Amino acid sequence of the Bb fragment from human complement Factor B

Alignment of the cyanogen bromide-cleavage peptides

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The alignment of all the CNBr-cleavage peptides of fragment Bb from human Factor B (a component of the alternative pathway of complement) was determined. This was derived from cleavage of the fragment Bb at arginine residues by using trypsin and clostripain. Details of the isolation and amino acid sequences of these peptides are given. Together with previously published *N*-terminal sequences of the CNBr-cleavage peptides [Christie & Gagnon (1982) *Biochem. J.* **201**, 555–567], this provides the amino acid sequence of the *N*-terminal half of fragment Bb.

Factor B is cleaved at a single Arg–Lys bond by Factor $\bar{D}$ during the activation of complement. This results in an *N*-terminal Ba fragment and a C-terminal Bb fragment with molecular weights of approx. 30 000 and 60 000 respectively (Kerr, 1979; Lesavre *et al.*, 1979; Niemann *et al.*, 1980). The Bb fragment is the catalytically active component of the complex proteinases of the alternative pathway of complement, C3 convertase C3b,Bb and C5 convertase C3b,Bb,C3b (for reviews see Fothergill & Anderson, 1978; Porter, 1979; Müller-Eberhard & Schreiber, 1980; Reid & Porter, 1981).

Partial amino acid sequence studies have showed Factor B to be a novel type of serine proteinase with a catalytic chain of approximately twice the size of proteinases previously studied (Christie *et al.*, 1980; Mole & Niemann, 1980). Although the *N*-terminal sequence of the Bb fragment showed no homology with the well-conserved *N*-termini of the activated forms of serine proteinases, considerable homology was found in the C-terminal portion of the molecule, which contained the essential active-site residues.

Details of the isolation and *N*-terminal sequences of nine CNBr-cleavage peptides from Factor B have been reported (Christie & Gagnon, 1982). These sequences accounted for 359 residues of Factor B and enabled full definition of the sequence containing the site of Factor $\bar{D}$ cleavage and the localization of a free thiol group in the Bb fragment. Since amino acid analyses of the Ba and Bb fragments indicated that all but one methionine

residue are located within the Bb portion of the molecule, the alignment of the two CNBr-cleavage peptides of the Ba fragment was deduced. Details of the isolation of the tryptic peptides and the alignment of the CNBr-cleavage peptides of the Bb fragment are given in the present paper. The complete sequence of the large C-terminal CNBr-cleavage peptide from Factor B, CB-II (molecular weight approx. 30 000), is described in the following paper (Christie & Gagnon, 1983). This work provides the complete sequence of 505 residues of the catalytically active Bb fragment of Factor B.

Materials and methods

Materials and general methods

The materials used and procedures performed have been described previously (Christie & Gagnon, 1982).

Tryptic digest of the Bb fragment

Reduced and *S*-[¹⁴C]carboxymethylated succinoylated Bb (890 nmol) in 0.1 M-NH₄HCO₃ (10 ml) was incubated with Tos-Phe-CH₂Cl-treated trypsin (0.28 mg) for 2 h at 37°C. A further 0.28 mg of enzyme was added, the incubation repeated and the digest freeze-dried. The digest was redissolved in 0.1 M-NH₄HCO₃ (1.5 ml) and centrifuged. The pellet was extracted with a further 1.5 ml of 0.1 M-NH₄HCO₃, and the combined supernatants (3 ml) were applied to a column of Sephadex G-50 (superfine grade) equilibrated with 0.1 M-NH₄HCO₃. The flow rate was 15 ml/h, and 2.0 ml fractions were collected. Peptides were detected by their absorbance at 280 nm. The radioactivity of samples

Abbreviations used: h.p.l.c., high-pressure liquid chromatography. The nomenclature of complement components is that recommended by the World Health Organisation (1968).

(15 μ l) from the column fractions was determined by scintillation counting. Peptides were further purified by ion-exchange chromatography on DEAE-Sephacel and by h.p.l.c. as described in the Results section.

