Completion of the amino acid sequence of the $\alpha 1$ chain from type I calf skin collagen

Amino acid sequence of $\alpha 1(I)B8$

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The complete amino acid sequence of the 279-residue CNBr peptide CB8 from the $\alpha 1$ chain of type I calf skin collagen is presented. It was determined by sequencing overlapping fragments of CB8 produced by *Staphylococcus aureus* V8 proteinase, trypsin, Endoproteinase Arg-C and hydroxylamine. Tryptic cleavages were also made specific for lysine by blocking arginine residues with cyclohexane-1,2-dione. This completes the amino acid sequence analysis of the 1054-residues-long $\alpha(I)$ chain of calf skin collagen.

The amino acid sequence of the $\alpha 1$ chain of type I calf skin collagen has been determined, with the exception of 279 residues which correspond to CNBr peptide CB8 (Rauterberg *et al.*, 1972a,b; Fietzek & Kühn, 1975; Fietzek *et al.*, 1972a,b, 1973; Wendt *et al.*, 1972). This peptide, isolated from rat (Balian *et al.*, 1971, 1972) and chick (Highberger *et al.*, 1982) skin collagens has, however, been sequenced. In order to carry out meaningful investigations into the tertiary structures of collagen (Hofmann *et al.*, 1978) and comparisons with other types of collagen (Hofmann *et al.*, 1980) it was necessary to have the complete amino acid sequence of $\alpha(I)$ from one species. Here we present the sequence of $\alpha 1(I)CB8$ isolated from calf skin collagen, thus completing the sequence of the 1054-residues-long $\alpha 1$ chain.

Methods

$\alpha 1(I)CB8$ was prepared as previously described (Rauterberg & Kühn, 1971).

Trypsin cleavage

Cleavage of 600 mg of CB8 was carried out in 0.2 M-NH₄HCO₃, pH 7.8, with 6 mg of trypsin [tosylphenylalanylchloromethane ('TPCK')-treated; Worthington; 206 units/mg] at 37°C for 4 h. The peptide mixture was separated on a column (4 cm ×

145 cm) of Bio-Gel P4 (Bio-Rad; 200–400 mesh) equilibrated with 0.1 M-acetic acid.

Blocking of arginine residues

Arginine residues were rendered insensitive to trypsin cleavage by blocking with cyclohexane-1,2-dione, essentially as previously described (Patthy & Smith, 1975). The peptide CB8 was incubated for 2 h under N₂ at 35°C in 0.15 M-cyclohexane-1,2-dione in 0.25 M-borate buffer, pH 9. The reaction mixture was desalted over Bio-Gel P2 (100–200 mesh) into 0.1 M-acetic acid and freeze-dried. The blocked peptide (200 mg) was cleaved with 4 mg of trypsin in 0.25 M-borate buffer, pH 7.8, at 37°C for 4 h. The reaction mixture was separated on Bio-Gel P4 as described above. Before sequencing, peptides were deblocked by incubating in a 0.5 M-hydroxylamine for 8 h at 37°C.

Cleavage with *Staphylococcus* V8 proteinase

Cleavage of peptide CB8 (50 mg) with 2.5 mg of *S. aureus* V8 proteinase (Miles Laboratories; 510 units/mg) was carried out in 0.2 M-NH₄HCO₃, pH 7.8, at 37°C for 16 h. The reaction mixture was applied directly to a column (2 cm × 145 cm) of Sephadex G50 (superfine grade) equilibrated with 0.2 M-NH₄HCO₃.

Cleavage with hydroxylamine

A fresh solution of hydroxylamine hydrochloride in cold water (3.48 g/13 ml) was adjusted to pH 9.5 with 12.5 M-NaOH in an ice bath. To this solution,

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

5 ml of 1 M- K_2CO_3 was added and the pH of the mixture adjusted to 9.0 with 6 M-HCl while it was still cooling in an ice bath.

