Guilfoyle, T. J., & Key, J. L. (1977) *NATO Adv. Study Inst. Ser., Ser A 12*, 37–63.
Guilfoyle, T. J., & Jendrisak, J. J. (1978) *Biochemistry 17*, 1860–1866.
Guilfoyle, T. J., & Malcolm, S. (1980) *Dev. Biol. 78*, 113–125.
Guilfoyle, T. J., Lin, C. Y., Chen, Y. M., Nagao, R. T., & Key, J. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 69–72.
Guilfoyle, T. J., Lin, C. Y., Chen, Y. M., & Key, J. L. (1976) *Biochim. Biophys. Acta 418*, 344–357.
Gurley, W. B., Lin, C. Y., Guilfoyle, T. J., Nagao, R. T., & Key, J. L. (1976) *Biochim. Biophys. Acta 425*, 168–174.
Hardin, J. W., Clark, J. H., Glasser, S. R., & Peck, E. J., Jr. (1976) *Biochemistry 15*, 1370–1374.
Jaehning, J. A., Stewart, C. C., & Roeder, R. G. (1975) *Cell (Cambridge, Mass.) 4*, 51–57.
Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14*, 4639–4645.
Jendrisak, J. J., & Burgess, R. R. (1977) *Biochemistry 16*, 1959–1964.
Jungmann, R. A., & Kranias, E. G. (1977) *Int. J. Biochem. 8*, 819–830.
Key, J. L. (1969) *Annu. Rev. Plant Physiol. 20*, 449–474.
Key, J. L., Lin, C. Y., Gifford, E. M., & Dengler, R. (1966) *Bot. Gaz. (Chicago) 127*, 87–94.
Kumar, B. V., McMillian, R. A., Medoff, G., Gutwein, M., & Kobayashi, G. (1980) *Biochemistry 19*, 1080–1087.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lin, C. Y., Chen, Y. M., Guilfoyle, T. J., & Key, J. L. (1976) *Plant Physiol. 58*, 614–617.
Linn, T., Greenleaf, A., Shorenstein, R., & Losick, R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1865–1869.
Mauck, J. C. (1977) *Biochemistry 16*, 793–797.
O'Brien, T. J., Jarvis, B. C., Cherry, J. H., & Hanson, J. B. (1968) *Biochim. Biophys. Acta 169*, 35–43.
Pong, S. S., & Loomis, W. F., Jr. (1973) *J. Biol. Chem. 248*, 3933–3939.
Spindler, S. R., Duester, G. L., D'Alessio, J. M., & Paule, M. R. (1978) *J. Biol. Chem. 253*, 4669–4675.

A Major Intermolecular Cross-Linking Site in Bovine Dentine Collagen Involving the $\alpha 2$ Chain and Stabilizing the 4*D* Overlap†

Paul G. Scott

ABSTRACT: Approximately 20% of the radioactivity incorporated into the dentine collagen of unerupted bovine molars after reduction with tritiated sodium borohydride was recovered in a cyanogen bromide peptide fraction of M_r 61 000 following chromatography on agarose A5m. After rechromatography on agarose A1.5m, this fraction was resolved into ten components by gel isoelectric focusing. Of these components, nine (the most acidic) were tritiated and contained the reduced cross-links dihydroxylysinonorleucine and hydroxylysinonorleucine. The amino acid compositions were consistent with the identification of each of these components as α2CB3,5 linked to one or two small peptides. By limited Edman degradation, with and without prior digestion with pyroglutamate aminopeptidase (EC 3.4.11.8), these small peptides were identified as α1CB0,1 and α2CB1, occurring in a ratio of approximately 2:1. Specific cleavage with cathepsin D revealed that all the cross-link was associated with the C-terminal one-third of the α2 chain, thus fixing the displacement of the participating molecules at 4*D*. The content of the known reducible cross-links present in these peptides, calculated from the specific activity of the reductant, was sufficient to account for only 10–20% of the cross-linking actually found, suggesting that stabilization is mainly through nonreducible cross-links of as yet undetermined structure. By quantitative analysis of homoserine content and semiquantitative amino-terminal analyses, it was determined that virtually all of the α2 chain of bovine dentine collagen is cross-linked in this manner. One cross-link per molecule in this location could make a major contribution to the mechanical stability of the insoluble collagen fibrils in this tissue.

In the form in which it fulfills a structural and mechanical role in the organism, collagen consists of fibers composed of many individual collagen monomers aligned in a parallel, polarized fashion. The presence of covalent intermolecular cross-links between these monomers is believed to be responsible for the insolubility of fibrillar collagen in nondenaturing solvents and for the physical properties (for example, tensile strength) which are essential for function in vivo. All vertebrate collagens so far studied contain cross-links of the Schiff base type formed by reaction of lysine or hydroxylysine with aldehydes resulting from oxidative deamination of the ϵ-amino group of lysine or hydroxylysine (Tanzer, 1976). This reaction is catalyzed by the enzyme lysyl oxidase (Pinnell & Martin, 1968; Siegel & Martin, 1970; Siegel et al., 1970). The cross-link formed from hydroxylysine and δ-hydroxy-α-aminoadipic δ-semialdehyde (usually referred to as hydroxyallysine) predominates in bone and dentine (Mechanic et al., 1971; Davis & Bailey, 1971). This may rearrange to the corresponding keto amine, hydroxylysino-5-ketonorleucine (Robins et al., 1973; Mechanic et al., 1974; Robins & Bailey, 1975). This species is isolated after reduction with sodium borohydride as dihydroxylysinonorleucine (DHLNL). Hydroxylysinonorleucine (HLNL) is also found but at lower levels. Both these reducible cross-links are generally considered to be intermediates which are subsequently converted to stable nonreducible forms (Robins et al., 1973) by processes which are, as yet, incompletely understood.

