acid residue is substituted by a neutral residue, a change of from one to two charges has been detected.

Most unstable hemoglobins have been detected because of their association with hemolytic disease (Motulsky and Stamatoyannopoulos, 1968). Hemoglobin Tacoma was discovered as a result of screening of blood samples for evidence of electrophoretic abnormalities. The instability of Hb Tacoma in the absence of hematologic findings suggests that there may be other unstable hemoglobins without significant clinical manifestations. Studies on *in vitro* chemical lability, therefore, are indicated in all hemoglobin variants for better understanding of the relationship of structure to function in the hemoglobin molecule. Some of these relationships of structure to function which can be deduced from studies of mutant hemoglobins such as Hb Tacoma have been described recently by Perutz and Lehmann (1968).

Acknowledgments

We wish to thank Miss Marie Duerst for performing the amino acid analyses.

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Isolation and Characterization of the Cyanogen Bromide Peptides from the $\alpha 2$ Chain of Rat Skin Collagen*

Peter P. Fietzek and Karl A. Piez

ABSTRACT: The $\alpha 2$ chain of rat skin collagen was cleaved at methionyl bonds with cyanogen bromide. The digest was fractionated by ion-exchange and molecular sieve chromatography and six peptides were isolated in approximately equimolar amounts. Each peptide represents a unique portion of the $\alpha 2$ chain as shown by chromatographic properties, amino acid analysis, and molecular weight. Together they account for all the amino acids of the $\alpha 2$ chain and its molecular weight of about 95,000. The peptides include a tripeptide, a tetradecapeptide, a peptide of 30 residues, and three large peptides each of about mol wt 30,000.

For studies of the primary structure of the collagen molecule, it has been shown that cleavage of methionyl peptide bonds with CNBr is a very useful method (Bornstein and Piez, 1965, 1966; Bornstein *et al.*, 1966). The collagen molecule contains three polypeptide chains, each having a molecular weight of about 95,000. Two of the chains, the $\alpha 1$ chains, in collagens from higher animals appear to be identical while the third, the $\alpha 2$ chain, differs in amino acid composition and chromatographic behavior from the two $\alpha 1$ chains (see Piez, 1967). Butler *et al.* (1967) have reported the cleavage with CNBr of the seven methionyl peptide bonds in $\alpha 1$ from rat skin collagen. Eight unique peptides were obtained, which account for all the

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amino acids and the molecular weight of the α1 chain. The present status of investigations on the α1 chains from a total of five collagens has been summarized (Piez *et al.*, 1968).

Two CNBr peptides from the α2 chain of rat skin collagen have been reported, α2-CB1, a tetradecapeptide from the cross-link region at the NH_2-terminal end of the α2 chain, and α2-CB2, a peptide of 30 amino acids (Bornstein and Piez, 1966). In this paper we report the isolation of the remaining peptides derived from the cleavage of the α2 chain from rat skin collagen with CNBr. The nomenclature of the CNBr peptides from collagen has been discussed (Miller *et al.*, 1969).

Material and Methods

Source and Preparation of α2. Salt and acid-extracted collagens were prepared as previously described (Bornstein and Piez, 1966) from the skins of 80–100-g Sprague–Dawley rats. For most preparations the rats were made lathyritic by feeding a diet containing 0.1% β-aminopropionitrile fumarate for 3 weeks. No difference was found between α2 chains obtained from salt or acid-extracted collagen or from normal or lathyritic rats except in the proportion of lysine- and aldehyde-containing forms of α2-CB1 already reported (Bornstein and Piez, 1966). Therefore, the results are given without indicating the source of the collagen.

