Analysis of cyanogen bromide peptides of type I collagen from a patient with lethal osteogenesis imperfecta

Overhydroxylation of lysine residues is found all along the collagen chains

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The CNBr peptides of type I collagen from bone of a patient with lethal osteogenesis imperfecta and age-matched controls were isolated by molecular-sieve chromatography and their amino acid compositions were determined. No differences were found between the compositions of the peptides from the patient and those from the controls, except for an increase in the degree of hydroxylation of lysine in all the peptides from the patient. Type I collagen CNBr peptides from chick-embryo skin [Barnes, Constable, Morton & Kodicek (1971) *Biochem. J.* **125**, 925–928] and guinea-pig scar tissue [Shuttleworth, Forrest & Jackson (1975) *Biochim. Biophys. Acta* **379**, 207–216] also have an increased degree of hydroxylation of lysine with an otherwise normal amino acid composition, and it was believed that this could be an embryonic form of collagen. As a similar collagen was present in the bones of the patient studied, it seems possible that the same 'embryonic' collagen is synthesized during development, in repair processes and also in genetic disorders of collagen metabolism.

Increased amounts of hydroxylysine have been reported in tissues and collagen from patients with osteogenesis imperfecta (Bleckmann *et al.*, 1971; Eastoe *et al.*, 1973; Heathcote *et al.*, 1976; Kirsch *et al.*, 1981; Traelstad *et al.*, 1977). In five of these patients (Kirsch *et al.*, 1981; E. Kirsch, T. Krieg, K. Remberger, P. Meinecke, H. J. Klose & P. K. Müller, unpublished work), type I collagen has been isolated and it could be shown that the increased amounts of hydroxylysine were accompanied by a corresponding decrease in lysine residues, i.e. the degree of hydroxylation of lysine was increased. The amino acid composition was otherwise normal. Further studies on type I collagen from one of these patients (Kirsch *et al.*, 1981) showed that it formed normal segment-long-spacing crystallites and had the same mid-point melting temperature as control type I collagen. It was cleaved by mammalian collagenase at the normal cleavage point and the pattern of its CNBr peptides did not differ from that of control type I collagen. All these findings argue against a large structural defect, but do not exclude small alterations in amino acid composition or sequence. In the present study we pursued the investigations on the primary structure of this type I collagen and prepared the CNBr peptides. Analysis of these smaller peptides permits a more sensitive

investigation of the structure and would show whether the observed overhydroxylation of lysine residues is located in a certain domain of the molecule or uniformly distributed along the molecule.

Materials and methods

Patient

The patient was a 3-month-old baby with osteogenesis imperfecta who died of respiratory distress caused by multiple fractures of his ribs. A detailed description of the patient and the two age-matched controls is given by Kirsch *et al.* (1981).

Materials

Agarose A 1.5 m (200–400 mesh) and Bio-Gel P2 (200–400 mesh) were bought from Bio-Rad, Munich, Germany. CNBr (crystallized) was obtained from Merck-Schuchardt, Hohenbrunn bei München, Germany. Acrylamide and *NN'*-methylbisacrylamide were products of Serva, Heidelberg, Germany.

Methods

Isolation of α -chains. Type I collagen was obtained from calvarial bone of the patient and from

long bones of the age-matched controls and the α -chains isolated as described by Kirsch *et al.* (1981).

Cleavage with CNBr

The α -chains of type I collagen from patient and control were dissolved in 70% (v/v) formic acid and treated with CNBr as described by Epstein *et al.* (1971). After digestion for 6 h the solubilized peptides were desalted on Bio-Gel P2 (200–400 mesh) into 0.1 M-acetic acid and freeze-dried.

Molecular-sieve chromatography

The CNBr digest was chromatographed on agarose A 1.5 m under denaturing conditions (buffer: 1 M-CaCl₂/0.05 M-Tris/HCl, pH 7.4). The material eluted in the central portions of each peak was collected and desalted on Bio-Gel P2 (200–400 mesh).

Polyacrylamide-gel electrophoresis

The components of the individual peaks were identified by 12–18% (w/v)-polyacrylamide-slab-gel electrophoresis (Laemmli, 1970).

Amino acid analysis

Amino acid analysis of peptides after hydrolysis in 6 M-HCl and under N₂ for 24 h was performed with a Durrum D 500 analyser.