Information on both the number and the location, as well as on the chemistry, of the cross-links is essential for a complete understanding of their role in collagen structure and metabolism. Two cross-linking sites are well established for type

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I collagen on the basis of the known sequence (Fietzek & Kühn, 1976) and by isolation of double-chain peptides from insoluble collagens. The first is between α1 amino-terminal residue N9 and α1 helical residue 930. Peptides believed to be joined by a cross-link in this location have been isolated from rat tail tendon (Kang, 1972) and bovine skin (Dixit & Bensusan, 1973) and dentine (Volpin & Veis, 1973). A less well-characterized peptide which may be analogous has been isolated from chick bone (Eyre & Glimcher, 1973a). The second established site is between residue C17 in the α1 chain carboxy-terminal sequence and residue 87 in the α1 chain helical sequence. Corresponding peptides have been isolated from bovine bone (Eyre & Glimcher, 1973b) and skin (Becker et al., 1975) and from rabbit bone, tendon, and skin (Henkel et al., 1976). Although the same cross-linked peptides have been isolated from several different tissues, there is preliminary evidence for tissue-specific variation in location of cross-links (Kuboki & Mechanic, 1974; Scott & Veis, 1976). Some of these observations could, however, reflect differences in cross-link chemistry and stability, rather than location.

The collagen of unerupted bovine dentine is especially suitable for an extensive study of location, chemistry, and number of intermolecular cross-links because this tissue does not normally turn over, contains essentially only one genetic type of collagen (type I; Scott & Veis, 1976), and exhibits a relatively simple profile of reducible cross-links (Mechanic et al., 1971; Bailey & Peach, 1968). In addition, the unusual chemical and physical stability of this collagen suggests a relatively high degree of intermolecular cross-linking (Veis & Schlueter, 1964).

Materials and Methods

Preparation of Reduced CNBr Peptides. Bovine dentine collagen was prepared from the unerupted molars of 18 month old animals as described by Volpin & Veis (1973b). About 400 mg of fragmented collagen was hydrated at 4 °C overnight with 0.15 M sodium chloride/0.1 M sodium dihydrogen phosphate, adjusted to pH 7.4. Tritiated sodium borohydride (Amersham-Searle; 500 mCi/mmol) was added to the stirred suspension (weight ratio 1:40) which was then left at room temperature. After 1 h, a second aliquot of reductant was added, and after a further 1 h the reductant was destroyed by lowering the pH to 4.5 with glacial acetic acid. The reduced collagen was dialyzed against 6 changes of 0.1 M acetic acid at 4 °C over 6 days. Cyanogen bromide peptides were prepared from the reduced collagen (Volpin & Veis, 1973b) and chromatographed on two coupled 2 × 100 cm columns of agarose A5m (Bio-Rad) eluted with 1 M $CaCl_2$/0.05 M Tris-HCl, pH 7.5 (Piez, 1968). The columns were calibrated for molecular weight determination (Scott & Veis, 1976).

Analysis for Reducible Cross-Links. Aliquots of each fraction obtained by agarose chromatography were hydrolyzed at 105 °C for 22 h with redistilled, constant-boiling HCl containing phenol (0.2% w/v) and chromatographed on a 0.9 × 31 cm jacketed column of JEOLCO amino acid analyzer resin, maintained at 55 °C and eluted with pH 5.28 sodium citrate buffer of 0.35 M sodium ion concentration. On this system, dihydroxylysinonorleucine elutes immediately following phenylalanine and is succeeded by hydroxylysinonorleucine. Both are well separated from hydroxylysine. A standard ninhydrin detection system is employed with the total effluent being collected in 1.7-mL fractions after passing through the colorimeter. Scintillation fluid (Unisolve; Koch-Light Labs Ltd., Colnbrook, Bucks, England) was added (10 mL), and samples were counted in a MK III scintillation counter (Searle Analytic, Inc.).

Two-Dimensional Gel Electrophoresis. Acrylamide gel rods were prepared from "electrophoresis-grade" reagents (Bio-Rad) several days before required. The gels (2.5 × 110 mm), acrylamide cross-linked with *N,N'*-methylenebis(acrylamide) (5% T, 0.17% C), were preeluted by dialysis against 100 volumes of distilled water, 4 changes over 4 days, and then dialyzed against 6 volumes of 2.33 M urea/4.66% Servalyt, pH 2–11 (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany), at 4 °C overnight. The gels were then sucked back into glass tubes; the bottoms of the tubes were sealed with dialysis tubing, and the tubes were placed in a gel rod apparatus containing 0.06 N H_2SO_4 as anolyte (upper compartment) and 0.04 N NaOH/0.02 N $Ca(OH)_2$ as catholyte. The gel tubes were filled with 10% (w/v) sucrose, and the gels were prefocused at constant current (0.3 mA/tube) for 1 h when the voltage gradually rose to 600 V. Samples of approximately 50 μg of peptide dissolved in 20 μL of freshly prepared 4 M urea were heated to 50 °C for about 15 min. Aliquots (10 μL) were loaded under fresh 10% (w/v) sucrose, and gels were focused for 3 h at 600 V at room temperature. The pH gradients were then measured with a microelectrode. Gels for the second dimension were prepared in a Bio-Rad Model 220 apparatus and consisted of 1.5-mm thick slabs of acrylamide (12% T) cross-linked with *N,N'*-methylenebis(acrylamide) (0.4% C). A continuous buffer system of 0.05 M Tris/0.0375 M borate/0.1% sodium dodecyl sulfate ($NaDodSO_4$), pH 8.6, was employed. Rods after electrofocusing were sealed onto the top edge of the slab gels with 1% (w/v) agarose in the above buffer. Bromophenol blue (0.00025%, w/v, final concentration) was added to the upper buffer chamber. Electrophoresis was at 50 V for 30 min followed by 250 V for 1 h 45 min. Slabs were stained for 5 h in stain 1 of Fairbanks et al. (1971), destained overnight in 10% (w/v) acetic acid in a diffusion destainer, and then shrunk in two changes of 50% (w/v) methanol over 2 days at 4 °C and dried between sheets of dialysis membrane in a slab dryer.