Peptide CB8 was dissolved in this solution (100 mg/25 ml) and incubated for 2 h at 45°C. The reaction mixture was desalted over Bio-Gel P2 into 0.1 M-acetic acid and freeze-dried. Hydroxylamine peptides were separated on a column (2.5 cm × 145 cm) of Agarose 1.5 m (Bio-Rad; 200–400 mesh) equilibrated with 0.05 M-Tris/HCl, pH 7.5, containing 1 M- $CaCl_2$.

Specific cleavage at arginine residues

Staphylococcal proteinase peptide 3 (SP3) was cleaved with an arginine-specific proteinase isolated from mouse submaxillary glands (Boehringer Mannheim; 150 units/mg). Cleavage was carried out in 0.2 M-Tris/HCl, pH 8.0, containing 3 mM glycine as stabilizer, for 15 h using an enzyme/substrate ratio of 1:100. As two peptides of similar size were expected, the reaction mixture was acidified with acetic acid and chromatographed on a column (2.5 cm × 15 cm) of phosphocellulose (Whatman P12) equilibrated with 5 mM-acetate buffer, pH 3.6, and eluted with a gradient of 0–0.7 M-NaCl in 2 litres of the equilibration buffer.

Amino acid analysis

Peptides were hydrolysed in 6 M-HCl at 110°C under N_2 for 24 h. The hydrolysates were analysed by a Durrum D500 amino acid analyser.

Sequence analysis

Amino acid sequences were determined automatically with a Beckman 890B or 890C sequenator. For most of the sequences, the amino acid phenylthiohydantoin derivatives were identified by using t.l.c., but more recently h.p.l.c. separations were used (Henkel & Glanville, 1982).

Results

Sequence analysis of CB8 identified the first 19 amino acids (Fig. 1, Table 1). Separation of the staphylococcal-proteinase (SP) peptides on Sephadex G-50 is shown in Fig. 2. Fractions 1 and 2 were rechromatographed on phosphocellulose (not shown), yielding peptides SP3, SP2 and SP8 respectively. Fractions 3 and 4 were rechromatographed on Bio-Gel P4, yielding SP4, SP5, SP6 from fraction 3, and SP1 and SP7 from fraction 4. Amino acid compositions of all SP peptides are shown in Table 2. Peptides 2, 3, 4, 5, 6 and 8 were sequenced, giving the positions indicated in Table 1 and diagrammatically shown in Fig. 1. Peptides were aligned by using the homologous sequence of $\alpha 1(I)$ CB8 from rat skin collagen. SP2 could also be positioned at the *N*-terminal as its sequence overlapped the *N*-terminal sequence of CB8. SP8 was positioned at the *C*-terminal, as it contained homoserine. Peptides produced by trypsin cleavages of CB8 after blocking arginine residues were separated on Bio-Gel P4 as shown in Fig. 3. Six fractions were

Fig. 1. Schematic diagram showing the relative positions of peptides produced by digestion of CB8 with *Staphylococcus aureus* V8 proteinase (SP), trypsin (T), trypsin after blocking arginine residues (Tb), hydroxylamine (HA) and mouse submaxillary-gland proteinase (MS)

The numbers are residue positions in the triple-helical portion of the $\alpha 1(I)$ chain of collagen. CB8 occupies positions 124–402. The thick bars represent the sequenced regions of the peptides. Bar A represents the sum of the sequences of the SP and Tb peptides.

Table 1. *Sequence data for individual peptides*

Key to programs: Quadrol, Beckman program numbers 122974 and 060275; DMAA, Beckman program number 111374; Poly. Polybrene.

