Chemical characterization of cyanogen bromide fragments from the β-chain of human complement factor C3

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The isolated β-chain of human complement factor C3 (C3β) was fragmented by cyanogen bromide. Nine fragments were defined by gel filtration and high-pressure liquid chromatography, and characterized with respect to their M_r, amino acid composition and N-terminal amino acid sequence. Approx. 30% of the primary structure of C3β was determined. Alignment of the 3 N-terminal fragments allowed determination of 61 of the amino terminal residues of C3β. This region demonstrated 40% homology with the sequence in the N-terminal segment of the α-chain of the cobra venom factor.

Complement *Human C3* *Cyanogen bromide fragmentation* *Sequence* *Homology*

1. INTRODUCTION

The complement factor C3 is one of the central components in the complement cascade. Functionally, C3 is of crucial importance for activation of both the classical and alternative pathway. The molecule is composed of two polypeptide chains designated α (M_r 120000) and β (M_r 75000) [1,2], which are combined by disulfide bridges and non-covalent forces.

In addition to the interaction with several other complement proteins, activated C3 (C3b) is also able to elicit a covalent bond to other molecules, and to attach to cell receptors (review [3]). Many of these functions are closely associated with the α-chain (C3α). Concerning the β-chain (C3β), very little is known about its structure and virtually nothing about its function. The M_r, amino acid composition and a short N-terminal sequence have been published [4,5]. In contrast to C3α, C3β is fairly resistant to proteolytic enzymes [1,4,6]. To understand the functional role of C3β further knowledge about the structure is necessary. We describe here the preparation and characterization of cyanogen bromide fragments from C3β.

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were purchased: Sephadex G-75 superfine, Sephadex G-200, QAE–Sephadex A-50, SP–Sephadex C-50 (Pharmacia, Uppsala); controlled pore glass (CPG 10) and 3-aminopropyltriethoxysilane (Serva, Heidelberg); DITC (Rathburn, Walkerburn, England); guanidine hydrochloride Aristar® (BDH, Poole, England); phenylmethylsulfonyl fluoride (Sigma, St. Louis).

2.2. *Isolation of human C3*

Fresh frozen plasma was thawed and phenylmethylsulfonyl fluoride was added to a concentration of 0.25 mg/ml. The plasma was adsorbed twice with QAE–Sephadex A-50 (15%,

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Abbreviations: DITC, *p*-phenylenediisothiocyanate; HPLC, high-pressure liquid chromatography; C3α, α-chain of human C3; C3β, β-chain of human C3; CVF, cobra venom factor; CVFα, α-chain of CVF

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v/v) under continuous stirring for 1 h at 4–6°C. The adsorbed gel portions were then layered on top of a thin bed of fresh QAE–Sephadex A-50 in separate columns and washed with20 mM Tris–HCl buffer with 0.11 M NaCl and 2 mM EDTA (pH 7.0). C3 was eluted by 0.2 M NaCl. C3 was assayed in the column effluent by hemolytic titration [7,8]. The C3 pools were combined and dialysed against a buffer of 20 mM phosphate with 40 mM NaCl (pH 6). Insoluble material was removed by centrifugation and the supernatant applied on an SP–Sephadex C-50 column (5 × 30 cm) equilibrated in the same buffer. C3 was separated from C5 and factor H by elution with a gradient composed of 1.5 l of starting buffer *vs* 1.5 l of 20 mM phosphate buffer with 0.1 M NaCl (pH 7.5). Final purification of C3 was achieved by gel filtration on Sephadex G-200 in 0.02 M phosphate buffer with 0.15 M NaCl (pH 7.2).

2.3. *Isolation of the β-chain of human C3 (C3β)*

Purified human C3 was treated with methylamine as in [8] and then reduced and carboxymethylated [9]. The material was dialysed against 1.0 M acetic acid, lyophilized and redissolved in 0.1 M pyrophosphate with 6 M guanidinium hydrochloride. Maleylation was carried out essentially as in [10], and the maleylated C3 was transferred to 20 mM Tris–HCl with 0.25 M NaCl (pH 8.0). C3β was obtained by chromatography on a DEAE–Sephacel column (2.5 × 35 cm) equilibrated in the same buffer and eluted by applying a gradient of 500 ml starting buffer *vs* 500 ml of 20 mM Tris buffer with 0.75 M NaCl. The prepared C3β was found to be devoid of α-chain material as found by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE), amino acid analysis and immunodiffusion. Unblocking of the maleyl groups of C3β was achieved in 0.1 M glycine buffer (pH 3.0).

Full details of the separation of C3, C4, C5, factor H and the C3 chains will be published elsewhere.