Preparative Gel Isoelectric Focusing. Gels (5 × 100 mm) were prepared as described for two-dimensional electrophoresis. Samples of approximately 400 μg were dissolved in 50 μL of 2 M urea and focused as above. After the pH gradient was read, the gel was immersed in 15% (w/v) trichloroacetic acid at 4 °C for at least 2 h. Records of the precipitated bands were made by scanning at 440 nm in a Gilford Model 252 spectrophotometer equipped with a linear transporter. Protein-containing sections of the gel were cut out, macerated by forcing through a fine syringe needle into a silanized glass vial, and eluted at 37 °C overnight with 1 mL of 0.02% (w/v) sodium azide. After the supernatants were centrifuged, they were decanted, and the elution was repeated. Combined extracts were lyophilized, redissolved in 200 μL of water, and run on a 1.5 × 6 cm column of Bio-Gel P10 (100–200 mesh) eluted with 1.0 M formic acid. Peptides emerged at the void volume of this column and were usually substantially free from ampholyte. The solutions were stored at −20 °C in silanized glass tubes.

Digestions with L-*Pyroglutamyl-Peptide Hydrolase.* Aliquots of the rechromatographed agarose fraction 7RP (3 mg; see Results) were dissolved in 1 mL of 0.1 M sodium phosphate (pH 8.0)/0.1% β-mercaptoethanol containing 0.5 mg of enzyme protein (Sigma Chemical Co.) and incubated at 37 °C for 16 h. The digested peptide was desalted on Bio-Gel P10 and lyophilized.

Limited Edman Degradation. Duplicate aliquots of 7RP (2–5 nmol) were subjected to two or three cyles of degradation as described by Tarr (1977) with coupling in 50% (v/v)

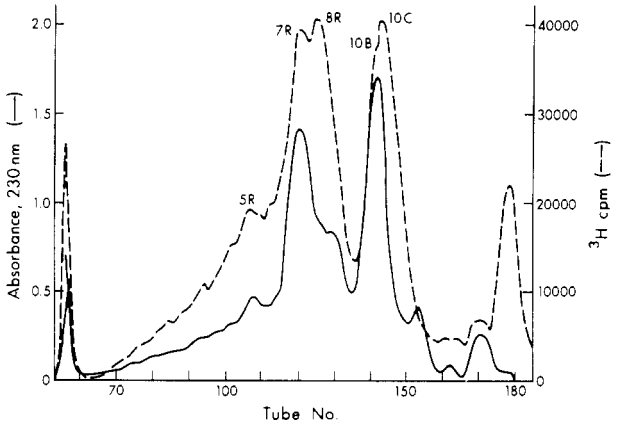

FIGURE 1: Chromatography of CNBr peptides of reduced bovine dentine collagen on agarose A5m. A sample (100 mg) of the peptide mixture was dissolved in 2 mL of 1 M $CaCl_2$/0.05 M Tris-HCl, pH 7.5, with heating at 40 °C for 30 min and applied to two coupled 2 × 100 cm columns. Fractions (3.5 mL) were collected, and 0.1-mL aliquots were taken for counting of tritium activity (as cpm/0.1 mL of aliquot).

pyridine (Bruton & Hartley, 1970). Amino acids were identified on the amino acid analyzer following back-hydrolysis from the anilinothiazolinones at 150 °C, 4 h, in 5.7 N HCl/0.1% (w/v) $SnCl_2$ (Lai, 1977). Results were not corrected for possible hydrolytic losses.

Amino Acid Analysis. Samples were hydrolyzed as described for cross-link analysis and analyzed in duplicate on a Beckman 121 MB automatic amino acid analyzer.

To convert homoserine lactone to homoserine, the hydrolysates were freed of HCl, dissolved in 0.1 N NaOH, allowed to stand 10 min at room temperature, and then acidified by adding an equal volume of 0.114 N HCl. A loading 4–5 times normal was applied to the analyzer. Glycine and alanine contents were determined in runs at normal loading (2–3 μg) and the data combined. Determinations of reducible cross-link contents were carried out on both acidic and basic (2 N NaOH, 22 h, 105 °C) hydrolysates with the amino acid analyzer. The total column effluent was collected after passing through the colorimeter and counted as above.

Digestion with Cathepsin D. Cathepsin D was obtained from bovine thymus and purified as described by Scott & Pearson (1978). Digestions were performed on samples of 7RP dissolved in sodium phosphate buffer (0.15 M), pH 4.0, at 750 μg/mL with an E/S ratio of 1:30. After incubation at 45 °C, 2.5 h, digestion was terminated by addition of pepstatin (Protein Research Foundation, Osaka, Japan) to a final concentration of 10 μg/mL. Samples of digested peptide were examined by electrophoresis on 12% gels (with $NaDodSO_4$ as described by Laemmli (1970). Preparative scale isolation of fragments was accomplished by chromatography on a 1 × 120 cm column of agarose A1.5m, as described for isolation of CNBr peptides.

Results

Five major peaks of radioactivity are apparent in the agarose A5m elution profile of the CNBr peptides of reduced bovine dentine collagen (Figure 1). These are numbered to show the relationship to the previously reported results for unreduced collagen (Scott & Veis, 1976). The peak labeled 7R corresponds to an apparent M_r of 61 000 and elutes in the position expected for α2CB3,5. After rechromatography on agarose A1.5m, to give a component now called 7RP, the two-dimensional electrophoresis pattern shown in Figure 2 was obtained. The M_r 61 000 material was resolved into seven major