Peptide	Length (residues)	Position	Program	Quantity sequenced (nmol)	Residues identified
CB8	279	124–402	Quadrol	400	19
SP2	45	132–176	DMAA		40
SP3	112	177–288	Quadrol	1000	45
SP4	20	289–308	Quadrol + Poly.		20
SP5	24	309–332	Quadrol + Poly.		24
SP6	15	333–353	DMAA		12
SP8	49	354–402	DMAA	500	40
Tb2	45	175–219	DMAA + Poly.	400	30
Tb3	33	220–252	DMAA	500	16
Tb4	12	253–264	DMAA		8
Tb6	20	271–290	DMAA		19
Tb8	15	328–342	DMAA		8
Tb9	18	343–360	DMAA		17
Tb10	14	361–374	DMAA		14
Tb11	28	375–402	DMAA		26
T4	30	145–174	DMAA	500	29
T7	27	193–219	DMAA + Poly.	400	27
T12	20	271–290	Quadrol		20
T19	18	343–360	Quadrol + Poly.		12
T22	6	397–402	DMAA + Poly.	500	4
HA2	162	223–384	Quadrol	500	44
MS4	53	238–291	Quadrol + Poly.		35

Fig. 2. *Separation of peptides, produced by digesting CB8 with Staphylococcus aureus V8 proteinase, on Sephadex G-50*

The column (2 cm × 145 cm) was equilibrated with 0.2M-NH₄HCO₃, pH 7.8. Fractions collected are denoted by bars and the peptides isolated from these fractions are shown above the peaks. The void volume of the column was 160 ml.

Fig. 3. *Separation of peptides, produced by digesting CB8 with trypsin after blocking arginine residues with dicyclohexane-1,2-dione, on Bio-Gel P4*

The column (4 cm × 145 cm) was equilibrated in 0.1M-acetic acid. Fractions collected are denoted by bars and the peptides isolated from these fractions shown above the peaks. The void volume of the column was 600 ml.

pooled as shown and each was rechromatographed on phosphocellulose. The peptides isolated from each fraction are indicated in Fig. 3, and their amino acid compositions are given in Table 3. Peptides Tb1, Tb5 and Tb7 were not isolated in pure form. The positions identified by sequencing each peptide

are given in Table 1 and shown schematically in Fig. 1. Exact positioning of Tb peptides was possible because of overlapping with the SP peptides with the exception of Tb4. Combining the sequences of SP and Tb peptides, as shown schematically in Fig. 1(a),

Table 2. *Amino acid compositions of the staphylococcus V8 proteinase peptides of CB8*

Values are amino acids per peptide. Numbers in parentheses are expected values based on the sequence data.

	SP1	SP2	SP3	SP4	SP5	SP6	SP7	SP8	Sum SP1-8
Hyp	0.4 (1)	4.6 (5)	11.7 (12)	2.5 (3)	1.9 (2)	2.6 (2)	1.0 (1)	5.8 (7)	30.5 (33)
Asp	—	2.1 (2)	4.8 (6)	—	1.1 (1)	—	—	2.3 (2)	10.3 (11)
Thr	—	1.7 (2)	2.0 (2)	—	—	—	—	1.7 (2)	5.4 (6)
Ser	—	—	4.0 (4)	—	0.9 (1)	0.9 (1)	—	1.9 (2)	7.7 (8)
Hse	—	—	—	—	—	—	—	0.9 (1)	0.9 (1)
Glu	1.2 (1)	1.7 (1)	10 (11)	2.0 (2)	1.1 (1)	1.2 (1)	0.9 (1)	3.4 (2)	21.5 (20)
Pro	0.9 (1)	6.4 (5)	14 (13)	2.0 (2)	2.2 (2)	1.2 (2)	—	5.4 (5)	32.1 (30)
Gly	3.0 (3)	15 (15)	40 (38)	6.7 (7)	8.5 (9)	4.6 (5)	2.3 (2)	16 (16)	96.1 (96)
Ala	0.5	8.0 (9)	13 (15)	2.1 (2)	3.3 (3)	2.1 (2)	1.0 (1)	5.4 (5)	35.4 (37)
Val	—	0.9 (1)	0.7 (1)	—	1.0 (1)	—	—	1.2 (1)	3.8 (4)
Ile	—	—	1.5 (2)	—	—	—	—	—	1.5 (2)
Leu	0.7 (1)	—	—	1.0 (1)	—	—	—	1.8 (2)	3.5 (4)
Tyr	—	—	—	—	—	—	—	—	—
Phe	—	0.9 (1)	1.0 (1)	—	1.0 (1)	—	—	—	2.9 (3)
His	—	—	—	—	—	—	—	—	—
Hyl	—	0.4	0.3	—	—	0.2	—	—	0.9
Lys	—	0.6 (1)	3.8 (4)	1.1 (1)	1.0 (1)	0.8 (1)	—	1.8 (2)	9.1 (10)
Arg	0.9 (1)	3.2 (3)	2.6 (3)	2.0 (2)	2.2 (2)	0.9 (1)	1.0 (1)	1.8 (2)	14.6 (15)
Totals	7.6 (8)	45.5 (45)	109.4 (112)	19.4 (20)	24.2 (24)	14.5 (15)	6.2 (6)	49.4 (49)	276.2 (279)