Results

As Figs. 1 and 2 show, the elution profiles on agarose A 1.5 m of CNBr digests of the $\alpha 1(I)$ and $\alpha 2(I)$ chains were similar for patient and controls. The components of the individual peaks, as identified by slab-gel electrophoresis, were also the same for patient and controls (Figs. 3 and 4). α_1 -CB6, α_1 -CB3, α_2 -CB3–5 and α_2 -CB4 were each found to be the main component of a given peak, whereas α_1 -CB7 and α_1 -CB8 were found in the same peptide preparation. Further purification of the peptides was, however, not possible, because of the small amounts of material available. But since the components of the corresponding peaks from patient and controls were the same, i.e. the 'contaminants' of a given peptide preparation were the same, we carried out amino acid analysis on portions of the peaks. The amino acid composition of these partially purified preparations of α_1 -CB7 and α_1 -CB8, α_1 -CB6, α_1 -CB3, α_2 -CB3–5 and α_2 -CB4 from patient and control type I collagen is given in Table 1. It can be seen that the amino acid composition of the peptides from the patient's collagen does not differ from that of the controls, except in the values for hydroxylysine and lysine. In all the peptides from the patient, more hydroxylysine and correspondingly fewer lysine residues are found. This difference is ex-

Fig. 1. Molecular-sieve chromatography on agarose A 1.5 m under denaturing conditions of CNBr peptides of $\alpha_1(I)$ from bone of controls (a) and an osteogenesis-imperfecta patient (b)

Peaks 1–8 were collected, desalted, and identified by slab-gel electrophoresis.

Fig. 2. Molecular-sieve chromatography on agarose A 1.5 m under denaturing conditions of CNBr peptides of $\alpha_2(I)$ from bone of controls (a) and an osteogenesis-imperfecta patient (b)

Peaks 1–3 were collected, desalted and identified by slab-gel electrophoresis.

Fig. 3. Slab-gel electrophoresis of CNBr peptides of $\alpha_1(I)$ from bone of controls (a) and an osteogenesis-imperfecta patient (b)

The peptides were isolated by molecular-sieve chromatography on agarose A 1.5 m (Fig. 1) and the number of the corresponding peak is given for lanes (a) 2–8 and (b) 2–7. Lanes (a) 1 and (a) 9 represent CNBr peptides of $\alpha_1(I)$ and $\alpha_2(I)$ from controls, and lane (b) 1 represents CNBr peptides of $\alpha_1(I)$ from the patient.

Fig. 4. Slab-gel electrophoresis of CNBr peptides of $\alpha_2(I)$ from bone of controls (a) and an osteogenesis-imperfecta patient (b)

The peptides were isolated by molecular-sieve chromatography on agarose A 1.5 m (Fig. 2) and the number of the corresponding peak is given for lanes (a) 2–4 and (b) 2 and 3. Lanes (a) 1 and (a) 5, (b) 1 and (b) 4 represent CNBr peptides of $\alpha_1(I)$ and $\alpha_2(I)$ from controls and the patient respectively.

pressed in the degree of hydroxylation of lysine (%) in Table 2. Peaks 7 and 8 from Figs. 1(a) and 1(b), which contained peptides smaller than α_1 -CB3, were not further investigated.

Discussion

Type I collagen from some patients with osteogenesis imperfecta (Bleckmann *et al.*, 1971; Eastoe

et al., 1973; Heathcote *et al.*, 1976; Kirsch *et al.*, 1981; Trelstad *et al.*, 1977) contains increased amounts of hydroxylysine. This increased degree of hydroxylation could be due to a defect in the primary structure which delays helix formation and so allows the enzyme lysyl hydroxylase to act longer. Indeed, skin fibroblasts from a patient with severe osteogenesis imperfecta have been reported to

Table 1. *Amino acid compositions of CNBr peptides of type I collagen from bone of an osteogenesis-imperfecta (OI) patient and two age-matched controls (C)*

The values correspond to residues per peptide and are rounded off to the nearest whole number. Actual values are listed for residues present as less than ten residues and for hydroxyproline, proline, hydroxylysine and lysine, since partial hydroxylation can give rise to non-integral values for these amino acids. The values represent the average results from three or more analyses.

Amino acid	Peptide ...	Composition (residues/peptide)									
		α_1 -CB7 + -CB8		α_1 -CB6		α_1 -CB3		α_2 -CB3-5		α_2 -CB4	
		C	OI	C	OI	C	OI	C	OI	C	OI
Hyp		55.3	55	18.9	17.9	15.2	15.5	55.5	56.8	31.9	31.9
Asp		22	22	8.3	8.3	6.1	6.4	31	30	14	14
Thr		11	10	3.6	3.4	1.5	1.8	14	13	6	6
Ser		19	19	8.4	8.8	5.0	5.3	22	22	12	11
Hse		2.2	1.8	—	—	1.0	0.8	—	—	1.3	0.9
Glu		38	39	13	14	13	13	43	42	24	24
Pro		69.1	70	29	27	17	17	75.8	78.4	37	37
Gly		177	176	63	62	48	47	212	213	105	103.7
Ala		68	67	20	21	18	17	69	70	36	37
Cys		—	—	—	—	—	—	—	—	—	—
Val		10	11	3.5	4.2	3.2	3.5	21	20	10	11
Met		—	—	—	—	—	—	0.9	0.9	—	—
Ile		3.8	4.3	2.3	1.9	0.9	1.0	11	11	4.5	5.2
Leu		9.6	9.7	4.4	4.2	3.4	3.7	19	19	11	11
Tyr		—	—	—	—	—	—	1.9	1.5	—	—
Phe		5.4	5.4	1.7	1.9	2.3	2.3	5.4	5.1	3.2	3.2
His		1.6	1.1	1.0	1.2	0.4	0.7	9.0	7.7	2.3	2.2
Hyl		2.5	5.2	0.9	1.3	0.6	1.6	4.3	6.6	2.4	4.1
Lys		17.3	14.6	4.0	3.6	4.5	3.5	15.4	13.1	7.0	5.3
Arg		27	27	10	10	6.6	6.6	34	34	16	16
Total		539	538	192	192	147	147	644	644	324	324

