IDENTIFICATION OF THREE GENETICALLY DISTINCT COLLAGENS BY CYANOGEN BROMIDE CLEAVAGE OF INSOLUBLE HUMAN SKIN AND CARTILAGE COLLAGEN

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SUMMARY. Cyanogen bromide cleavage of insoluble newborn human skin and cartilage in 70% formic acid liberates in good yield peptides derived from collagen. Characterization of these peptides and comparison with those previously derived from soluble human skin collagen permit the identification of an additional type of collagen in skin and yet another type in cartilage. The results further indicate that the new types of collagen are comprised of chains homologous to the α1 chain of soluble human skin collagen.

Cyanogen bromide (CNBr) cleavage of the α1 and α2 chains prepared from soluble collagens is an extremely useful procedure in the study of these large polypeptide chains of approximately 95,000 molecular weight. Examination of the CNBr peptides derived from soluble collagens of various lathyritic chick tissues has been instrumental in demonstrating that extracts of sternal cartilage contain an additional type of α1 chain which is not present in collagen extracted from bone or skin (1). Recently, the CNBr peptides from the α1 and α2 chains of soluble newborn human skin collagen have been isolated and characterized (2,3). Extension of these studies to the collagens in other human tissues, however, is difficult due to the relative insolubility of bone and cartilage collagens and the impossibility of utilizing experimental lathyrism as a means of enhancing collagen solubility in human subjects. We have elected, therefore, to apply the technique of CNBr cleavage to insoluble collagen and to characterize these collagens at the level of the peptides so released. In the present paper, we report the application of this technique to insoluble human skin and cartilage collagens and demonstrate in each of these tissues the presence of previously unstudied types of collagen. In accordance with the

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nomenclature previously adopted (1), the α1 chain of soluble human skin collagen is termed α1(I), the α1 chain which is apparently restricted to cartilage collagen is described as α1(II), and the new α1 chain detected in insoluble skin collagen is called α1(III).

METHODS. Skin from the trunk and limbs and epiphyseal cartilage were removed from infants who died of respiratory failure soon after birth. The tissues were mechanically cleaned of surrounding fat, muscle, bone and articular cartilage and cut into small pieces. Skin was extracted at 4°C with two portions of 1 M NaCl, 0.05 M Tris, pH 7.4 for one week, and subsequently with five changes of 0.5 M acetic acid over four weeks. During this period, the epidermis floated away from the dermis and the latter tissue was extracted with acetone and anhydrous ether, each for one day, and then lyophilized. Epiphyseal cartilage slices were extracted at 4°C for three days with 1 M NaCl, 0.05 M Tris, pH 7.4. This extraction procedure was then repeated three times with fresh solvent. Following the last extraction interval, the cartilages were rinsed thoroughly with water, lyophilized, and ground to a free-flowing powder in a Wiley Mill employing a 40 mesh sieve. The collagen remaining in each tissue after these procedures was operationally defined as insoluble collagen. In the present study, soluble human skin collagen was isolated and purified from the initial two acetic acid extracts as previously described (3). The extraction procedure for epiphyseal cartilage failed to solubilize any collagen, therefore, no soluble collagen was available from this tissue.

For cleavage with CNBr, 200-400-mg aliquots of dry tissue were suspended in 50 ml of 70% formic acid. The suspensions were flushed with nitrogen and a weight of CNBr equal to twice the weight of dry tissue was added. Flasks containing the suspensions were stoppered and incubated at 30°C for 4 hours with constant stirring and any particulate matter remaining was removed by centrifugation at 50,000 x g for 30 minutes in a refrigerated centrifuge. The solubilized peptides were obtained free of CNBr and formic acid by passing the clear supernatants over a 6 x 30 cm column of Bio-Gel P-2 (100-200 mesh)

equilibrated with 0.1 N acetic acid. The portion of the P-2 eluant containing the peptides was lyophilized. Acetic acid-soluble human skin collagen was digested with CNBr in the same manner without prior separation of the chains on carboxymethyl (CM-) cellulose.