Samples of the α2 chain were obtained by chromatography of 200–300 mg of denatured collagen at 40° on a 25 × 90 mm CM-cellulose column (Whatman CM32) as described previously (Piez *et al.*, 1963). Separation was achieved with a linear gradient of 400 ml of starting buffer (0.06 M sodium acetate–0.06 M acetic acid, pH 4.8) and 400 ml of limit buffer (starting buffer containing 0.1 M sodium chloride). The column was eluted at a rate of 200 ml/hr and the effluent was monitored continuously at 230 mμ in a Gilford Model 2000 spectrophotometer. The column effluent was collected in 10-ml fractions. The fractions containing the α2 chains were combined, lyophilized, redissolved in 0.1 M acetic acid, desalted at room temperature on a 3 × 40 cm column of Bio-Gel P-2, 100–200 mesh (Bio-Rad Laboratories), equilibrated with 0.1 M acetic acid, and relyophilized. In some cases the samples were rechromatographed on CM-cellulose-columns under the same conditions.

Cleavage with CNBr. Cleavage with CNBr was performed as described previously (Bornstein and Piez, 1966). Samples of α2 weighing from 50 to 200 mg were dissolved in 0.1 M acetic acid to give a concentration of 10 mg/ml and 6 N HCl was added to pH 1.5. The collagen solution was flushed with nitrogen and the same weight of CNBr as of collagen (a 200-fold molar excess relative to methionine) was added. After shaking the flask to dissolve the crystals, incubation was carried out at 30° for 4 hr. The reaction mixture was diluted tenfold with water, lyophilized, and redissolved in 50 ml of water and relyophilized for complete removal of CNBr.

Chromatography of CNBr Peptides on Phosphocellulose. The CNBr digest was chromatographed on a 25 × 60 mm column of phosphocellulose essentially as described earlier (Bornstein and Piez, 1966). Samples weighing 25–100 mg were dissolved in 10 ml of starting buffer (0.001 M sodium acetate adjusted to pH 3.6 with acetic acid) and pumped onto the column equilibrated with the same buffer. Chromatography was performed at 40° in the same buffer with a linear gradient of NaCl from 0 to 0.3 M. The total volume of the gradient was 800 ml and the flow rate was 180 ml/hr. The larger or more basic peptides, which were not eluted by this gradient, were removed as one peak by eluting with 0.5 M NaCl. The column effluent was continuously monitored as described above and 8-ml fractions were collected. Appropriate fractions were combined, concentrated by lyophilization, and then desalted on Bio-Gel P-2.

Chromatography of CNBr Peptides on CM-cellulose. The peptides that were eluted with 0.5 M NaCl as a single peak were chromatographed on a 16 × 80 mm column of CM-cellulose equilibrated with 0.004 M sodium formate adjusted to pH 3.6 with formic acid. Chromatography was performed at 40° and a flow rate of 190 ml/hr. Elution was with the same buffer and a linear gradient of sodium chloride from 0.06 to 0.15 M NaCl over a total volume of 800 ml. Samples weighing 25–100 mg were dissolved in 10 ml of starting buffer and pumped onto the column. The column effluent was monitored as described above. To separate larger amounts, a 25 × 90 mm column and a total gradient volume of 2000 ml were employed. Fractions comprising a given peak were combined, lyophilized, desalted, and lyophilized. In some cases the peptides were rechromatographed under the same conditions.

Molecular Sieve Chromatography. To separate α2-CB0 from all other peptides, a 1.7 × 80 cm Bio-Gel P-4 column equilibrated with 0.1 M acetic acid was used. The peptides were dissolved in 1.5 ml of 0.1 M acetic acid and applied to the column. Elution was at room temperature and a flow rate of 60 ml/hr. The effluent was monitored as described above and 3-ml portions were collected. Appropriate fractions were combined and lyophilized.

Amino Acid Analysis. Samples were hydrolyzed in 2 ml of constant-boiling HCl at 108° for 24 hr under nitrogen in sealed tubes. The acid was removed in a

