve the NH_2-terminal portion of collagen.

Preliminary evidence has been presented (Volpin and Veis, 1973) that the CNBr digest of bovine dentin collagen contains a cross-linked fragment involving α1CB0, 1, and α1CB6. The present paper describes evidence for the occurrence of a second, more prevalent, double-chain peptide involving α1CB6.

Materials and Methods

Preparation and Isolation of CNBr Peptides. Bovine dentin collagen was prepared from the unerupted molars of 18-month-old animals and cleaved with CNBr as previously described (Volpin and Veis, 1973). Agarose chromatography was carried out on two coupled 2 × 100 cm columns of Agarose A5 m (Bio-Rad Labs), eluted with 1 M $CaCl_2$–0.05 M Tris-HCl, pH 7.5 (Piez, 1968). The columns were calibrated for molecular weight determinations with the known CNBr peptides of bovine dentin collagen (Volpin and Veis, 1973) and with α, β and γ components of soluble collagen. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out as described by Furthmayr and Timpl (1971), using 11 × 0.5 cm gels of 7.4% acrylamide, stained according to Fairbanks et al. (1971). These were scanned at 560 nm on a Gilford 2400S spectrophotometer fitted with a linear transporter permitting quantitation of resolved components (Scott et al., 1976). When required, 1-mm slices were prepared, digested in NCS tissue solubilizer (Amersham/Searle) and counted in a Nuclear Chicago Isocap 300 scintillation counter.

Reduction of CNBr Peptides. For reduction, the peptide fraction was dissolved in 0.2 M sodium phosphate buffer, pH 7.4 with heating at 40 °C for 10 min. After cooling to 15 °C, tritiated sodium borohydride (Amersham/Searle, 820 mCi/mmol) was added, at a reductant:protein ratio of approximately 1:20 (w/w). After 1 h, a second aliquot was added, followed 1 h later by acidification to pH 4.0–4.5 with glacial acetic acid. The solution was then desalted on a column of Bio-Gel P2 (Bio-Rad Labs) eluted with 0.1 M acetic acid.

Cleavage of Cross-Link Peptides. Periodate degradation was carried out on peptide fractions dissolved in 0.2 M sodium phosphate buffer, pH 5.0, to which was added an equal volume of 0.05 M sodium metaperiodate in the same buffer. After 2 h in the dark at room temperature, sufficient cold sodium borohydride was added to simultaneously destroy unreacted periodate and to reduce any aldehydes generated. After standing for 1 h, the solution was acidified with glacial acetic acid (to pH 4.5) and desalted on Bio-Gel P2.

Component Analyses. Amino acid analyses were performed in duplicate on a Jeolco JL6AH automatic amino acid analyzer after hydrolysis with 6 N HCl, in vacuo, at 110 °C for 22 h.

The system described by Mechanic (1974) was employed for determination of the nature of reduced cross-link components and precursors.

Results

Figure 1 shows the elution profile of the CNBr peptides chromatographed on agarose A-5m. Each of the components indicated in Figure 1 is labeled on the basis of identifications previously made in this laboratory (Volpin and Veis, 1973; Scott and Veis, 1976a,b). The shoulder on the α2CB3-5 peak, labeled fraction 8, did not correspond in weight or composition to any peptide obtainable from single α1 or α2 chains, as previously noted (Scott and Veis, 1976b). Sodium dodecyl sulfate gel electrophoresis of the mixture of CNBr peptides gave better resolution in the region migrating just ahead of α2CB3-5, and material corresponding to fraction 8 is distinctly separated from α2CB3-5 and the uncleaved peptide α1CB5-8-3 (Figure 2). The dashed line in Figure 1 indicates the agarose elution of re-chromatographed fraction 8. Material from the center of this peak, purified fraction 8, labeled 8p in Figure 1, migrates as a single main component on gel electrophoresis, the dashed line of Figure 2, with only a minor contaminating amount of α1CB5-8-3.