Clostripain digest of the Bb fragment

Unmodified Bb fragment (10 mg) was subjected to limited proteolysis by incubation in 20 mM- NH_4HCO_3 (10 ml) with clostripain (125 μ g) for 2 h at 37°C and the digest freeze-dried. The digest was redissolved in 0.1 M- NH_4HCO_3 (2.0 ml) and applied to a column (2 cm $\times$ 90 cm) of Sephadex G-75 equilibrated with 0.1 M- NH_4HCO_3 . A fragment Bb,Cl-3, corresponding to the *N*-terminal portion of Bb with a molecular weight of approx. 30 000, was eluted between 141 and 168 ml. The reduced and *S*-[^3H]carboxymethylated succinoylated fragment Bb,Cl-3 (2 mg) in 20 mM- NH_4HCO_3 (1 ml) was further digested by incubation with clostripain (25 μ g) for 2 h at 37°C. The digest was then subjected to gel filtration on a column (2 cm $\times$ 90 cm) of Sephadex G-75 in 0.1 M- NH_4HCO_3 . Peptides Bb,Cl-3,Cl-1 and Bb,Cl-3,Cl-2 were eluted at 142–160 ml and 162–192 ml respectively. Peptides Bb,Cl-3,Cl-1 and Bb,Cl-3,Cl-2 from two such preparations were pooled before sequence analysis. Clostripain was activated immediately before use by incubation of the enzyme (0.5 mg/ml) in 20 mM- NH_4HCO_3 /10 mM-dithiothreitol, pH 8.0, for 1 h at 37°C.

V8-proteinase digest of peptide Bb,T-1e

Peptide Bb,T-1e (80 nmol) was dissolved in 50 mM- NH_4HCO_3 /2 mM-EDTA, pH 7.8 (0.4 ml), and digested with V8 proteinase (15 μ g) for 4 h at 37°C. The digest was injected on to a μ -Bondapak C_{18} column equilibrated with 0.1% NH_4HCO_3 /acetonitrile (95:5, v/v). A linear gradient of 60 min was pumped to the column at 1 ml/min to give a final solvent composition of 0.1% NH_4HCO_3 /acetonitrile (60:40, v/v). Peptides Bb, T-1e, SP-5 and Bb,T-1e,SP-14 were eluted after 15 and 25 min respectively.

V8-proteinase digest of peptide Bb,Cl-3,Cl-1

Peptide Bb,Cl-3,Cl-1 (40 nmol) in 50 mM- NH_4HCO_3 /2 mM-EDTA, pH 7.8 (0.5 ml), was digested with V8 proteinase (7 μ g) for 2 h at 37°C. The digest was injected on to a Radial-pak C_{18} column (Waters Associates) equilibrated with 0.1% NH_4HCO_3 /acetonitrile (95:5, v/v). A linear gradient of 60 min was applied to the column at 1 ml/min to give a final solvent composition of 0.1% NH_4HCO_3 /acetonitrile (45:55, v/v). Two peaks, denoted SP-8 and SP-9, were eluted between 23 and 26 min from the time of injection. This material had identical amino acid compositions and these frac-

tions were pooled before amino acid sequence analysis.

V8-proteinase digest of peptide CB-V

Peptide CB-V (approx. 100 nmol) in 50 mM- NH_4HCO_3 /2 mM-EDTA, pH 7.8 (0.5 ml), was digested with V8 proteinase (25 μ g) for 2 h at 37°C. The digest was then subjected to h.p.l.c. as described above for the digest of peptide Bb,Cl-3,Cl-1. Peptide CB-V,SP-14 was eluted 33 min from the time of injection.

Results

We have already reported details of the isolation and *N*-terminal sequences of the CNBr-cleavage peptides from Factor B (Christie & Gagnon, 1982). The present study showed that all but one methionine residue of Factor B were located in the Bb portion of the molecule. The succinoylated Bb fragment has now been subjected to digestion with trypsin to provide a complementary set of peptides necessary to the completion of the sequence of the Bb fragment. Automated sequence analysis of tryptic peptides and clostripain peptides containing methionine established the alignment of the CNBr-cleavage peptides of the Bb fragment.

Isolation of tryptic peptides from the succinoylated Bb fragment

The tryptic digest of reduced and *S*-[^{14}C]carboxymethylated succinoylated Bb fragment was initially fractionated by gel filtration on a column (2 cm $\times$ 90 cm) of Sephadex G-50 (superfine grade) equilibrated with 0.1 M- NH_4HCO_3 (Fig. 1a). Fractions were pooled as indicated and designated T-1–T-6, and freeze-dried.