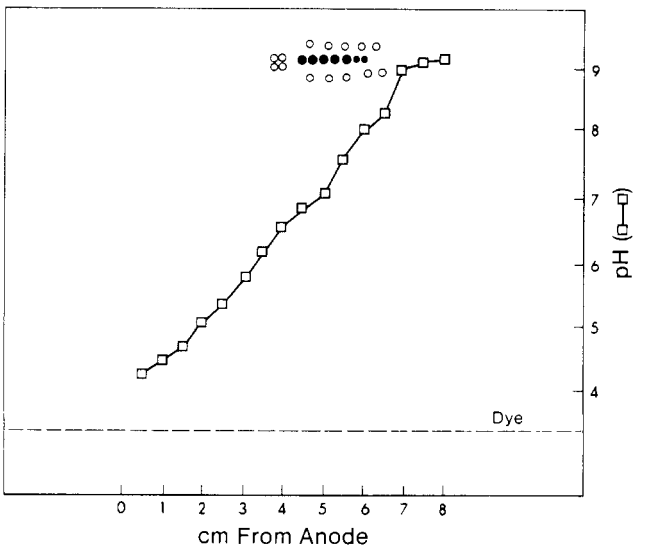

FIGURE 2: Two-dimensional electrophoresis of agarose fraction 7RP. Open and closed circles represent, respectively, minor (weakly stained) and major (strongly stained) components, traced directly from the slab gel. Details given in text.

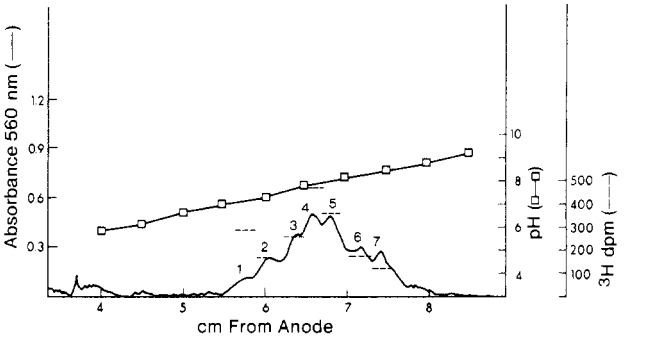

FIGURE 3: Densitometric scan along axis of major components in two-dimensional electrophoresis of agarose fraction 7RP. Sections (4-mm diameter) containing spots corresponding to components 1–7 inclusive were punched out of the slab gel and digested in 0.3 mL of NCS solubilizer (Amersham-Searle) at 37 °C overnight. Scintillation fluid was added after acidification with acetic acid and tritium activity, as dpm/component (---), determined.

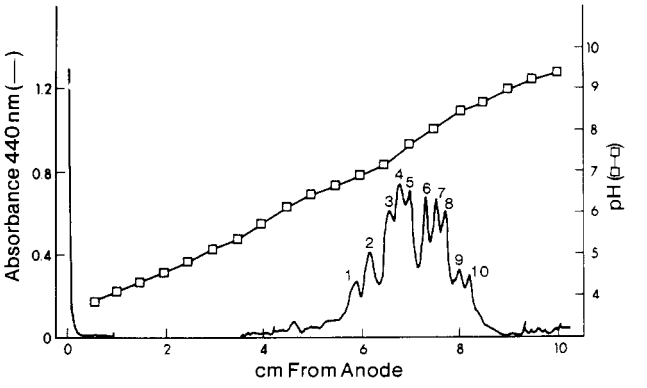

FIGURE 4: Preparative gel isoelectric focusing on agarose fraction 7RP. Details given in text. Components 1–9 inclusive were found to be tritiated.

components, with isoelectric points ranging from 6.5 to 8.5. Only small amounts of contaminating peptides of higher and lower molecular weights were apparent. After densitometric scanning along the axis of the major components (Figure 3), 4-mm-diameter sections were cut out with a gel punch, and the radioactivity in each was determined. The results suggest that each component is cross-linked. In order to confirm this,

Table I: Amino Acid Compositions (Residues/1000 Total) of Agarose Fraction 7RP, Bovine α2CB3,5, and Peptides Isolated by Isoelectric Focusing

amino acid	α2CB3,5[a]	7RP	isoelectric focusing fractions[b] 3	4	5	6	7
Hyp	83.4	81.6	76.9	76.2	71.5	71.3	78.8
Asp	51.1	52.7	52.5	49.3	47.6	51.2	46.5
Thr	17.3	16.8	19.5	22.3	28.6	23.3	20.0
Ser	35.1	33.5	39.0	53.5	52.3	60.4	43.7
Glu	69.1	71.7	69.8	80.1	83.3	85.3	79.3
Pro	113.5	115.2	101.2	102.9	102.6	99.0	105.6
Gly	335.9	344.5	329.4	312.7	319.5	313.4	324.0
Ala	104.8	110.2	128.1	120.1	119.4	117.7	126.6
Val	28.7	27.6	27.6	24.4	27.6	26.7	24.9
Met							
Ile	20.0	19.2	19.7	20.4	19.0	19.0	18.7
Leu	29.2	29.4	20.9	26.6	22.8	22.9	23.3
Tyr	4.1	3.9	2.0	1.7	1.3	3.5	2.1
Phe	15.7	16.4	12.4	16.0	13.5	14.5	14.6
Hyl	9.5	11.3	16.4	13.4	12.6	11.5	12.4
Lys	23.4	19.5	22.5	20.2	20.3	21.1	21.1
His	8.5	7.3	6.2	9.8	9.5	10.6	6.9
Arg	50.5	54.4	54.9	49.4	47.3	47.4	50.0
Hse		1.6[c]	1.0	1.1	1.3	1.3	1.5
reducible cross-link[d]		0.32	0.11	0.14	0.21	0.12	0.14

[a] Prepared from soluble bovine type I collagen by gel chromatography and ion-exchange chromatography on carboxymethylcellulose (Volpin & Veis, 1973a). [b] See Figure 4. [c] A second batch of reduced collagen gave 7RP with identical Hse content. [d] Sum of dihydroxylysinonorleucine and hydroxylysinonorleucine.

isolation of pure peptides for direct analysis was attempted by preparative gel isoelectric focusing (Figure 4). This one-dimensional separation shows slightly greater resolution by the isoelectric point than was obtained on the two-dimensional system. Components 1–9 inclusive were tritiated, but only components 3–7 were obtained in sufficient yield to permit complete amino acid analyses to be obtained (Table I). These were generally very similar to that of unfractionated 7RP and to un-cross-linked α2CB3,5 (prepared from soluble type I collagen of calf skin), although some showed rather high levels of serine, glutamic acid, and alanine, presumably resulting from slight contamination with ampholytes or other reagents. These do not obscure the obvious close relationship of these components to α2CB3,5.