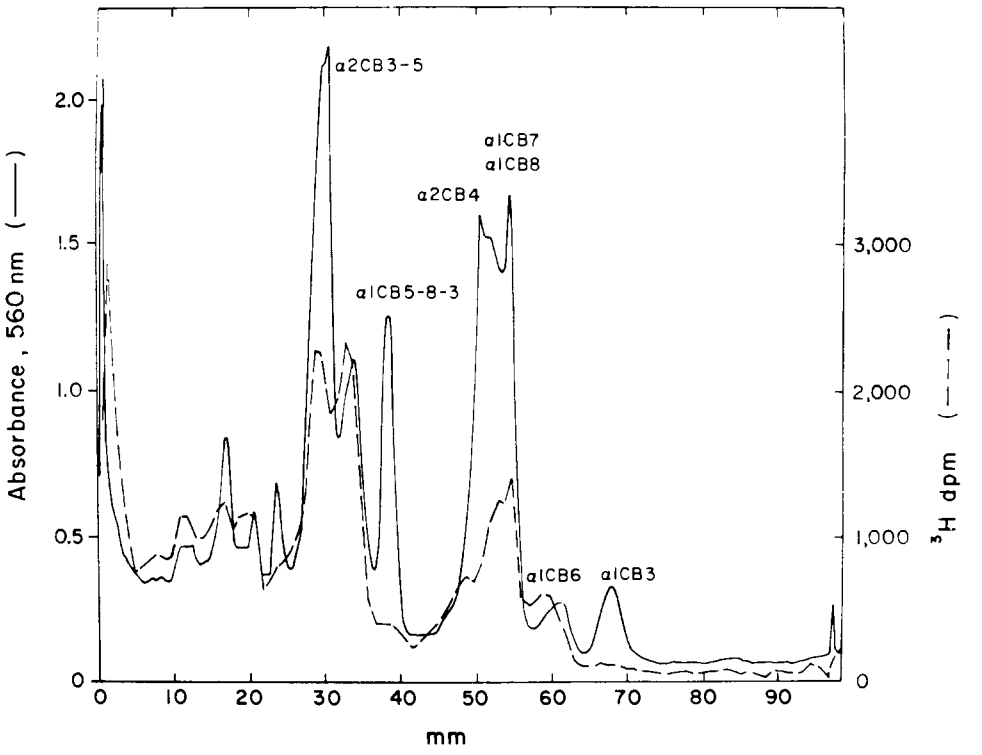

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of CNBr peptides produced from bovine dentin collagen after reduction with tritiated sodium borohydride. Peak corresponding to agarose fraction 8p (migration distance 33 mm) contains a significant proportion of the total counts and is labeled at high apparent specific activity.

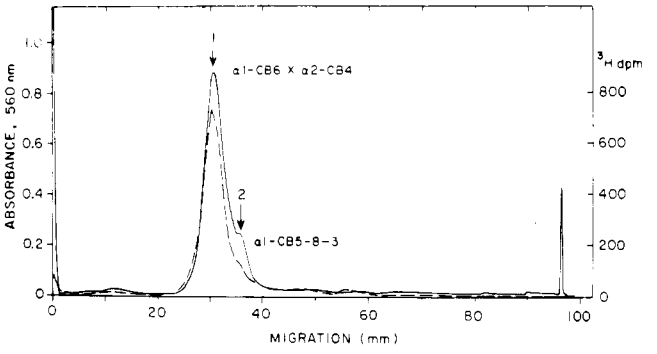

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of agarose fraction 8p after reduction with tritiated sodium borohydride. Only trace amounts of tritium were incorporated into the small proportion of contaminating α1CB5-8-3 (peak 2).

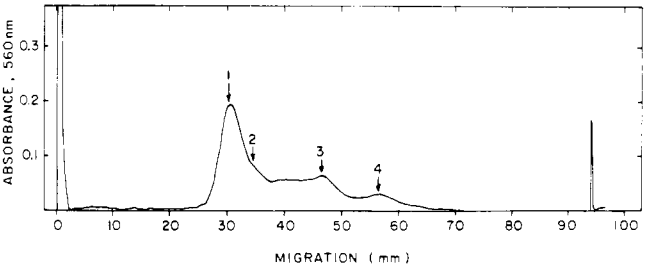

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis of unreduced agarose fraction 8p after oxidation with sodium metaperiodate (same sample as that used for reduction (Figure 4).

Reduction of the collagen with tritiated borohydride before CNBr digestion introduces tritium into many peptides but fraction 8 is labeled at high specific activity (dpm/unit area of staining) and accounts for a significant proportion of the total activity (Figure 3). The isolated 8p can be reduced directly and the activity corresponds almost entirely to the main component (Figure 4). No products of reductive peptide bond cleavage (Paz et al., 1970) were apparent. These data (Figures 3 and 4) show that fraction 8p contains a readily reducible bond or group.