Isolation and amino acid composition of peptides from the T-1 pool (Fig. 1a)

The tryptic peptides present in pool T-1 were further purified by ion-exchange chromatography on a column (1 cm $\times$ 25 cm) of DEAE-Sephacel, equilibrated with 0.25 M- NH_4HCO_3 and developed with a linear gradient of NH_4HCO_3 (0.25–1.0 M) in a total volume of 300 ml (Fig. 1b). Nine pools were made as indicated and designated DE-1–DE-9. Five peptides were subsequently isolated from these pools, and these peptides were designated T-1a–T-1e. Amino acid analysis indicated that pools DE-1 and DE-2 contained the same peptide (T-1a), and no further purification was performed. Peptides from other fractions were purified further by h.p.l.c. on a μ -Bondapak C_{18} column (3.9 mm $\times$ 300 mm) equilibrated with 0.1% NH_4HCO_3 /acetonitrile (90:10, v/v). A linear gradient of 30 min was pumped to the column at 1 ml/min to give a final solvent composition of 0.1% NH_4HCO_3 /acetonitrile (70:30, v/v).

Fig. 1. Separation of the peptides from the tryptic digest of the succinoylated Bb fragment

(a) The freeze-dried digest was dissolved in 0.1 M-NH₄HCO₃ (3 ml) and applied to a column (2 cm $\times$ 90 cm) of Sephadex G-50 (superfine grade) equilibrated with 0.1 M-NH₄HCO₃. The flow rate was 15 ml/h, and 2 ml fractions were collected. The radioactivity present in portions (15 μ l) of column fractions was determined by scintillation counting. Fractions were pooled as indicated, designated T-1–T-6 and freeze-dried. —, A_{280} ; ----, radioactivity. (b) The freeze-dried material corresponding to pool T-1 above was redissolved in 0.25 M-NH₄HCO₃ (3 ml), and applied to a column (1 cm $\times$ 25 cm) of DEAE-Sephacel equilibrated with the same buffer and developed with a linear gradient of NH₄HCO₃ (0.25–1.0 M) in a total volume of 3000 ml at a flow rate of 12 ml/h. Portions (20 μ l) of column fractions (2.5 ml) were taken for counting of radioactivity. Nine pools were made and designated in order of their elution DE-1–DE-9. —, A_{280} ; ----, radioactivity.

Two peptides (T-1b and T-1c) were isolated from pools DE-3–DE-5 by this procedure. Similarly, a further two peptides (T-1d and T-1e) were isolated from pools DE-7 and DE-8. Peptides were not isolated from pools DE-6 and DE-9 in an acceptable yield.

Three of the five peptides isolated from the T-1 pool of the tryptic digest of succinoylated fragment Bb were found to contain methionine. The amino acid compositions of two of these, peptides T-1c and T-1e, are presented in Table 1. The third methionine-containing peptide (T-1d), isolated in only a 12% yield, was found on further analysis to be derived partly from a chymotryptic-like cleavage (see below). Yields of peptides T-1a and T-1b and their positions in the sequence of fragment Bb estimated

from their amino acid compositions are presented in Table 2.

Isolation and amino acid compositions of the peptides from pools T-2–T-6 of the tryptic digest of succinoylated fragment Bb (Fig. 1a)

Peptides from pools T-2–T-6 of the tryptic digest of fragment Bb were purified directly by h.p.l.c. Water (0.9 ml) and 10% (v/v) trifluoroacetic acid (0.1 ml) was added to the freeze-dried material, and a portion (0.3 ml) was centrifuged. The soluble material was injected on to a μ -Bondapak C₁₈ column equilibrated with 0.1% trifluoroacetic acid/solvent B (95:5, v/v); solvent B was methanol/acetonitrile/propan-2-ol (1:1:1, by vol.). A linear gradient of 45 min was applied to the column at

Table 1. Amino acid compositions and details of automated Edman degradation of peptides used to complete the alignment of the CNBr-cleavage peptides of fragment Bb

Compositions are given as mol of residues/mol of peptide and were calculated from 24 h-hydrolysis values. Values less than 0.2 have been omitted. Values for any particular peptide are normalized with respect to the residue marked with an asterisk (*). Cysteine was determined as *S*-carboxymethylcysteine. For peptide CB-V,SP-14, methionine was determined as homoserine. Abbreviation: N.D., not determined.