The CNBr peptides were chromatographed initially on CM-cellulose using conditions described in the Legend for Figure 1. Appropriate fractions were combined, lyophilized and desalted. Further fractionation and purification of the known peptides from soluble skin collagen was achieved as previously described (3). Isolation and purification of new peptides derived from insoluble skin and cartilage collagens was achieved by rechromatography on CM-cellulose and agarose molecular sieve chromatography on a 1.8 x 230-cm column of Bio-Gel

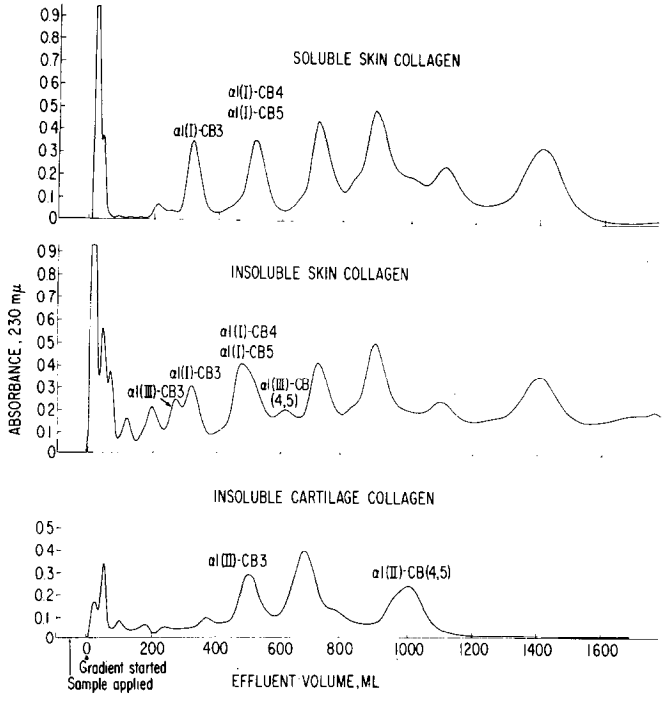

Fig. 1. CM-cellulose chromatograms of the CNBr peptides derived from soluble human skin, insoluble human skin, and insoluble human cartilage collagens. Elution was achieved with a linear salt gradient established by 1 ℓ of starting buffer (0.02 N sodium citrate, 0.02 N NaCl adjusted to pH 3.6 with citric acid) and 1 ℓ of limit buffer (starting buffer containing 0.14 N NaCl) in a two-chamber constant level device. Columns were eluted at a flow rate of 200 ml/hr. Quantities of peptides applied were: soluble skin, 250 mg; insoluble skin, 250 mg; insoluble cartilage, 150 mg.

A-1.5 (200-400 mesh) (4). Molecular weights of the isolated peptides were determined from their elution volumes on a calibrated agarose column identical to that just described (4,5). Amino acid analyses were performed on an automatic amino acid analyzer (6) after hydrolysis and preparation of the samples as previously described (4).

RESULTS AND DISCUSSION. During incubation of insoluble dermis with CNBr in 70% formic acid essentially all of the tissue was rendered soluble within 2 hours and approximately 90% of the dry weight could be recovered as lyophilized material in the P-2 eluant. Although only 35% of the dry weight of epiphyseal cartilages was recovered in this fraction, amino acid analyses of the total tissue, the solubilized fraction, and the residue after digestion indicated that approximately 95% of the total tissue collagen had been recovered. Amino acid analyses of the material solubilized from both tissues also indicated that methionyl bond cleavage, as estimated from methionine and homoserine content, was greater than 90%.

Figure 1 depicts representative CM-cellulose chromatograms of the CNBr peptides prepared from whole soluble skin (top), insoluble skin (middle), and insoluble cartilage (bottom) collagens. The peptide pattern for soluble skin collagen is that expected for peptides derived from a 2:1 molar ratio of $\alpha 1$ and $\alpha 2$ chains (3). Peaks containing $\alpha 1$(I)CB3, $\alpha 1$(I)-CB4, and $\alpha 1$(I)-CB5, the peptides of interest in this study, are so designated. The chromatogram of the peptides from insoluble skin collagen is similar to that of soluble skin collagen but contains several additional peaks. Among the additional peaks are two which, on the basis of molecular weight and amino acid composition (Table I), have been designated $\alpha 1$(III)-CB3 and $\alpha 1$(III)-CB(4,5) to indicate probable homology with sequences represented by $\alpha 1$(I)-CB3 and $\alpha 1$(I)-CB4 plus $\alpha 1$(I)-CB5. Although $\alpha 1$(III)-CB3 and $\alpha 1$(III)-CB(4,5) are relatively large peptides, the number and magnitude of the amino acid differences observed when compared with $\alpha 1$(I)-CB3 and $\alpha 1$(I)-CB4 plus $\alpha 1$(I)-CB5 clearly indicate a different primary structure. The most reasonable explanation of these data