Dihydroxylysinonorleucine and hydroxylysinonorleucine were the only radioactive components identified in acid or base hydrolysates of 7RP (Figure 5A,B). These two components occurred in a ratio of approximately 5:1, reflecting that normally found for whole dentine collagen (Mechanic et al., 1971) and indicating that hydroxylation of the participating lysine residues was almost complete. The total reducible cross-link content in 7RP calculated from the specific activity of the tritiated sodium borohydride (data from the supplier) and the recovery of hydroxyproline from the amino acid analyzer was 0.21 mol/mol of peptide. An even lower value (0.13) was obtained by using the specific activity determined by an independent calibration method (Paz et al., 1970).

The total cross-link content of 7RP can be calculated from the homoserine present. Since α2CB3,5 contains no methionine and 7RP is not significantly contaminated with any uncleaved CNBr peptides (Scott & Veis, 1976), as may be judged from the amino acid composition (Table I) and the specific and complete cleavage by cathepsin D (Figure 6), the number of residues of homoserine per peptide is equal to the number of carboxy terminals present in addition to that of α2CB3,5. Duplicate analyses of this preparation of 7RP gave 1.13 ± 0.02 residues Hse/peptide (1.6 residues/1000). A second preparation of reduced collagen of a different batch gave 1.12 Hse/peptide, and a preparation from nonreduced dentine collagen gave 1.19 Hse/peptide. These values are consistent

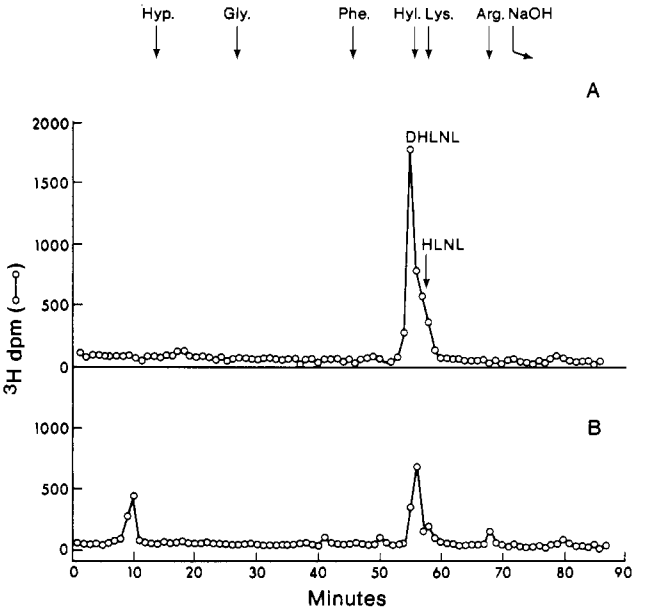

FIGURE 5: Elution of radioactive components present in acid (A) and base (B) hydrolysates of agarose fraction 7RP, from Beckman 121MB amino acid analyzer. Details given in text. The elution positions of reference amino acids and the commencement of regeneration with NaOH are indicated by the straight and curved vertical arrows, respectively. DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine. Radioactivity is expressed as dpm/tube.

with the ratio of amino terminals found in the Edman degradation (see below).

Without prior digestion with pyroglutamate aminopeptidase, only glycine could be identified at the amino terminus in the Edman degradation of 7RP. After digestion, both leucine and phenylalanine were obtained, in addition to glycine (Table II). The two-dimensional electrophoresis pattern of digested 7RP did not show any new peptides, suggesting that the new amino terminals were not exposed as a result of peptide-bond hydrolysis at sites other than at terminal pyroglutamic acid. This was confirmed by subjecting a sample of pure un-cross-linked α2CB3,5 to the same procedure, when only glycine was ob-

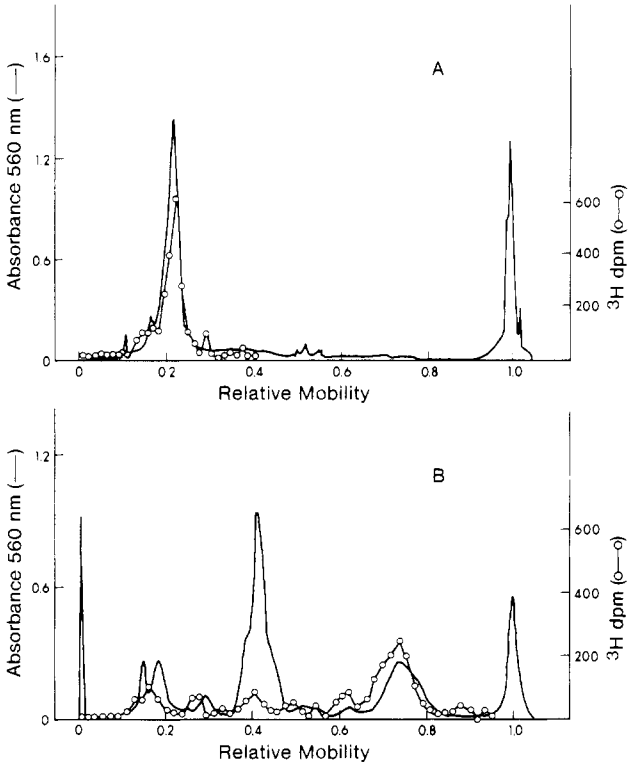

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of agarose fraction 7RP before (A) and after (B) digestion with cathepsin D, as described in text. After the fractions were scanned, 1-mm slices were digested with NCS, and tritium activity, as dpm/slice (O), was determined. Relative mobility is referred to bromophenol blue tracker dye.