Table 2. *Hydroxylation of lysine in CNBr peptides of type I collagen from bone of an osteogenesis-imperfecta (OI) patient and age-matched controls (C)*

The number of hydroxylysine residues in the various peptides is given here as a percentage of the sum of hydroxylysine and lysine residues. Each value represents the average result from three or more analyses.

	Hydroxylation of lysine (%)	
	C	OI
α_1 -CB7 + -CB8	12.5	26.2
-CB6	17.9	26.9
-CB3	12.2	30.9
α_2 -CB3-5	21.8	33.5
-CB4	25.3	43.2

synthesize an abnormal type I procollagen (Peltonen *et al.*, 1980a,b), and there is some evidence that the skin fibroblasts of a patient with lethal osteogenesis imperfecta synthesize two pro- α_1 (I) chains whose CNBr peptide pattern is abnormal (Barsh & Byers, 1981). Unfortunately it is not known whether the

collagen of these patients contain increased amounts of hydroxylysine.

In a previous study (Kirsch *et al.*, 1981) we investigated the primary structure of type I collagen from a patient with lethal osteogenesis imperfecta whose collagen contained about twice as much hydroxylysine as control collagen. Its segment-long-spacing pattern, mid-point melting temperature and sensitivity to mammalian collagenase were, however, normal, as were the chromatographic behaviour and CNBr peptide pattern of its α -chains. The amino acid composition of the α -chains only differed from that of the controls with respect to the degree of hydroxylation of lysine. In the present study we isolated by molecular-sieve chromatography the larger CNBr peptides of type I collagen from the same patient and two age-matched controls. The amino acid composition of the various peptides from the controls were found to be similar to the ones obtained by Click & Bornstein (1970) and Epstein *et al.* (1971) with human skin collagen. Only in the analysis of our α_1 -CB3 preparation we found threonine, isoleucine and histidine which are absent in the α_1 -CB3 from skin, but this is probably explained by the fact that our preparation contained

some α_1 -CB6. Comparing the peptides from the patient and controls, we found no differences in the amino acid composition except for a high degree of hydroxylation of lysine in the peptides derived from the patient. The increase was of the order of 50–150% and would have been higher if we had been able to derive the control peptides from calvarial bone as in the case of the patient instead of from long bones, whose collagen has a somewhat higher degree of hydroxylation of lysine (16.3%) than that of calvarium (11.7%) (Kirsch *et al.*, 1981). This increase was, furthermore, not limited to some regions of the helix as might have been the case if there had been a single exchange in the amino acid composition or sequence, but was seen in all the peptides investigated. This uniformity suggests that the mechanism controlling the rate of formation of the whole helix is affected or, alternatively, that the activity of the enzyme lysyl hydroxylase is higher. Higher activities of this enzyme have indeed been found in the skin of human fetuses (Antinnen *et al.*, 1973) whose collagen also contains high amounts of hydroxylysine (Kirsch *et al.*, 1981).

Collagen with an increased degree of hydroxylation of lysine has, however, been found not only in embryonic and foetal tissues (Barnes *et al.*, 1971, 1974; Kirsch *et al.*, 1981), but also in fractures (Glimcher *et al.*, 1980) and scar tissue (Barnes *et al.*, 1975; Shuttleworth *et al.*, 1975) and in vitamin D-deficient bone (Barnes *et al.*, 1973). The CNBr peptides of type I collagen have also been isolated in two of these cases: in chick embryo skin (Barnes *et al.*, 1971) and guinea-pig scar tissues (Shuttleworth *et al.*, 1975), and they have been shown to differ from the peptides of control skin collagen only with respect to the degree of hydroxylation of their lysine residues, which was higher. The similar findings here with CNBr peptides of type I collagen from an osteogenesis-imperfecta patient supports the postulate that in some stress situations, genetic or acquired, the body synthesizes 'embryonic' type I collagen. In osteogenesis imperfecta, the high degree of hydroxylation of lysine the collagen of some patients would thus represent the persistence

of an embryonic state, caused by a failure to synthesize adequate amounts of normal type I collagen.

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