Table I

Amino Acid Compositions of Selected CNBr Peptides Derived from Human Soluble Skin, Insoluble Skin, and Insoluble Cartilage Collagen[a)]

	α1-(CB3)			α1-CB(4,5)		
	I	II	III	I[b]	II	III
4-Hydroxyproline	15	16	19	9(9.1)	11	12
Aspartic acid	6(5.8)	4(4.1)	8(7.9)	6(5.9)	4(3.7)	4(3.8)
Threonine	0	3(2.9)	4(3.9)	1(1.0)	1(1.1)	1(0.8)
Serine	3(2.8)	3(3.1)	1(0.8)	2(1.9)	3(2.7)	4(3.9)
Glutamic acid	15	14	12	6(6.1)	6(5.9)	6(6.3)
Proline	15	17	19	8(8.2)	7(7.3)	7(6.8)
Glycine	49	49	49	28	28	28
Alanine	21	14	15	7(7.2)	6(6.0)	9(9.0)
Valine	4(4.2)	2(2.2)	1(1.0)	0	2(2.2)	1(1.0)
Methionine	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	1(0.9)
Leucine	3(3.1)	6(6.3)	5(4.9)	3(3.0)	2(2.1)	1(0.9)
Tyrosine	0	0	0	0	1(1.1)	0
Phenylalanine	3(3.0)	3(3.1)	1(1.3)	1(1.0)	1(0.9)	1(0.9)
Hydroxylysine[c]	0.2	2.5	0.3	1.3	2.9	0.2
Lysine[c]	4.8	4.2	7.0	3.7	2.9	1.6
Histidine	0	0	0	1(1.2)	1(1.0)	1(0.9)
Arginine	6(6.1)	7(6.8)	4(3.8)	5(5.0)	4(4.3)	5(5.1)
Homoserine	1(0.8)	1(0.8)	1(0.8)	2(1.8)	1(0.8)	1(0.8)
Total	146	146	146	84	84	84

a. Residues per peptide rounded off to the nearest whole number. Actual values are listed where less than 10 residues are found. A value of zero indicates less than 0.2 residue.

b. In α1(I) the sequence homologous to the region represented by α1-CB(4,5) in α1(II) and α1(III) contains a methionyl residue giving rise α1-CB4 and α1-CB5. The analysis shown is the sum of both.

c. Values for lysine and hydroxylysine have not been rounded off since there is evidence for partial hydroxylation giving rise to noninteger values (4,7).

is that the new peptides are derived from a genetically distinct α1 chain, designated α1(III). Based on the relative amounts of α1(I)-CB3 and α1(III)-CB3 in insoluble collagen, it can be estimated that α1(III) represents approximately one-third of the total collagen in newborn human skin. Since peptides derived from a new type of α2 chain were not found, and by analogy to the situation in chick cartilage (1), it seems likely that human skin collagen is a mixture of molecules with the chain compositions, $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(III)]_3$.

The CM-cellulose chromatogram of the peptides from insoluble human cartilage collagen differs from the other two and is much simpler due to the apparent absence of peptides derived from α1(I), α1(III), and α2. The peptides

designated α1(II)-CB3 and α1(II)-CB(4,5) by homology have been isolated from the indicated regions (Figure 1) and their amino acid compositions are also listed in Table I for comparison with their homologues from α1(I) and α1(III). These results indicate that essentially all of the collagen in newborn human epiphyseal cartilage is comprised of yet another type of α1 chain, α1(II), and that the chain composition of native molecules in this tissue may be characterized as $[\alpha 1(II)]_3$. A similar conclusion with regard to the collagen in lathyritic chick sternal cartilage has been derived from studies on soluble collagen extracted from the latter tissue (8,9). In addition, the CNBr peptides derived from chick α1(II)(10) are closely homologous to those derived from human α1(II) and differ from chick α1(I) peptides in much the same manner as illustrated here for human α1(I) and α1(II) peptides. These comparisons suggest that cartilage collagens represent a series of closely homologous collagens specifically adapted to the function of cartilaginous structures.

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