2.4. *Preparation of cyanogen bromide fragments of C3β*

Lyophilized C3β (0.6 μmol) was dissolved in 7 ml of 100% formic acid, which was subsequently diluted with 3 ml distilled water. Digestion was performed at room temperature with 0.3 mmol cyanogen bromide for 24 h, whereafter the sample was diluted with 50 vols of distilled water and lyophilized. It was then suspended in 10 ml of 50% acetic acid. Insoluble material was collected by centrifugation at 15000 × *g* (pool 2). The supernatant (pool 1) was chromatographed on a 4.9 cm^2 × 100 cm column of Sephadex G-75 superfine in 1.0 M acetic acid at a flow rate of 10 ml/h, and the effluent was monitored at 280 nm.

2.5. M_r *determination of cyanogen bromide fragments*

M_r determinations were performed by chromatography on TSK 2000 (7.5 × 600 mm) in 6 M guanidinium hydrochloride in 25 mM ammonium acetate (pH 6.5), with a flow rate of 33 μl/min. The column eluate was monitored at 230 nm. Reduced and carboxymethylated proteins and cyanogen bromide fragments from cytochrome *c* were used as standards and the M_r values were calculated as in [11].

2.6. *Amino acid analysis and sequence determination*

Amino acid analysis was performed as in [12]. Manual Edman degradation was done by the direct phenylthiohydantoin method [13]. Automatic Edman degradation on solid phase was performed on an LKB 4020 solid-phase peptide sequencer. Cyanogen bromide peptides with a C-terminal homoserine were immobilized on aminopropyl glass as in [14]. Peptides without C-terminal homoserine were coupled to DITC-glass [15]. The PTH amino acids were identified by HPLC on a Nucleosil ODS column (4 × 300 mm) in 6 mM sodium acetate buffer (pH 4.9), using a gradient of acetonitrile, except for PTH-valine and PTH-methionine which were determined by back hydrolysis [16].

3. RESULTS AND DISCUSSION

The chromatogram of pool 1 on Sephadex G-75 is shown in fig.1. The peaks were analysed by amino acid analysis (table 1), and manual and automatic Edman degradation. Three cyanogen bromide fragments were obtained in homogeneous form: CN2, CN3 and CN8. Their N-terminal sequences are shown in fig.2. CN8 was completely

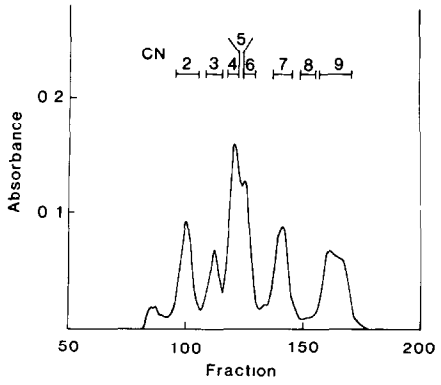

Fig.1. Gel filtration on Sephadex G-75 superfine of the acid-soluble cyanogen bromide peptides of human C3β (pool 1) in 1.0 M acetic acid. The column eluate was monitored at 280 nm. Pooling was performed as indicated.

sequenced, 27 residues were determined in CN2 and 41 residues in CN3.

The third peak contained 3 different fragments (CN4, CN5, CN6), yielding a triple sequence on automatic Edman degradation. However, by separate analyses of the ascending and descending parts of the peak (see fig.1) double sequences were obtained for each analysis, one of which was dominant in each pool. By determination of the recovery of the individual PTH-amino acids it was found that the ascending part contained 60–70% of CN4 and the descending part 60–70% of CN6. The sequence of CN6 was completed with a C-terminal homoserine in position 30, while the sequence of CN4 could be determined up to residue 31. The PTH-amino acids in the triple sequence in the central part of the peak occurred in approximately equimolar proportions, therefore the se-