tained as the amino-terminal residue. At the second cycle, new amino terminals of alanine, proline, and aspartic acid were found in 7RP. No useful information could be obtained at the third cycle. These results, in conjunction with the published sequences of bovine $\alpha 1$ and $\alpha 2$ chains (Fietzek & Kühn, 1976), are sufficient to positively identify α1CB0,1 (sequence: pGlu-Leu-Ser...) and α2CB1 (sequence: pGlu-Phe-Asp...) as the small peptides attached to α2CB3,5 in this cross-linked peptide fraction. [Anilinothiazolinone serine is converted to alanine on acid hydrolysis (Lai, 1977).] The ratio of leucine to phenylalanine obtained at the first cycle after digestion was approximately 2:1, reflecting the ratio of $\alpha 1/\alpha 2$ in the intact bovine dentine collagen. The sum of the yields of these two amino acids (Table II) exceeds that of glycine, suggesting that the ratio (α1CB0,1 + α2CB1)/α2CB3,5 is slightly greater than one. Virtually identical results were obtained in a second experiment on a different preparation of 7RP.

The fragmentation pattern with cathepsin D can be used to localize the attachment of the small peptides. This enzyme has a very restricted action against denatured type I collagen, cleaving only within the carboxy-terminal extrahelical region of the $\alpha 1$ chain (Scott & Pearson, 1978) and between residues 782 and 783 in the $\alpha 2$ chain (Scott & Pearson, 1981). The reducible cross-links (radioactivity) are associated with the smaller (carboxy-terminal) fragment of 7RP following digestion with cathepsin D and $NaDodSO_4$ gel electrophoresis (Figure 6), or chromatography on agarose (results not shown). This fragment was also found to contain most of the homoserine in 7RP.

Discussion

There is a paucity of published information on the absolute numbers of intermolecular cross-links present in collagen fibers.

Table II: Composition of Back-Hydrolysates (Residues Percent) Obtained for Anilinothiazolinone Amino Acids Released in Edman Degradation of 7RP

amino acid	7RP digested with pyroglutamate aminopeptidase		undigested 7RP
	cycle 1	cycle 2	cycle 1
Hyp			
Asp	4.7 ± 0.2[a]	6.5 ± 0.8	4.6 ± 0.2
Thr	2.2 ± 0.6	2.0 ± 0.9	2.1 ± 0.5
Ser	4.5 ± 0.3	3.5 ± 0.3	6.8 ± 0.3
Hse			
Glu	4.7 ± 0.7	4.5 ± 0.4	7.8 ± 0.2
Pro		9.2 ± 3.8	
Gly	32.6 ± 2.1[b]	43.6 ± 3.5	59.1 ± 1.0
Ala	10.6 ± 1.1	19.6 ± 2.3	6.3 ± 0.2
Cys			
Val			
Met			
Ile		4.0 ± 1.1	3.9 ± 0.2
Leu	23.7 ± 0.5	4.2 ± 1.0	3.7 ± 0.2
Tyr			
Phe	14.3 ± 0.2	2.9 ± 0.7	
Hyl			
Lys			3.0 ± 0.6
His			0.5 ± 0.5
Arg			

[a] Average and range for duplicate experiments. [b] Major N-terminal amino acids identified are underlined.

This probably results from uncertainty concerning the nature of the nonreducible cross-links and the significance, in quantitative terms, of the known reducible cross-links. From the results described here, we conclude that most, if not all, of the $\alpha 2$ chains of bovine dentine collagen are linked to either α1CB0,1 or α2CB1. Three different lines of evidence support this. Both the homoserine content and the ratio of amino-terminal amino acids suggest that the sum of the contents of α1CB0,1 and α2CB1 exceeds that of α2CB3,5 in 7RP. This fraction contains almost all of the α2CB3,5 of bovine dentine collagen (P. G. Scott, unpublished experiments). Nine of the ten peptides separated by isoelectric focusing are tritiated, and at least six, and probably all, contain reducible cross-links. The preparative isoelectric focusing procedure employed here is capable of resolving peptides differing by one unit of charge (P. G. Scott, unpublished experiments), leading to the conclusion that each of the isolated species is a pure peptide. Some of the complexity in the isoelectric focusing profile may result from variation in amide content, since un-cross-linked α2CB3,5 isolated from bovine skin shows four or five components differing slightly in apparent isoelectric point. Dentine collagen has been reported to have covalently attached phosphate groups on the $\alpha 2$ chain (Volpin & Veis, 1973b). This could generate further isoelectric forms.

It has been proposed (Robins & Bailey, 1973) that under certain circumstances treatment of collagen with sodium borohydride may lead to the induction of reducible cross-links in some tissues. The finding that the analogous fractions isolated from bovine dentine collagen with and without prior reduction contained a similar amount of homoserine (Scott & Veis, 1976; present results) suggests that the level of cross-linking reported here is truly representative of the level in vivo. Cross-linked peptides involving α2CB3,5 and "small peptides" have been reported in corneal collagen (Harding, 1978) and scleral collagen (Crabbe & Harding, 1979). These were not, however, characterized further.