Periodate treatment of unreduced 8p leads to a decrease in the relative amount of the major component (1, Figure 5), no change in the contaminating uncleaved peptide (2, Figure 5) and the appearance of two new components (3 and 4, Figure 5). From the relative areas of these component regions it appears that 30–40% of the main component has been cleaved by the periodate. For preparative purposes a large quantity of 8p was reduced with [^{3}H]borohydride, cleaved with periodate, and then chromatographed on agarose. In this large scale preparation experiment it was evident that several higher molecular weight species had been formed (A, B, and C of Figure 6). This polymerization only occurred when the peptide concentration was greater than about 1 mg/ml in the reaction mixture and was not encountered during small scale experiments (e.g., Figure 5). Components D, E, and F of Figure 6 correspond to components 1, 3, and 4 visualized on the sodium dodecyl sulfate gel electrophoresis (Figure 5). The higher weight components probably arose from the polymerization of some peptides with the formaldehyde and/or norvaline aldehyde resulting from the periodate oxidation of hydroxylysine, and cross-linkages. It is interesting to note that peaks E and F were not equivalent in ^{3}H-specific activity. Peak F was more heavily labeled.

Molecular weights were estimated for 8p and its periodate digestion products by both sodium dodecyl sulfate gel electrophoresis and agarose chromatography (Table I). Both α1 and α2 chain CNBr peptides migrate according to molecular weight on agarose columns, but α1 and α2 chain peptides of the same weight migrate at different rates on sodium dodecyl sulfate gel electrophoresis (Furthmayr and Timpl, 1971).

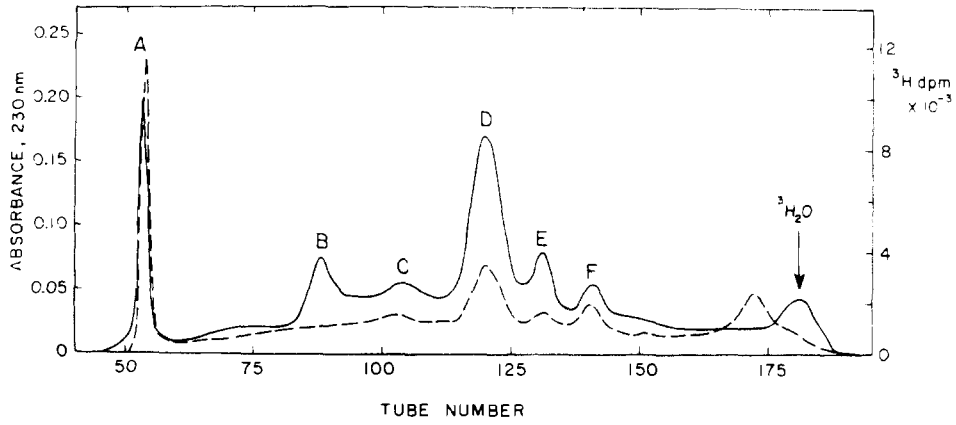

FIGURE 6: Agarose A-5m chromatography of products of sequential reduction with tritiated sodium borohydride, oxidation with sodium metaperiodate and reduction with unlabeled sodium borohydride of 8p. Material from peaks E and F was pooled, desalted, lyophilized, and analyzed for amino acid composition.

TABLE I: Molecular Weights Determined for Agarose Fraction 8p and Periodate Degradation Products.

	$NaDodSO_4$ Gel Electrophoresis	Agarose Chromatography
Untreated	49 000[a]	
	52 000[b]	
Periodate treated	1[c] 50 000[a]	D[d] 46 000
	53 000[b]	
	3[c] 26 800[a]	E[d] 28 000
	30 600[b]	
	4[c] 18 300[a]	F[d] 18 000
	21 800[b]	

[a] Determined from calibration for α1 chain peptides. [b] Determined from calibration for α2 chain peptides. [c] Component designation, Figure 5. [d] Component designation, Figure 6.

Therefore, the weights determined by the gel electrophoresis technique may suffer from an error in assignment of chain type. Agarose chromatography yields a molecular weight of 46 000 for 8p and sodium dodecyl sulfate gel electrophoresis, a range of 49 000–53 000 depending upon the calibration used. The two degradation fragments have weights of 28 000 (E) and 18 000 (F) via agarose or 26 000 to 30 600 (E) and 18 300–21 800 (F) via electrophoresis. In either case, the molecular weights of the two degradation products effectively account for that determined for the intact peptide 8p. Moreover, they were formed in a molar ratio 1:1. The basic conclusion from this phase of the study is that 8p is a peptide composed of one chain each of E and F joined by a periodate sensitive linkage.

The amino acid compositions of 8p and E and F are presented in Table II and compared with the composition expected for the cross-linked peptide (α1CB6 × α2CB4) based on previously published amino acid compositions of α1CB6 and α2CB4 from bovine dentin collagen (Volpin and Veis, 1973). These peptides were chosen for comparison because of the reduced amounts of α1CB6 and α2CB4 present in the dentin CNBr digest, the presence of histidine in 8p and the molecular weights of the components involved. Component E shows good agreement with the analysis of α2CB4, component F with α1CB6, and the sum with that predicted for (α1CB6 × α2CB4). The major differences in composition may be ascribed directly to the effects of periodate.