Table 1

Amino acid composition of C3β and cyanogen bromide fragments

	CN2	CN3	CN4	CN6	CN7a	CN7b	CN8	CN9	C3β
Trp[a]	N.D.	N.D.	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	3.2
Lys	4.8	5.6	1.7	2.0 (3)	0.4	1.0 (1)	–	–	38.7
His	1.9	4.2	0.6	0.7 (1)	0.3	0.8 (1)	–	–	9.3
Arg	6.0	1.2	1.3	1.5 (1)	1.5 (1)	1.8 (2)	0.2	–	29.0
Cys[b]	0.6	0.1	0.1	0.3	–	–	–	–	4.4
Asx	9.3	6.6	5.7	2.0	1.2 (1)	1.0 (1)	1.1 (1)	0.4	51.2
Thr	9.6	6.9	1.6	2.3 (3)	1.9 (2)	0.9 (1)	0.2	–	46.6
Ser	8.0	3.5	3.6	2.3 (2)	1.8 (2)	0.4	0.5	0.9 (1)	45.5
Glx	13.7	6.0	5.8	3.3 (3)	2.4 (3)	0.9 (1)	0.4	0.4	79.6
Pro	7.8	2.3	2.4	2.9 (4)	1.0 (1)	–	1.0 (1)	1.0 (1)	41.1
Gly	6.8	3.2	2.3	1.9 (2)	0.2	0.4	0.3	0.3	45.5
Ala	6.2	4.4	0.7	1.6 (1)	0.6	1.8 (2)	0.2	–	30.9
Val[c]	9.4	9.3	3.7	2.3 (1)	–	0.2	0.3	–	68.7
Met/Hse	1.0	0.6	0.4	0.4 (1)	0.4 (1)	0.4 (1)	0.6 (1)	0.6 (1)	11.1
Ile[c]	2.9	0.7	2.0	1.6 (3)	2.3 (3)	1.7 (2)	–	–	29.4
Leu	10.1	7.0	5.8	2.3	2.0 (2)	1.0 (1)	1.0 (1)	0.3	57.5
Tyr	2.3	0.8	0.9	1.4 (2)	1.7 (1)	2.7 (3)	–	–	24.4
Phe	1.9	1.7	0.7	1.2 (2)	–	–	0.9 (1)	0.3	25.7
Residues	102.3[d]	62.8[d]	39.7[d]	(30)	(17)	(16)	(5)	(3)	641.5

[a] Determined after hydrolysis in 3 M toluene-*p*-sulfonic acid
[b] Determined as carboxymethylcysteine
[c] Determined after hydrolysis for 72 h
[d] Estimated after gel filtration in 6 M guanidinium hydrochloride

Figures within parentheses represent numbers of amino acid residues estimated from amino acid analyses and sequence determinations. N.D., not determined

	1									10										20										30										40
CN2	V	F	V	T	N	P	D	G	S	P	A	Y	R	V	P	V	A	V	Q	G	E	D	T	V	Q	S	L													
CN3	V	L	E	A	H	D	A	Q	G	D	V	P	V	T	V	T	V	H	D	F	P	G	K	K	L	V	L	S	S	E	K	T	V	L	T	P	A	T	N	Q	Q
CN4	X	N	I	E	N	P	E	G	I	P	V	K	Q	D	S	L	S	S	Q	N	Q	L	G	V	L	P	L	S	W	D	I										
CN5[b)]	X	A	L	P	Y	S	T	V	G	N	S	N	N	Y	L	H	L																									
CN6	X	Q	A	E	R	S	G	I	P	I	V	T	S	P	Y	Q	I	H	F	T	K	T	P	K	Y	F	K	P	G	M											
CN7a	Y	S	I	I	T	P	N	I	L	P	L	E	S	E	E	T	M																									
CN7b	D	R	A	H	E	A	K	I	R	Y	Y	T	Y	L	I	M																									
CN8	P	F	D	L	M																																					
CN9	S	P	M																																							

Fig.2. N-terminal amino acid sequence of cyanogen bromide fragments of C3β. X denotes that the N-terminal amino acid could not be ascertained. Manual Edman degradation of a pool containing CN4, CN5 and CN6 yielded only PTH-valine. The sequence of CN5 was deduced from the same pool, whereby the sequences of CN4 and CN6 were subtracted from the obtained triple sequence.

quence of CN5 was deduced up to step 15 by subtracting the sequences of CN4 and CN5.

Manual Edman degradation revealed that the fourth peak contained two fragments (CN7a and CN7b). These were further purified with HPLC on a 5μ Lichrosorb RP8 column (4 × 250 mm), operated at 35°C with a linear gradient of acetonitrile in 0.10% trifluoroacetic acid (not shown). The two fragments were well separated from each other as determined by amino acid analysis and manual Edman degradation. The completed sequences of the fragments are shown in fig.2.

Three amino acids in equimolar proportions were detected by amino acid analysis in the total volume eluate of the G-75 column: homoserine, proline and serine (CN9, fig.1). By manual Edman degradation PTH-serine was obtained, suggesting the sequence Ser-Pro-Hse of the peptide. This sequence agrees with the reported N-terminal sequence of C3β [5], thus CN9 most probably represents the N-terminal cyanogen bromide fragment.