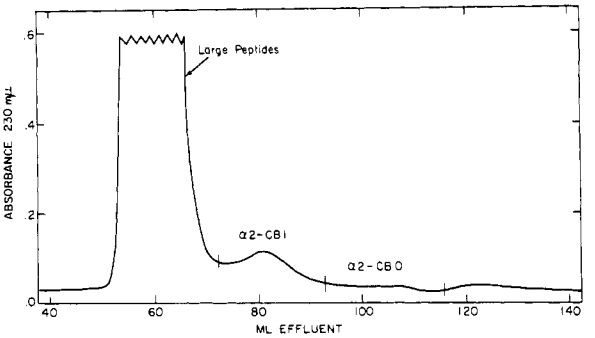

FIGURE 1: Phosphocellulose elution pattern of the CNBr peptides derived from the α2 chain of rat skin collagen. Elution was performed in 0.001 M sodium acetate (pH 3.6) using a linear gradient of sodium chloride from 0 to 0.3 M. The total volume of the gradient was 800 ml.

rotary evaporator at about 50° under vacuum. The dried samples were dissolved in water and a volume containing 0.1–1 mg was used for amino acid analysis on a single-column automatic analyzer equipped for high-speed analysis (Miller and Piez, 1966). To resolve homoserine, the column was kept at 50° for the first 45 min and at 60° thereafter. Losses of threonine, serine, and tyrosine and the incomplete release of valine were corrected by the application of factors previously determined for collagen (Piez *et al.*, 1960). As in the case of the α1 chain from rat skin collagen (Butler *et al.*, 1967), it was found that methionine was completely recovered if less than 10% conversion into the sulfoxides occurred.

Molecular Weight Determination. The molecular weights of the larger CNBr peptides were determined by molecular sieve chromatography on a 1.5 × 106 cm column of Bio-Gel A-1.5, 200–400 mesh (Bio-Rad Laboratories), as described by Piez (1968). Minimal molecular weights were also calculated from the amino acid compositions using values for those amino acids present in small amounts to determine a factor to convert micromoles into equivalents.

Results

Phosphocellulose Chromatography of CNBr Peptides. A typical chromatogram obtained by chromatography of the CNBr peptides is shown in Figure 1. α2-CB1 and α2-CB2 have been described by Bornstein and Piez (1966). Eluting with α2-CB1 was a tripeptide, designated α2-CB0. It was found when the fractions containing α2-CB1 were chromatographed on Bio-Gel P-2 or P-4 (see later) and effluent fractions were lyophilized, hydrolyzed, and analyzed. It does not have sufficient absorbance at 230 mμ to be detected in the manner used for the other peptides and therefore was

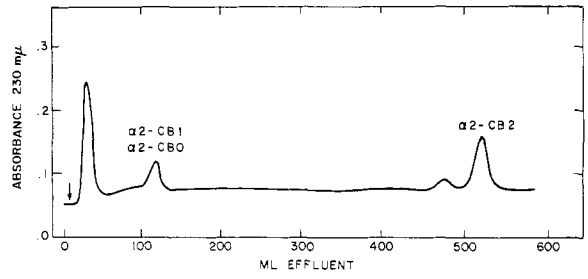

FIGURE 2: CM-cellulose elution pattern of the CNBr peptides from the α2 chain of rat skin collagen after removal of α2-CBO, 1, and 2 on a phosphocellulose column. Elution was performed in 0.004 M sodium formate (pH 3.6) using a linear gradient of 0.06–0.15 M sodium chloride. The total volume of the gradient was 800 ml.

TABLE I: Amino Acid Compositions[a] of CNBr Peptides of the α2 Chain of Rat Skin Collagen.