Table 3. *Amino acid compositions of the tryptic peptides obtained by cleaving CB8 after blocking arginine residues*

Values are amino acids per peptide. Numbers in parentheses are expected values based on the sequence data.

B indicates the presence of a blocked arginine residue in a peptide which could not be accurately quantified.

	Tb2	Tb3	Tb4	Tb6	Tb8	Tb9	Tb10	Tb11
Hyp	3.6 (4)	3.6 (4)	1.9 (2)	2.0 (2)	1.5 (1)	2.9 (3)	1.7 (2)	4.1 (4)
Asp	2.0 (2)	1.0 (1)	0.8 (2)	—	0.9	—	1.0 (1)	1.1 (1)
Thr	—	—	—	1.0 (1)	—	—	0.8 (1)	1.0 (1)
Ser	0.9 (1)	2.0 (2)	1.4 (1)	—	0.8	0.9 (1)	1.7 (2)	—
Hse	—	—	—	—	—	—	—	1.2 (1)
Glu	5.6 (6)	1.2 (1)	1.1 (1)	3.6 (4)	1.3 (1)	2.1 (2)	—	2.1 (2)
Pro	6.7 (6)	4.9 (5)	—	1.9 (3)	1.8 (3)	0.6	1.3 (1)	4.5 (4)
Gly	16.0 (16)	11.0 (11)	4.6 (4)	7.0 (7)	3.8 (5)	5.4 (6)	4.8 (5)	8.0 (9)
Ala	5.9 (6)	4.8 (5)	1.3 (1)	1.2 (1)	2.2 (3)	3.5 (3)	—	3.2 (3)
Val	0.1 (1)	—	—	—	—	—	—	1.0 (1)
Ile	—	0.9 (1)	—	1.0 (1)	—	—	—	—
Leu	—	—	—	—	—	0.9 (1)	0.9 (1)	—
Tyr	—	—	—	—	—	—	—	—
Phe	—	0.9 (1)	—	—	—	—	—	—
His	—	—	—	—	—	—	—	—
Hyl	0.3	—	—	—	—	—	—	—
Lys	0.8	0.9 (1)	1.0 (1)	1.1 (1)	0.9 (1)	1.1 (1)	0.9 (1)	—
Arg	B (1)	B (1)	—	—	B (1)	B (1)	—	B (1)
Totals	42.6 (45)	31.3 (33)	12.1 (12)	18.8 (20)	13.7 (15)	17.4 (18)	13.1 (14)	26.2 (28)

left unidentified regions between residues 172 and 175, 236 and 252, 261 and 270 and 401 and 402. In order to clarify these positions, three more cleavages were necessary. It had been previously shown that treatment of rat CB8 with hydroxylamine produced two major fragments by cleavage of an Asn-Gly bond. This bond was found to be present in Tb3, and therefore cleavage with hydroxylamine should en-

able the sequence determination of residues 236-270. Peptide HA2 was prepared and separated from HA1 and uncleaved CB8 by using previously described methods (Bornstein, 1970). Sequencing of HA2 gave positions 223-266 as indicated in Figs. 1 and 5 (below), leaving positions 267-270 still unclear. Inspection of the sequence around this area indicated that this region could only be elucidated by

using a specific cleavage at arginine residues. In order to simplify the separation of the required peptide, SP3 was first isolated and purified and this peptide was cleaved with an arginine-specific proteinase. The four peptides expected were 7, 9, 45 and 51 amino acid residues long. The required peptide, MS4, was 51 residues long and therefore the cleavage mixture was separated directly on phosphocellulose (not shown). The amino acid composition of MS4 is given in Table 3 and the positions sequenced shown in Table 1 and Fig. 5 (below).