Chromatography of cyanogen bromide-digested C3β on TSK 2000 in guanidine hydrochloride is shown in fig.3A. For comparison, pool 2 (acid-insoluble material) was run in the same way (fig.3B), and 3 peaks were generated (in addition to the total volume). These peaks coincide with the first 3 eluting peaks of the total digest (fig.3A), and represent most probably the acid-insoluble

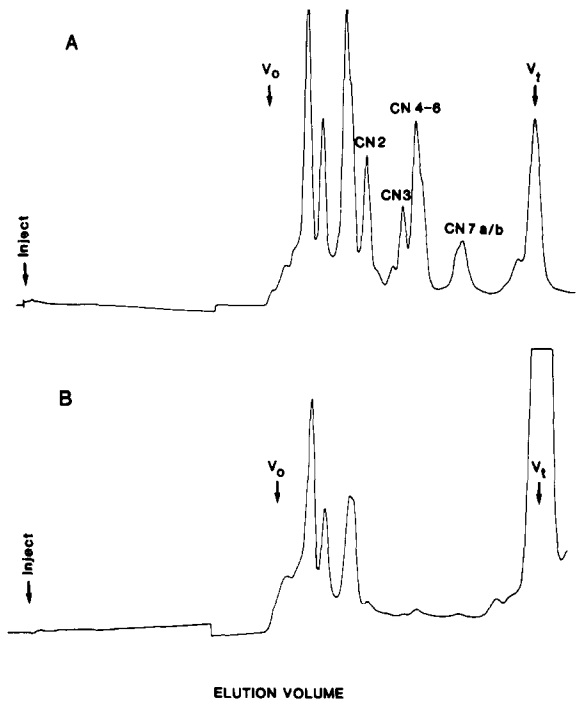

Fig.3. Chromatography in 6 M guanidinium hydrochloride on TSK 2000. The column eluate was monitored at 230 nm. V_o, void volume; V_t, total volume. (A) Cyanogen bromide-digested C3β. (B) Acid-insoluble fragments from cyanogen bromide-digested C3β (pool 2).

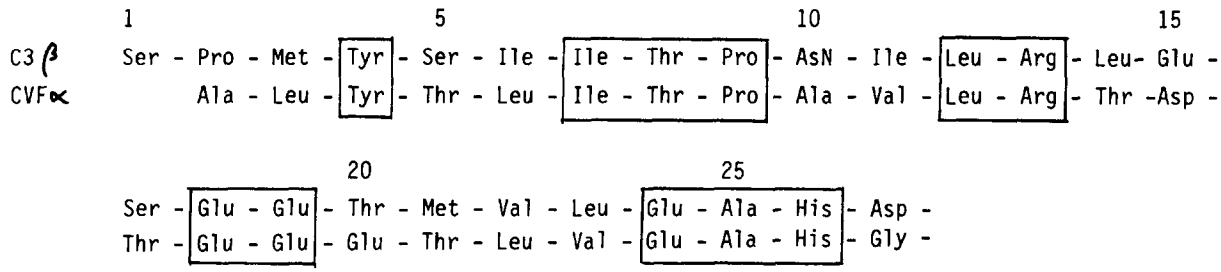

Fig.4. Comparison of the N-terminal amino sequence of the α-chain of CVF and human C3β. The N-terminal sequence of CVFα is reported in [13] and the amino terminal sequence of C3β is obtained by alignment of cyanogen bromide peptides CN9, CN7a and CN3. Identical residues in the two chains are boxed in. The numbers refer to amino acid residues in C3β; the N-terminal amino acid in CVFα is located at no.2.

cyanogen bromide fragments. Since C3β contains 11 methionines, as has been found by us (table 1) and others [4,5], 12 cyanogen bromide fragments would be expected, suggesting 3 acid-insoluble fragments besides the 9 acid-soluble fragments, which would agree with the number of peaks obtained in fig.3B. The elution positions of CN2, CN3, CN4-6 and CN7a/b were estimated after comparison with the elution pattern of the fragments from Sephadex G-75. The M_r values obtained were 11300 (CN2), 6800 (CN3), 4300 (CN4-6) and 1400 (CN7a/b). The acid-soluble fragments account for approx. 50% of the M_r of C3β. All contained homoserine, which indicates that the C-terminal fragment of C3β might be found among the acid-insoluble fragments. None contained significant amounts of cysteine, suggesting that all the cysteine residues are located in the acid-insoluble fragments.

From the reported N-terminal amino acid sequence of C3β [5], the alignment CN9-CN7a can be made. If this combined sequence is compared with the reported N-terminal sequence of the chains of CVF [9], a homology of 40% is found between the first 19 amino acid residues of the α-chain in CVF (CVFα), and residues 2–20 of C3β (fig.4). This confirms the structural relationships between C3β and CVFα as suggested by immunological methods [17]. Furthermore, residues 20–25 in CVFα are so similar to the amino terminal residues in CN3 that the latter can be assumed to represent the third cyanogen bromide fragment from the amino terminal end of C3β, which makes it possible to deduce the N-terminal amino acid sequence of C3β up to residue 61. We could not detect any sequence homology between our C3β fragments and the amino terminal sequences of the other two chains of CVF (CVFβ and CVFγ), which is in accordance with the statement that these chains are immunologically related to C3α. Since the α-chain of CVF most probably corresponds to the β-chain of cobra C3 [17] further studies of the primary structure of CVFα and human C3β will give valuable information about the phylogenetic development of the C3 molecule.

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