Peptide ...	Amino acid compositions (mol of residue/mol)						
	Bb,T-1c	Bb,T-3-7b/8b	Bb,T-1e	Bb,T-1e,SP-5	Bb,T-1e,SP-14	Bb,Cl-3,Cl-1,SP-8/9	Bb,Cl-3,Cl-2
Asx	4.6	4.2	10.2	2.0	0.9	5.0	11.8
Thr	2.7	1.8	0.4	—	0.5	1.7	1.2
Ser	4.6	—	3.2	—	1.9	2.9	5.3
Glx	7.2	1.3	9.4	2.0*	2.0*	2.0*	7.8
Pro	1.4	1.1	1.0	—	—	2.3	3.1
Gly	5.1	3.2	3.3	—	1.2	2.6	4.8
Ala	4.3	—	2.5	—	—	2.0	3.5
Cys	2.1	—	1.0	—	0.5	—	1.2
Val	7.1	2.2	8.8	1.0	1.1	2.9	9.0
Met	1.2	1.7	2.4	0.8	0.5	0.9	2.1
Ile	3.1	4.4	2.0	0.8	—	0.9	3.3
Leu	2.0	2.2	6.1	—	1.7	2.4	6.8
Tyr	2.4	—	3.2	0.8	—	1.0	3.3
Phe	2.1	—	2.7	0.8	—	—	2.4
His	3.7	1.9	1.9	—	—	0.8	1.0*
Lys	7.1	—	4.3	—	0.5	3.5	7.1
Arg	2.0*	1.0*	1.0*	—	0.5	0.8	1.5
Total residues	63	25	64	9	12	32	75
Yield of peptide (%)	30	37	22	18	5	11	45
Amount used in automated sequence analysis (nmol)	110	120	118	60	15	15	90
Initial yield (%)	68	55	42	68	47	75	38
Repetitive yield (%)	92	90	N.D.	91	88	92	95

Table 2. Yields of tryptic peptides from reduced and carboxymethylated succinoylated fragment Bb, and their positions in the sequence

Peptide	Yield (%)	Position in sequence
T-1a	79	350–383
T-1b	50	288–346
T-1c	30	227–287
T-1d	12	50–120
T-1e	22	161–226
T-3-3a/4a	53	384–399
T-3-10a/11a	46	400–420
T-3-7b/8b	37	123–147
T-3-9b	54	481–505
T-4-3a/T-5-6a	32	438–449
T-4-4a/5a	34	421–437
T-4-11b/14b	49	452–471
T-5-2a/T-6-3a	53	157–160
T-5-3a/4a/5a	68	472–480
T-5-8a/3b	88	148–156
T-6-1a/2a	49	450–451, 121–122
T-6-4a	33	347–349

1 ml/min to give a final solvent composition of 0.1% trifluoroacetic acid/solvent B (45:55, v/v).

The acid-insoluble material present in the pellets obtained by centrifugation was recovered by freeze-

drying, and subsequently dissolved in 0.1% NH_4HCO_3 and injected on to a μ -Bondapak C_{18} column equilibrated with 0.1% NH_4HCO_3 /acetonitrile (95:5, v/v). A linear gradient of 60 min was applied to a column at 1 ml/min to give a final solvent composition of 0.1% NH_4HCO_3 /acetonitrile (45:55, v/v). One glycopeptide, apparently derived from a chymotryptic-like cleavage, was isolated from pool T-2. Its amino acid composition ($\text{Asp}_3, \text{Ser}_1, \text{Glu}_1, \text{Pro}_3, \text{Gly}_1, \text{Val}_1, \text{Arg}_1$) corresponded to positions 108–120 of the sequence.