Positions 172–175 and 401–402 were elucidated by using tryptic peptides T4 and T22, as shown in Fig. 1. The tryptic-digest separation is shown in Fig. 4. Peptides T4 and T22 were isolated from the peaks

indicated after rechromatographing the fractions on phosphocellulose. Amino acid compositions are given in Table 4. As can be seen from the composition of T22, this is the C-terminal tryptic peptide as it contains one homoserine residue. As five of the six residues of this peptide were sequenced, it was clear that the last residue, methionine, was residue 402.

The lysine residue at position 174 was not positively identified by sequencing. However, the identity of this residue was clear from the amino acid compositions of T4 and SP2. Two overlapping sequences, between residues 174–175 and 308–309, were not found. However, because of the extremely high homology of calf CB8 with rat CB8 (see the Discussion section) and restrictions on the length of CB8 imposed by homologies of the other CNBr peptides of the $\alpha 1(I)$ chain, it is highly unlikely that amino acids have been omitted. Additionally, an extra sequence between position 174 and 175 would require an arginine or a lysine residue to be present and this is clearly not the case, as can be seen from the amino acid composition of SP2. Similarly, additional sequences between 308 and 309 can be excluded on the basis of the amino acid composition of SP4.

Discussion

The complete amino acid sequence of $\alpha 1(I)$ CB8 from calf skin collagen is shown in Fig. 5. Compared with the corresponding sequences of rat skin and

Fig. 4. Separation of peptides, produced by digesting CB8 with trypsin, on Bio-Gel P4

See the legend to Fig. 3 for conditions. The fractions collected are denoted by bars and the peptides isolated from these fractions shown above the peaks. The void volume of the column was 600 ml.

Table 4. Amino acid compositions of some tryptic peptides of CB8, a hydroxylamine peptide and a peptide produced by the arginine-specific proteinase from mouse submaxillary gland

Values are amino acids per peptide. Numbers in parentheses are expected values based on the sequence data

	T4	T7	T12	T19	T22	HA2	MS4
Hyp	2.3 (3)	2.9 (3)	1.9 (2)	3.0 (3)	—	21.5 (23)	6.6 (4)
Asp	2.0 (2)	1.9 (2)	—	—	—	5.3 (6)	1.8 (3)
Thr	1.9 (2)	—	1.0 (1)	—	—	3.8 (4)	1.7 (2)
Ser	—	—	—	0.9 (1)	—	6.2 (7)	2.3 (3)
Hse	—	—	—	—	1.1 (1)	—	—
Glu	—	2.1 (2)	3.8 (4)	2.2 (2)	1.1 (1)	12.6 (12)	6.0 (6)
Pro	5.1 (4)	3.6 (4)	2.7 (3)	—	—	20.0 (16)	9.2 (8)
Gly	9.7 (10)	9.3 (9)	7.0 (7)	6.0 (6)	1.8 (2)	58 (55)	18 (18)
Ala	6.1 (6)	5.6 (6)	1.4 (1)	3.0 (3)	0.8 (1)	22 (19)	4.5 (3)
Val	0.8 (1)	—	—	—	0.8 (1)	3.1 (1)	0.4
Ile	—	—	1.0 (1)	—	—	2.0 (2)	0.9 (1)
Leu	—	—	—	1.0 (1)	—	3.6 (3)	—
Tyr	—	—	—	—	—	—	—
Phe	0.9 (1)	—	—	—	—	2.1 (2)	—
His	—	—	—	—	—	—	—
Hyl	0.5	0.2	—	—	—	0.4	—
Lys	0.5 (1)	0.8 (1)	1.0 (1)	1.0 (1)	—	6.1 (8)	3.3 (4)
Arg	—	—	—	0.8 (1)	—	10 (7)	0.6 (1)
Totals	29.8 (30)	26.4 (27)	19.8 (20)	17.9 (18)	5.6 (6)	176.7 (162)	55.3 (53)