Control experiments were carried out in which previously analyzed mixtures of α1 and α2 chain CNBr peptides were oxidized with periodate and then reduced with borohydride as in the case of the agarose fractions E and F of 8p. The major changes were the expected loss of hydroxylysine and an apparently equivalent increase in "homoserine". As noted in the discussion which follows, the combination of reactions produces hydroxynorvaline from the oxidized → reduced hydroxylysine and cross-links. In the amino acid analyzer system in use for these analyses hydroxynorvaline and homoserine coelute. Comparison of the analyses of 8p and the column headed $\sum$(E + F) in Table II shows that the sum of "apparent" homoserine and hydroxylysine remains constant in spite of treatment. The fact that some hydroxylysine persists after periodate oxidation is consistent with the previous observation (Volpin and Veis, 1973) that α2CB4 from dentin contains 1 to 2 residues and α1CB6 about 0.5 residue of glycosylated hydroxylysine. In the control experiments partial losses of tyrosine and histidine were also noted.

Fraction 8p reduced with [^{3}H]borohydride yielded a mixture of dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) together with lesser amounts of acidic, labeled products not related to known cross-links (Figure 7). The ratio ^{3}H dpm, DHLNL:HLNL, was ~11/1, indicating unequivocally that DHLNL is the major cross-linkage component in the cross-linked peptide (α1CB6 × α2CB4).

The two metaperiodate cleavage-product fractions, E and F of [^{3}H]borohydride reduced (α1CB6 × α2CB4), were analyzed for radioactivity after reduction with nonradioactive borohydride followed by hydrolysis to amino acids (Figure 8). In the system used for these analyses (Mechanic, 1974), hydroxynorvaline is well separated from homoserine. Both fractions contained [^{3}H]hydroxynorvaline, indicating that both had contained norvaline aldehyde (Mechanic et al., 1974). Surprisingly α2CB4 was more heavily labeled with hydroxynorvaline, 8261 dpm/nmol of peptide, than was α1CB6, 3330 dpm/nmol of peptide. A relatively greater proportion of the counts in α1CB6 were in species which eluted rapidly from the amino acid analyzer column, well before the reduced cross-links or precursors. Such nonspecific and as yet unidentified labeled components are typically seen in [^{3}H]borohydride reduced collagen preparations (Mechanic et al., 1971) and may be responsible for part of the general labeling noted as background in Figure 3.

Control reductions of non-cross-linked collagen α chains with [^{3}H]borohydride introduced a small amount of label in

TABLE II: Compositions of Fraction 8p and the Lower Molecular Weight Components Produced by Periodate Oxidation and Stabilization with Sodium Borohydride Reduction.[a]

Amino Acid	8p	Periodate Produced Fragments of 8p				Expected Composition
		E (α2CB4)[b]		F (α1CB6)[b]	Σ(E + F)	(α1CB6 × α2CB4)[b]
3Hyp	Present[c]			Present[c] (1.1)	Present[c]	1.1
4Hyp	51	32 (30)		20 (19)	52	49
Asp	25	13 (14)		8.9 (8.6)	22	23
Thr	9.0	6.0 (6.1)		3.6 (4.0)	9.6	10
Ser	16	11 (9.3)		8.2 (7.1)	19	16
Glu	36	21 (22)		14 (13)	35	35
Pro	57	35 (39)		26 (27)	61	66
Gly	169	101 (109)		66 (64)	167	173
Ala	62	36 (37)		22 (22)	58	59
Val	11	7.4 (10)		3.1 (3.3)	11	13
Met	0[d]	0 (0[d])		0 (0[d])	0	0
Ile	6.4	3.2 (3.7)		2.2 (1.9)	5.4	5.6
Leu	13	7.6 (9.4)		4.2 (4.0)	12	13
Tyr	1.7	0.3 (0[d])		0.4 (0[d])	0.7	0
Phe	6.2	4.0 (3.6)		1.7 (1.8)	5.7	5.4
Lys	9.4	5.4 (6.4)		2.7 (4.9)	8.1	11.3
Hyl	5.9	0.7 (4.1)		0.3 (1.4)	1.0	5.5
His	2.5	0.8 (2.2)		0.4 (0.8)	1.2	3.0
Arg	28	17 (17)		12 (11)	29	28
Hse	1.3	3.2[e] (1.0)		2.2[d]	5.4[e]	1.0
Total	510[f]	306[f] (322)		198[f] (195)	504[f]	518

[a] Actual values shown when less than ten residues per peptide. [b] Values for α1CB6 and α2CB4 from Volpin and Veis (1973). [c] Insufficient for quantitation. [d] Denotes less than 0.2 residue. [e] Sum of homoserine and hydroxynorvaline (see text). [f] Calculated from molecular weight (agarose and average residue weight).