Amino Acid	α2-CB0	α2-CB1[b]	α2-CB2[b]	α2-CB3	α2-CB4	α2-CB5	Total CNBr Peptides	α2[c]
4-Hydroxyproline	0	0	3	26	30	27	86	89
Aspartic acid	0	1	3	14	12	13	43	44
Threonine	0	0	1	5 (4.8)	8 (7.5)	8 (8.1)	22	22
Serine	0	2	1	14	13	14	44	44
Glutamic acid	0	1	1	24	25	20	71	71
Proline	0	2	3	33	31	33	102	109
Glycine	1 (1.0)	3	10	105	107	106	332	346
Alanine	0	1	2	36	37	30	106	106
Valine	0	1	1	8 (7.6)	13	12	35	35
Isoleucine	0	0	0	6 (5.9)	4 (4.1)	7 (7.1)	17	19
Leucine	1 (1.0)	0	1	7 (7.3)	13	13	35	36
Tyrosine	0	1	0	0	0	2 (1.5)	3	3 (3.2)
Phenylanine	0	0	0	4 (3.7)	4 (4.1)	3 (2.7)	11	10 (10.0)
Hydroxylysine[d]	0	0	0	2.4	3.7	2.8	8.9	9.5
Lysine[d]	0	1	0	9.0	6.8	5.9	23	26
Histidine	0	0	0	1 (1.0)	2 (2.3)	6 (5.6)	9 (8.9)	10 (9.5)
Arginine	0	0	3	15	17	17	52	54
Homoserine	1 (0.9)	1	1	1 (0.8)	1 (0.9)	0	5	5 (4.9)[e]
Total	3	14	30	310	328	320	1005	1039

[a] Residues per peptide. Values are rounded off to the nearest whole number. Actual values are in parentheses in those cases where less than 10 residues were found. A value of zero indicates less than 0.2 residues. [b] See Bornstein and Piez (1966). [c] Values are calculated on the basis of an average residue molecular weight of 91.5 and a molecular weight of 95,000 for the α2 chain. [d] Values for lysine and hydroxylysine are not rounded off where both are present and there are less than 10 residues since there is evidence for partial hydroxylation giving rise to noninteger values (Butler, 1968; Miller *et al.*, 1969). [e] Represents methionine in the case of α2.

not seen in earlier studies (Bornstein and Piez, 1966). The small peak eluting in front of α2-CB2 has the same amino acid composition as α2-CB2. Its size varied from preparation to preparation; it presumably represents a portion of α2-CB2 in which homoserine is present as the acid rather than the lactone.

CM-cellulose Chromatography of the Larger CNBr Peptides. Figure 2 shows a CM-cellulose chromatogram of the CNBr peptides that were eluted as a group from phosphocellulose with 0.5 M NaCl. The forepeak contains largely nonpeptide ultraviolet-absorbing material. Four main peaks were consistently observed. The first three labeled α2-CB3, α2-CB4, and α2-CB5 each contains a single unique peptide. The unlabeled peak following α1-CB5 represents products resulting from incomplete cleavage with molecular weights near 60,000. Peaks α1-CB3, 4, and 5 were purified by rechromatography on CM-cellulose under the same conditions.

Amino Acid Composition of CNBr Peptides. The amino acid compositions of the six CNBr peptides are shown in Table I. The values are given as residues per peptide. It was assumed that each peptide (with the exception of the COOH-terminal peptide) would contain one residue of homoserine.

With the exception of α2-CB1 each peptide contains one third glycine. Each peptide has a characteristic amino acid composition which distinguishes it from the other peptides as well as from the whole α2 chain. The smallest peptide, α2-CB0, contains one residue each of glycine, leucine, and homoserine. Peptide α2-CG1 contains one of the three tyrosine residues in the α2 chain; its sequence has been determined (Kang *et al.*, 1967). Peptide α2-CB2 is a small peptide relatively rich in polar amino acids. Peptide α2-CB5 contains no homoserine (indicating it is COOH terminal), two of the three residues of tyrosine, and six of the nine residues of histidine. Consistent with its location at the COOH terminus it has a composition very similar to the COOH-terminal peptide of about 24,000 molecular weight obtained by cleavage of native collagen by tadpole collagenase (Kang *et al.*, 1966). Peptides α2-CB3 and 4 are similar but contain significantly different amounts of threonine, valine, isoleucine, and histidine.

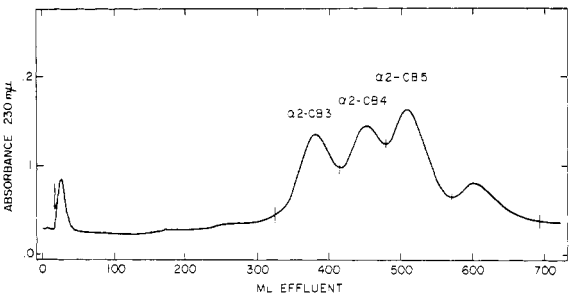

FIGURE 3: Molecular sieve chromatography (Bio-Gel P-4) of the CNBr peptides from the α2 chain of rat skin collagen. The elution was performed in 0.1 M acetic acid.