The content of reducible cross-links in peptides can theoretically be calculated from the specific activity of the reductant, if the radioactivity in the cross-linking amino acids

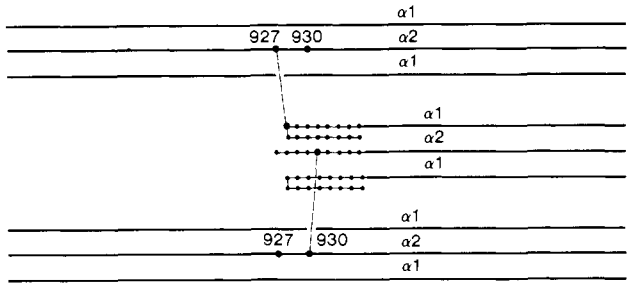

FIGURE 7: Suggested location of intermolecular cross-links involving the α2 chain helical site and amino-terminal extrahelical sequences (●) on molecule(s) related by an axial displacement of 4*D* (assumed to be 936 residues). The α1 chain amino-terminal sequence is shown as the hairpin structure suggested by Helseth et al. (1979). Molecular flexibility may permit slightly different arrangements, i.e., α1 residue N9 might be linked to α2 residue 930 and α2 residue N5 to α2 residue 927. Three molecules are shown for clarity. This is not intended to imply that there is direct evidence for this specific arrangement in vivo.

can be directly related to the amount of hydrolyzed peptide applied to the analyzer. The reliability of this procedure has been confirmed in our laboratory by the close agreement of results on bovine dentine collagen with those obtained by using the published ninhydrin color yields (Robins et al., 1973). Rereduction in solution of peptides prepared from dentine collagen reduced by the standard procedure described here does not lead to the incorporation of more tritium into DHLNL or HLNL (P. G. Scott, unpublished experiments). The finding that the reducible cross-links are sufficient to account for only 10–20% of the actual cross-linking present cannot therefore be simply ascribed to either analytical error or incomplete reaction with sodium borohydride. It is generally accepted that the reducible cross-links are intermediate forms which are converted into, or replaced by, nonreducible cross-links during maturation and aging (Robins et al., 1973). The mechanism of this process remains to be elucidated. Bailey et al. (1977) have suggested that oxidation may be a significant route for stabilization of reducible cross-links. Our results could possibly be explained in these terms. We are currently attempting to characterize the nonreducible cross-links joining these peptides.

The fragmentation pattern of 7RP digested with cathepsin D clearly establishes that the small peptides are associated with the carboxy-terminal one-quarter of the α2 chain. While the detailed three-dimensional structure of the collagen fibrils is not completely understood, there is general agreement that the axial displacement of the constituent collagen monomers is an integral multiple of *D*, the basic periodicity of 670 ± 30 Å, corresponding to 234 residues in the helical region [evidence reviewed by Bornstein & Traub (1979)]. The sequence of the bovine α2 chain (Hofmann et al., 1978) shows hydroxylysine in positions 927 and 930. For two molecules staggered by 4*D*, the (hydroxy)lysine residues in α1CB0,1 and α2CB1 on one molecule would lie opposite this region on the second. The (hydroxy)lysine residue on α2CB1 is apparently closer to the helix than that in α1CB0,1 by three residues (Fietzek & Kühn, 1976), leading to the tentative suggestion that α1CB0,1 could be linked to residue 927 and α2CB1 to residue 930 (Figure 7). The present evidence suggests that most, if not all, of the isolated peptides consist of two chains, that is, α2CB3,5 linked to either α1CB0,1 or α2CB1. The possibility of three-chain peptides in which α2CB3,5 is linked to both α1CB0,1 and α2CB1 cannot be excluded at this time. Staggering of molecules by integral multiples of *D* other than 4 would not align the amino-terminal peptides with α2CB3,5 in a manner consistent with the cathepsin D fragmentation pattern. Further, the α2 sequence shows no (hydroxy)lysine residue in a suitable position to be a partner in cross-link formation at either 2*D* or 3*D* (Hofmann et al., 1978). Intermolecular cross-links can modify several physical properties of collagen fibrils, including tensile strength (Bailey, 1968). The presence of one cross-link per molecule in the location described here, i.e., linking linear aggregates of molecules, could make a significant contribution to the tensile strength of the fibrils.

Intermolecular cross-links probably appear very early during the process of fibril formation (Boucek et al., 1979). Studies on the action of lysyl oxidase in vitro (Siegel & Fu, 1976) suggest that the enzyme has more activity on ordered aggregates of collagen molecules than on monomers in solution. The formation of cross-links between collagen monomers staggered by 4*D* could promote the process of fibril assembly in vivo through stabilization of noncovalent aggregates. The potential for specific, polarized, and noncovalent interactions between the extrahelical amino-terminal sequence of the α1 chain and the helical region of the α1 chain around residue 930 has been suggested to be responsible for the establishment of the 4*D* overlap during fibrillogenesis (Helseth et al., 1979). The covalent stabilization of this alignment in vivo may depend, at least in bovine dentine collagen, on the formation of intermolecular cross-links between the amino-terminal extensions and the α2 chains of adjacent molecules.

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References

Bailey, A. J. (1968) *Biochim. Biophys. Acta 160*, 447.
Bailey, A. J., & Peach, C. M. (1968) *Biochem. Biophys. Res. Commun. 33*, 812.
Bailey, A. J., Ranta, M. H., Nicholls, A. C., Partridge, S. M., & Elsden, D. F. (1977) *Biochem. Biophys. Res. Commun. 78*, 1403.
Becker, U., Furthmayr, H., & Timpl, R. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 21.
Bornstein, P., & Traub, W. (1979) *Proteins, 3rd Ed. 4*, 411.
Boucek, R. J., Noble, N. L., & Gunja-Smith, Z. (1979) *Biochem. J. 177*, 853.
Bruton, C. J., & Hartley, B. S. (1970) *J. Mol. Biol. 52*, 165.
Crabbe, M. J., & Harding, J. J. (1979) *FEBS Lett. 97*, 189.
Davis, N. R., & Bailey, A. J. (1971) *Biochem. Biophys. Res. Commun. 45*, 1416.
Dixit, S. N., & Bensusan, H. B. (1973) *Biochem. Biophys. Res. Commun. 52*, 1.
Eyre, D. R., & Glimcher, M. J. (1973a) *Biochem. J. 135*, 393.
Eyre, D. R., & Glimcher, M. J. (1973b) *Biochem. Biophys. Res. Commun. 52*, 663.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.
Fietzek, P. P., & Kühn, K. (1976) *Int. Rev. Connect. Tissue Res. 7*, 1.
Harding, J. J. (1978) *FEBS Lett. 87*, 251.
Helseth, D. L., Jr., Lechner, J. H., & Veis, A. (1979) *Biopolymers 18*, 3005.
Henkel, W., Rauterberg, J., & Stirtz, T. (1976) *Eur. J. Biochem. 69*, 223.
Hofmann, H., Fietzek, P. P., & Kühn, K. (1978) *J. Mol. Biol. 125*, 137.
Kang, A. H. (1972) *Biochemistry 11*, 1828.