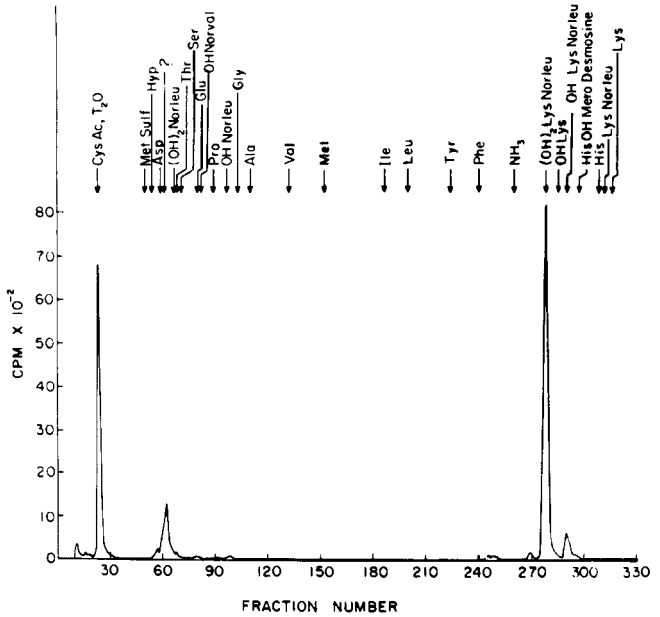

FIGURE 7: Chromatography of tritium-labeled amino acids from reduced 8p on the automated cross-link analysis system (Mechanic, 1974). The rapidly eluting species are not considered to be related to cross-links or cross-link precursors. The elution positions of amino acids in this system are indicated by the arrows at the top of the chromatogram.

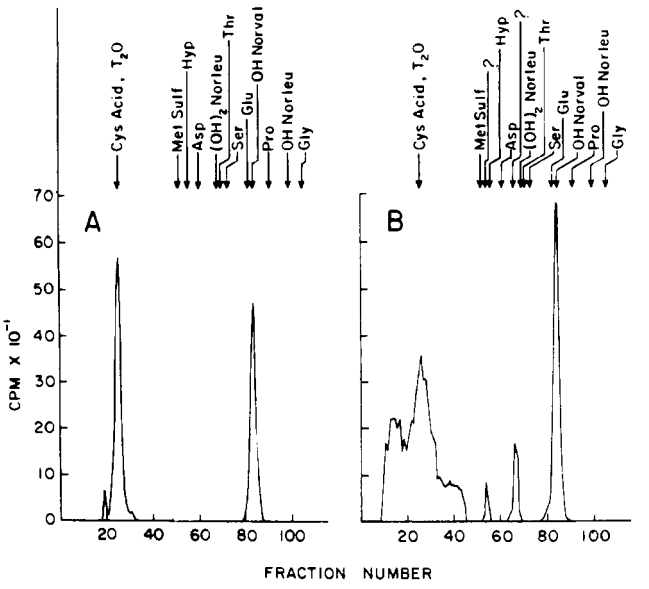

FIGURE 8: Chromatography of radioactive amino acids from the periodate-released fragments of 8p. (A) Hydrolysate of peak E, α2CB4. (B) Hydrolysate of peak F, α1CB6.

a nonspecific fashion but neither [^{3}H]hydroxynorvaline nor [^{3}H]homoserine, which could possibly arise from glutamine and asparagine, respectively, were detected on the cross-link analyzer.

Discussion

From the earliest studies of dentin (Schlueter and Veis, 1964) and bone collagen (Glimcher and Katz, 1965) swelling and solubility data made it evident that bone and dentin collagens were either very highly cross-linked or linked in a particularly stable fashion. Application of the CNBr digestion procedure to dentin and correlation of the resultant peptides with the known peptides of type I collagen show the presence of several peptide fractions which cannot be accounted for either as a single α1- or α2-chain peptide or as an uncleaved peptide (Scott and Veis, 1976b). The most prominent of these is the component labeled 8p in Figures 1 and 2. This had a molecular weight of 46 000 (Table I) on calibrated agarose