Molecular Weights of the Peptides. Table II shows the molecular weights of the CNBr peptides. The values were calculated from the amino acid composition and in the case of the larger peptides by molecular sieve chromatography. The values are in good agreement and the total of 94,000 is consistent with the known molecular weight of α2 of about 95,000.

Stoichiometry of CNBr Peptides. Although the six peptides reported above account for all the molecular weight and amino acids of the α2 chain, yields were estimated to rule out the possibility that repeating sequences were present which would yield more than 1 equiv of a peptide. This was done by chromatographing a single CNBr digest of α2 sequentially on Bio-Gel P-4, phosphocellulose, and CM-cellulose. On Bio-Gel P-4 (Figure 3), α2-CB0 and α2-CB1 separated from each other and from the other peptides. The larger peptides were then chromatographed on a phosphocellulose column to separate α2-CB2. The remaining peptides, α2-CB3, 4, and 5 in the 0.5 M wash, were then separated on a CM-cellulose column. To minimize losses, the peptides were not further purified. In each case the whole peptide sample was hydrolyzed and an

TABLE II: Molecular Weights of the CNBr Peptides from the α2 Chain of Rat Skin Collagen.

Peptide	No. of Amino Acids	Amino Acid Anal.	Molecular Sieve Chromatography[a]
α2-CB0	3	289	289
α2-CB1	14	1,365	1,365
α2-CB2	30	2,826	2,826
α2-CB3	310	28,024	29,000
α2-CB4	328	30,012	31,000
α2-CB5	320	29,404	29,500
Total	1,005	91,920	93,980

[a] The molecular weights of α2-CB0, α2-CB1, and α2-CB2 were calculated from their amino acid composition only.

TABLE III: Stoichiometry of CNBr Peptides from α2 of Rat Skin Collagen.

Column	Peptide	Equiv Recov[a]
Bio-Gel P-4	α2-CB0	0.9
	α2-CB1	1.0
Phosphocellulose	α2-CB2	0.8
CM-cellulose	α2-CB3	0.7
	α2-CB4	0.8
	α2-CB5	0.7

[a] Values are normalized to 1.0 for α2-CB1.

aliquot was analyzed on the amino acid analyzer. The yield of each was calculated from the homoserine content (homoserine plus homoserine lactine) except for the COOH-terminal peptide, α2-CB5, where the yield was calculated assuming two residues of tyrosine. The results (Table III) indicate that the peptides were present in equivalent amounts. The somewhat lower recoveries of α2-CB2, 3, 4, and 5 can be ascribed to the extra steps involved in their isolation.

Discussion

The isolation of six peptides from CNBr digests of the α2 chain completes the isolation of what appears to be all the CNBr peptides from rat skin collagen. A tentative order of the eight peptides from the α1 chains has been deduced (Piez *et al.*, 1968); this has not yet been done for the α2 chain except that α1-CB1 is NH_2 terminal and α1-CB5 is COOH terminal. These findings are an important first step in the elucidation of the complete chemistry of a collagen. Furthermore, they provide a useful method for the detailed comparison of collagens from different tissues and different species (see Piez *et al.*, 1968; Lane and Miller, 1969).

None of the peptides isolated from CNBr digests of α2 are the same as any of the peptides in digests of α1 and in both cases all of the peptides appear to be present in equimolar amounts. In digests of whole collagen, the peptides from α1 are present at twice the concentration of peptides from α2 (unpublished data) consistent with the known presence of two α1 and one α2 chains per molecule. These results suggest that α1 and α2 each consist of a single chain unique throughout its length. α1 and α2 are clearly different from each other and must therefore arise from different structural genes, but they are probably homologous as has been shown for other proteins containing dissimilar polypeptide chains.

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