Kuboki, Y., & Mechanic, G. L. (1974) *Connect. Tissue Res. 2*, 223.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lai, C. Y. (1977) *Methods Enzymol. 48*, 369.
Mechanic, G. L., Gallop, P. M., & Tanzer, M. L. (1971) *Biochem. Biophys. Res. Commun. 45*, 644.
Mechanic, G. L., Kuboki, Y., Shimokawa, H., Nakamoto, K., & Sasaki, S. (1974) *Biochem. Biophys. Res. Commun. 60*, 756.
Paz, M. A., Henson, E., Rombauer, R., Abrash, L., Blumenfeld, O. O., & Gallop, P. M. (1970) *Biochemistry 9*, 2123.
Piez, K. A. (1968) *Anal. Biochem. 26*, 305.
Pinnell, S. R., & Martin, G. R. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 708.
Robins, S. P., & Bailey, A. J. (1973) *Biochem. J. 135*, 657.
Robins, S. P., & Bailey, A. J. (1975) *Biochem. J. 149*, 381.
Robins, S. P., Shimokomaki, M., & Bailey, A. J. (1973) *Biochem. J. 131*, 771.
Scott, P. G., & Veis, A. (1976) *Connect. Tissue Res. 4*, 117.
Scott, P. G., & Pearson, C. H. (1978) *FEBS Lett. 88*, 41.
Scott, P. G., & Pearson, C. H. (1981) *Eur. J. Biochem.* (in press).
Siegel, R. C., & Martin, G. R. (1970) *J. Biol. Chem. 245*, 1653.
Siegel, R. C., & Fu, J. C. C. (1976) *J. Biol. Chem. 251*, 5779.
Siegel, R. C., Pinnell, S. R., & Martin, G. R. (1970) *Biochemistry 9*, 4486.
Tanzer, M. L. (1976) in *Biochemistry* of *Collagen* (Ramachandran, G. N., Ed.) p 137, Plenum Press, New York.
Tarr, G. E. (1977) *Methods Enzymol. 48*, 335.
Veis, A., & Schlueter, R. J. (1964) *Biochemistry 3*, 1650.
Volpin, D., & Veis, A. (1973a) *Biochemistry 10*, 1751.
Volpin, D., & Veis, A. (1973b) *Biochemistry 12*, 1452.

Structural Intermediates Trapped during the Folding of Ribonuclease A by Amide Proton Exchange†

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ABSTRACT: In the folding reaction of the slow-folding species (U_S) of ribonuclease A (RNase A), the slow isomerization of wrong proline isomers provides a suitable trap for kinetic folding intermediates at low temperatures (0–10 °C). Partly folded intermediates are known to accumulate before proline isomerization takes place, after which native RNase A is formed. We have been able to measure the protection from amide proton exchange which is provided by structure in the intermediates at different times along the folding pathway. Previous work has shown that, by labeling the amide protons of the unfolded protein before initiating refolding, an early folding intermediate can be detected. The new pulse-labeling method presented here can be used to label later folding intermediates. Our results indicate that, in conditions which strongly favor the native protein, intermediates are formed which provide protection against exchange. However, when folding is initiated in 2.5 M Gdn·HCl, 10 °C, pH 7.5, conditions in which folding goes to completion but there are no spectroscopically detectable intermediates, then no intermediates are detected by our method. Alternate minimal mechanisms for the folding of U_S are presented.

Previous work has shown that proline isomerization can be used as a kinetic trap for intermediates in the folding of RNase A.[1] The results can be summarized as follows. (1) There are two classes of unfolded RNase A: a fast-folding class, U_F, and a major (80%) slow-folding class, U_S (Garel & Baldwin, 1973, 1975a,b; Brandts et al., 1975; Garel et al., 1976; Hagerman & Baldwin, 1976). (2) The $U_F \rightleftharpoons U_S$ interconversion reaction in unfolded RNase A has been shown to be acid catalyzed and to have other specific properties of proline isomerization or of peptide bond isomerization (Schmid & Baldwin, 1978, 1979a), and it is probable that the slow-folding molecules of RNase A have one or more wrong proline isomers, as first proposed by Brandts et al. (1975). (3) There is recent evidence that, in the folding of RNase A at low temperatures, structural intermediates accumulate before proline isomerization takes place. An assay has been devised for wrong proline isomers which can be used during folding (Cook et al., 1979), and the results show that, at 0–10 °C, the folding reaction monitored by tyrosine absorbance occurs well in advance of proline isomerization. Schmid (1980) has found that folding monitored by tyrosine fluorescence follows the same kinetic progress curve as the assay for wrong proline isomers, demonstrating that the proline isomerization reaction can also be observed by a spectral technique. (4) Specific structure is formed in the next to last folding reaction monitored by tyrosine absorbance because a binding site for the specific inhibitor 2′-CMP is formed in this reaction (Cook et al., 1979; Schmid & Blaschek, 1980). Consequently, before proline isomerization takes place, a quasi-native intermediate, I_N, is formed in the folding reaction monitored either by tyrosine absorbance or by 2′-CMP binding. (5) A different intermediate, formed very early in folding, has been observed

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; RNase S, a derivative of RNase A cleaved at the peptide bond between residues 20 and 21; U_S and U_F, slow- and fast-folding species, respectively, of RNase A; Gdn·HCl, guanidinium chloride; Mops, morpholinopropanesulfonic acid; BPTI, bovine pancreatic trypsin inhibitor; 2′-CMP, cytidine 2′-phosphate.

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