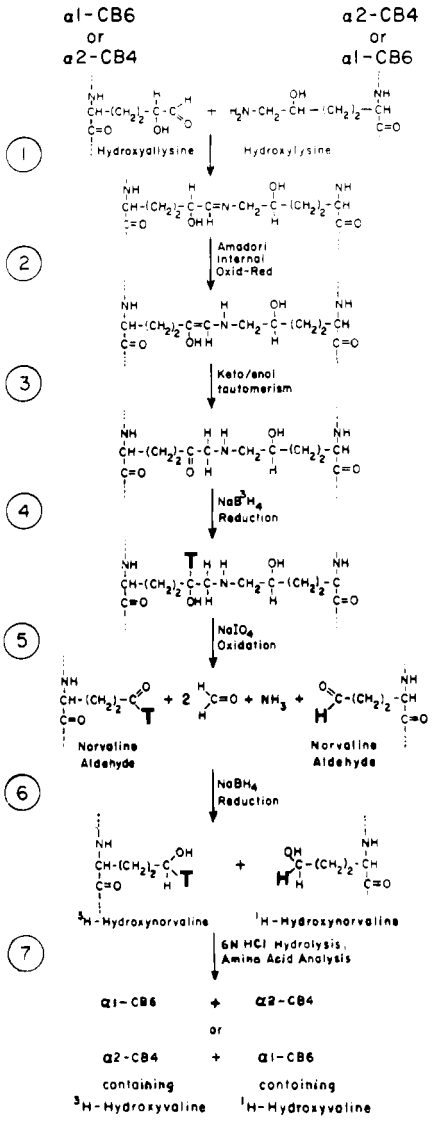

FIGURE 9: Reaction scheme to show probable route of formation ketimine cross-links, $\alpha 1CB6_{ald} \times \alpha 2CB4_{amino}$ and $\alpha 2CB4_{ald} \times \alpha 1CB6_{amino}$ (steps 1–3), reactions leading to isolation of the single-chain peptides (steps 4–6) and to release of [^{3}H]hydroxynorvaline (step 7).

columns and can be cleaved into two lower molecular weight peptides by periodate oxidation before or after borohydride reduction. In spite of small differences between the observed and expected amino acid compositions there seems little doubt that these periodate released peptides, E and F of Figure 6, are α2CB4 and α1CB6, respectively (Table II). These each have unique compositions which do not permit confusion with peptides of similar size which might have resulted from incomplete cleavage at methionine residues (Scott and Veis, 1976a,b). The peptides α2CB3 and α2CB5 are similar in molecular weight to α2CB4, but there are significant differences in amino acid composition (Volpin and Veis, 1971, 1973). Moreover, these peptides are not usually isolated separately in significant yield from insoluble bovine collagens, rather they appear as the larger uncleaved peptide, α2CB3-5 (Volpin and Veis, 1973).

The two-chain peptide contains one or more bonds readily reducible with tritiated sodium borohydride. Hydrolysis in 6 N HCl yields [^{3}H]DHLNL as the predominant (>90%) tritium labeled cross-linking component, conclusively establishing that the major reducible cross-linkages in (α1CB6 × α2CB4) are between two hydroxylysine side chains. The ability to isolate the (α1CB6 × α2CB4) peptide from a CNBr digest without prior reduction is not surprising. It has been shown that cross-links derived from hydroxylysine and hydroxyallysine may undergo rearrangement to form 5-keto-5′-hydroxylysinonorleucine which is stable in concentrated formic acid under the reaction conditions (Miller and Robertson, 1973). Direct quantitation (Mechanic et al., 1974) indicates this reaction is about 83% complete for dehydro-5,5′-dihydroxylysinonorleucine in dentin.

The low recovery of isolated α1CB6 and α2CB4 following metaperiodate oxidation and the large proportion of uncleaved 8p (Figure 6) make it difficult to conclude that 8p consists entirely of these two peptides linked by a single DHLNL precursor cross-linkage. However, rather inefficient (40%) cleavage with metaperiodate is not unexpected and side reactions of the aldehydes produced by the degradative oxidation can lead to the polymerization of peptides present in the reaction mixture, thus accounting for the production of the higher weight components, A, B, and C of Figure 6, and further reducing the yield of constituent single chain peptides.

The remaining question is: which peptide is the aldehyde donor? Both α1CB6 and α2CB4 were labeled following cleavage (Figure 6).

The reaction scheme for the proposed (α1CB6 × α2CB4) of 8p is presented in Figure 9. The first three steps show the presumed in vivo processes leading to formation of the acid-stable ketimine cross-linkage. This appears to be necessary in order for the cross-link to survive the 70% formic acid solvent system used in the initial CNBr degradation. Reduction with [^{3}H]borohydride introduces tritium at the C-5 position of the hydroxylysine of whichever peptide was the aldehyde donor in the original Schiff base reaction. Periodate oxidation then yields the reaction depicted in step 5 in which one obtains formaldehyde, tritiated-norvaline aldehyde from the aldehyde donor peptide, and unlabeled norvaline aldehyde in the amino donor peptide. Reduction with cold borohydride in step 6 produces stabilized [^{3}H]hydroxynorvaline or unlabeled hydroxynorvaline.