Fig. 5. Complete amino acid sequence of $\alpha 1(I)$ -CB8 from calf skin collagen expressed in the one-letter code for amino acids [see *Biochem. J.* (1969) **113**, 1–4]
‘+’ above P or K denotes 4-hydroxyproline and hydroxylysine respectively. Numbers above the sequence indicate the residue number within the triple-helical region of the $\alpha 1(I)$ chain. —, Residue identified by automatic sequencing of the peptide shown to the left; CB, CNBr-derived peptides; SP, *Staphylococcus* V8 proteinase-derived; T, tryptic; Tb, tryptic fragments of peptides with blocked arginine residues; HA, hydroxylamine-derived; MS, mouse submaxillary-gland proteinase-derived.

	137	138	140	146	150	162	171	177	182	191	215	222	248	276	278	300	306	311	323	330	335	338	339	384	390
Calf	A	P	P	N	T	A	V	G	P	V	E	N	P	T	I	A	P	G	V	A	A	P	A	N	P
Rat	P	P	S	D	V	T	A	A	A	V	Q	N	A	A	V	S	P	G	V	A	S	P	A	B	A
Chick	P	S	P	N	P	A	A	T	A	S	Q	T	P	A	V	S	A	A	I	P	S	A	V	D	A

Fig. 6. Positions in $\alpha 1(I)$ CB8 from calf, rat and chick skin collagen where the sequences differ
Numbers indicate residue number within the triple-helical region of the $\alpha 1(I)$ chains. ‘+’ above P denotes 4-hydroxyproline.

chick skin collagens, there is 94 and 92.5% identity respectively. Of the residues, 91% are identical in all three species. All substitutions are conservative and the positions in which differences occur are shown in Fig. 6. All proline residues in the Y-position of the

repeating triplet Gly-X-Y were hydroxylated, but none in the X-position, in accordance with the substrate specificity of prolyl 4-hydroxylase (Tryggvason *et al.*, 1977). The extent of proline hydroxylation varied between 50 and 100%. Of the

ten lysine residues present (two in the X- and eight in the Y-position), three in the Y-position were partially hydroxylated (position 174, 40%; position 219, 30%; position 342, 20%). No structural basis for the pattern of partial hydroxylation of lysine or proline was apparent.

In the sequence of rat CB8, positions 383 and 384 were reported as Glx-Asx. In calf these residues were clearly identified as Gln-Asn, giving rise to a second hydroxylamine cleavage point Asn-Gly at position 384–385. This explained the absence of homoserine in calf peptide HA2.

Blocking arginine residues with cyclohexane-1,2-dione before cleaving with trypsin and chromatographing the blocked peptides in acetic acid on Bio-Gel P4 was found advantageous. The peptides containing blocked arginine residues ran anomalously in this system. The more blocked arginine a peptide contained, the longer it was retained by the column. Thus peptide Tb10, which contained 14 amino acids but no arginine, was separated from Tb8, which contained 15 amino acids and one blocked arginine residue (Fig. 3). Similarly, Tb11, with 28 amino acids and one blocked arginine residue, was eluted after Tb6, which has 20 amino acids and no arginine. Tb1, with 27 amino acids and three blocked arginine residues, was eluted with Tb4 with only 12 amino acids and no arginine. The peptides were deblocked before rechromatographing or sequence determination. After sequence analysis the arginine phenylthiohydantion derivative could be identified in the normal way.

There are now four completely sequenced collagen α -chains: the $\alpha 1$ chains from calf and chick type I collagen and the $\alpha 1$ chains from calf and human type III collagen. In addition, the sequence of the N-terminal extension of the $\alpha 1$ chain of calf type I procollagen has been reported, giving a continuous sequence of 1194 residues for the calf α -chain plus extension (Hörlein *et al.*, 1979).

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