The finding that the peptides derived from both α1CB6 and α2CB4 contained [^{3}H]hydroxynorvaline indicated that both had contained reducible aldehydes before cross-link formation and that both peptides could act as aldehyde donors. Results of control experiments apparently preclude an artefactual origin of [^{3}H]hydroxynorvaline.

On agarose molecular sieve chromatography of the borohydride stabilized periodate cleavage products α1CB6, peptide F (Figure 6) had the highest ^{3}H-specific activity, suggesting that more hydroxyallysine was on the α1CB6 portion of the (α1CB6 × α2CB4). However, analyses in the cross-link detection system did not support this, a significantly greater proportion of the activity incorporated into α1CB6 being in acidic species not related to known cross-linking compounds or precursors (Figure 8). These data lead to the conclusion that both α1CB6 and α2CB4 serve as aldehyde donors in cross-link formation in dentin, a very striking conclusion in that α2CB4 is a helical region peptide, not usually considered to be susceptible to aldehyde formation. Deshmukh et al. (1973), however, have provided some evidence for the presence of aldehydes in the helical region of bone collagen and skin collagen (Deshmukh and Nimni, 1971). If both α1CB6 and α2CB4 can act as aldehyde donors, fraction 8p must be a mixture of two peptides of identical net composition and molecular weight but with different cross-linking positions.

Eyre and Glimcher (1973a) described a collagenase derived fragment of calf bone collagen which contained a cross-link

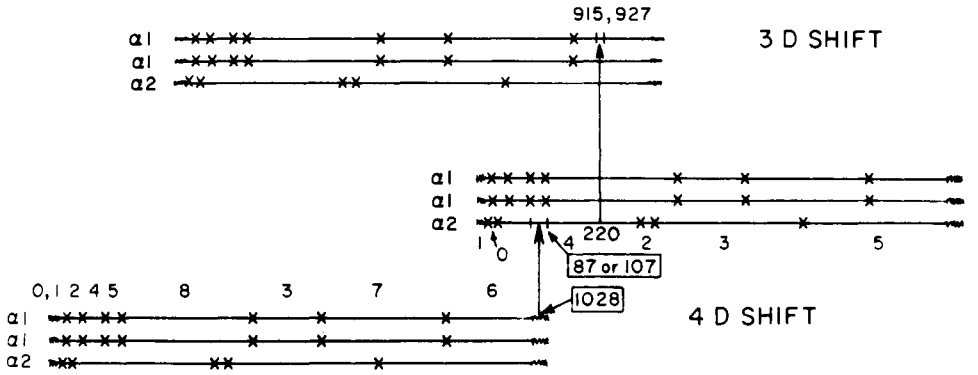

FIGURE 10: Possible location of cross-links involving α1CB6 and α2CB4 in the fibril system in relation to the "quarter-stagger" organization of tropocollagen monomers.

comprised of an hydroxyallysine located in the carboxyl-terminal region of an α1 chain, together with a glycosylated hydroxylysine in an adjacent α1 chain, probably the amino terminal end of the α1CB5 peptide. More recently (Becker et al., 1975) a cross-link peptide from the same location has been isolated from calf skin collagen. Eyre and Glimcher (1973a,b) suggested that an analogous cross-link amino group donor might be present on the α2 chain, and recent sequencing results (Fietzek and Rexrodt, 1975), showing hydroxylysine to be in helical position 87 of bovine type I collagen α2, support this idea. The α1CB6 aldehyde donor (α1CB6 × α2CB4) peptide from dentin may be of this type. The amino acid analysis data of Table II show that some hydroxylysine survives periodate oxidation and conversion by reduction to hydroxynorvaline, indicating that these residues may have their hydroxyl groups protected by glycosylation. Volpin and Veis (1973) have previously shown that α1CB6 and α2CB4 from dentin are partially glycosylated.

Studies in which the dentin was reduced with borohydride before cleavage with CNBr, rather than after, as in the experiments described above, indicated that (α1CB6 × α2CB4) was present in the same amount and became labeled to the same specific activity. It seems unlikely that the same cross-linked fragment would be generated artefactually under two quite different sets of conditions, and we may therefore suggest that a cross-link in this position serves an important stabilizing role in vivo.

A reducible component with electrophoretic mobility comparable to that of 8p in sodium dodecyl sulfate gels has been obtained in calf bone collagen (Eyre and Glimcher, 1973b) and we have observed its presence in rat incisor dentin collagen. However, the comparable fraction is absent in digests of insoluble bovine corium collagen, whether reduced or unreduced prior to CNBr cleavage (Scott and Veis, 1976b). We can suggest on the basis of the data presented here that the α1CB6 × α2CB4 cross-linked peptides are specific for mineralized tissues, being present in dentin, and perhaps bone, but not in skin collagen.

If α1CB6 is the aldehyde donor with the aldehyde being in the nonhelical end region, the aldehyde is on residue 1028 in the numbering system of Fietzek and Kühn (1974) and Rauterberg et al. (1972), in which residue no. 1 is the first helical region glycine. This aldehyde donor cross-linkage can be formed only from the 4D-end-overlap arrangement of a neighboring pair of molecules in which hydroxylysine residue 87, or less likely that at 108, on the α2CB4 (Fietzek and Rexrodt, 1975), is the aldehyde acceptor in the Schiff base (Figure 10). Gallop and Paz (1975) hypothesized that such a cross-linkage might exist, consistent with the long held view that it is the end-region aldehydes which are the donors in Schiff base intermolecular cross-linkages. In the reverse situation where α2CB4 is the aldehyde donor, hydroxyallysine at position 219 in α2CB4 might interact with helical region receptors at positions 915 or 927 on the helical region of α1CB6 in neighboring molecules shifted by 3D (Figure 10). That is, the α2CB4 aldehyde donor peptide must form a helical region–helical region intermolecular cross-linkage. Fujii et al. (1975) isolated a cross-link fragment peptide from calf bone collagen. This peptide was too small to be located definitively within the collagen sequence but the suggestion was made that it might join two helical regions involving α1 and α2 chains. Our present data leave no ambiguity in peptide or chain assignments, but our suggestions for cross-link placement assume skin and dentin collagens to possess the same, or very similar, sequences, an assumption supported by the available evidence (Butler, 1972).

Doyle et al. (1974) suggested that, since a pair of hydroxylysines in the α1 chain are separated by 234 residues, intermolecular cross-linkages between helical region hydroxylysines could stabilize the 1D stagger between adjacent molecules in the collagen fibril. The presence of the two α1CB6 × α2CB4 peptides in dentin shows that the stagger is stabilized at 3D and 4D shifts, making the 1D stagger stabilization hypothesis tenuous at best.

The presence of a helical region–helical region intermolecular cross-link in dentin may account, at least in part, for the unique stability of dentin to solubilization and swelling.

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An Enzyme Kinetics and ^{19}F Nuclear Magnetic Resonance Study of Selectively Trifluoroacetylated Cytochrome *c* Derivatives†

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ABSTRACT: The reaction of cytochrome *c* with ethyl thioltrifluoroacetate was carried out under conditions which led to the selective trifluoroacetylation of a small number of the 19 lysines. The mixture of derivatives was separated by ion-exchange chromatography and four different derivatives with well-resolved ^{19}F nuclear magnetic resonance (NMR) spectra were obtained. Peptide mapping techniques indicated that one of these derivatives contained a single trifluoroacetyl group at lysine 22, and another derivative was singly labeled at lysine 25. The trifluoroacetylated lysine 22 derivative was fully active toward both succinate-cytochrome *c* reductase (EC 1.3.99.1) and cytochrome oxidase (EC 1.9.3.1) while the trifluoroacetylated lysine 25 derivative was fully active toward the reductase, but had a threefold greater Michaelis constant in the cytochrome oxidase reaction. This supports the hypothesis that the cytochrome oxidase binding site is located in the heme crevice region, and that Lys-25 is important in the binding. ^{19}F NMR spectra of the cytochrome *c* derivatives bound to phospholipid vesicles were obtained. The reasonably narrow line widths (35-65 Hz) and good sensitivity of the trifluoroacetyl resonances indicated that they might be useful probes for the interaction of cytochrome *c* with intact mitochondria.

Although a wide variety of techniques have been used to study the reduction of cytochrome *c* by cytochrome *c* reductase and its oxidation by cytochrome oxidase, little is known about the mechanisms of these processes at the molecular level. The location of the reaction sites on cytochrome *c* for cytochrome *c* reductase and cytochrome oxidase is the subject of some controversy, particularly as to whether the sites are the same or different. A number of chemical modification and antibody binding studies indicate that the binding sites might be different (Takano et al., 1973; Margoliash et al., 1973; Smith et al., 1973) while Salemme et al. (1973) have suggested that both the oxidase and the reductase bind at the same site on the front of cytochrome *c* over the heme crevice. We report here on the preparation and characterization of several cytochrome *c* derivatives, two of which contained a single specifically trifluoracetylated lysine residue. We studied the effects of these modified lysine groups on the reactivity of cytochrome *c* with the reductase and the oxidase to characterize the reaction sites

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