Yields of the tryptic peptides isolated from pools T-3–T-6 of the tryptic digest of succinoylated fragment Bb (Fig. 1a) and their estimated position in the sequence are presented in Table 2. Peptides isolated by h.p.l.c. in trifluoroacetic acid- or NH_4HCO_3 -based solvent systems are denoted 'a' or 'b' respectively. Peptides isolated from the same h.p.l.c. run were numbered sequentially according to their order of elution. As indicated by Table 2, some peptides were present in more than one pool after gel filtration of the tryptic digest on Sephadex G-50 (Fig. 1a). Adjacent fractions from h.p.l.c. fractions frequently contained peptides of identical amino acid composition. This is attributed to the partial deamid-

ation or other uncharacterized differences resulting in differential mobility. In these cases, fractions containing peptides of identical amino acid composition were pooled as indicated in Table 2. Only peptide Bb,T-3-7b/8b from pools T-2–T-6 contained methionine, as indicated by its amino acid composition.

Alignment of the CNBr-cleavage peptides from the Bb fragment

We have previously aligned the CNBr-cleavage peptides CB-VII-3 and CB-IV at the *N*-terminus and peptide CB-II at the *C*-terminus of the Bb fragment (Christie *et al.*, 1980; Christie & Gagnon, 1982). The alignment of all the CNBr-cleavage peptides has now been established after isolation and sequencing of peptides derived from cleavage of the Bb fragment with trypsin and clostripain (Fig. 2).

Automated sequence analysis of peptides containing methionine from the tryptic digest of succinoylated fragment Bb

Four peptides Bb,T-1c, Bb,T-1d, Bb,T-1e and Bb,T-3-7b/8b from the tryptic digest of succinoylated fragment Bb were found to contain methionine (Tables 1 and 2).

Peptide Bb,T-1c. The *N*-terminal 41 residues of peptide Bb,T-1c were established by automated Edman degradation (Fig. 2 and Table 1). The sequence corresponding to residues 227–268 of the Bb fragment provides an overlap between the *C*-terminal sequence of peptide CB-VIa and the *N*-terminal sequence of peptide CB-II, aligning these two peptides in the order:

CB-VIa–CB-II

This confirms the results of previous work from the sequence of a clostripain-digest fragment (Christie *et al.*, 1980).

Peptide Bb,T-3-7b/8b. Automated sequence analysis enabled identification of 24 of the 25 residues of this peptide (Fig. 2 and Table 1). The sequence overlaps with the *C*-terminal sequence of the CNBr-cleavage peptide CB-VIb, which has previously been shown to contain 2 mol of homoserine/mol, indicating the presence of a methionyl bond resistant to CNBr (Christie & Gagnon, 1982). The sequence of peptide Bb,T-3-7b/8b revealed the presence of a Met–Thr bond and the sequence of a peptide Thr–Asp–Gly–Leu–His–Asn–Met that had not been isolated from CNBr digests of the Bb fragment. The sequence of peptide Bb,T-3-7b/8b overlapped with the *N*-terminal sequence of the CNBr-cleavage peptide CB-V. This aligns peptides CB-VIb and CB-V in the order:

CB-VIb–CB-V

Peptide Bb,T-1e. This peptide contained approx. 3 mol of methionine/mol (Table 1), and automated

sequence analysis showed it to result from cleavage of the Arg–Glu bond at position 160 of the Bb fragment (Fig. 2). Difficulty was encountered in sequencing this peptide, despite a good initial coupling yield and a single sequence Glu–Asp–being obtained from the first two cycles (Fig. 2 and Table 1). The tyrosine residue at position 3 of peptide Bb,T-1e, corresponding to position 163 of fragment Bb, reacted only partially with phenyl isothiocyanate (approx. 35%) under the conditions of the usual sequence programme. A further 28% reacted during cycle no. 4, with the result that both leucine and tyrosine were identified at this position. The poor reaction of the tyrosine residue may be a consequence of the succinoylation procedure, as no such difficulties were encountered during sequence analysis of the unmodified CNBr-cleavage peptide CB-V. Similar problems were not, however, encountered with other peptides after succinoylation. Despite the difficulties resulting from overlapping repetitive sequences, it was possible to assign tentatively residues to the sequence of peptide Bb,T-1e, to extend the sequence of peptide CB-V and align this peptide with the CNBr-cleavage peptide CB-VII-2 in the order:

CB-V–CB-VII-2

To overcome the difficulties encountered in sequencing peptide Bb,T-1e, the remaining amount of peptide (80 nmol) was digested with V8 proteinase, and the peptides were isolated by h.p.l.c. as described in the Materials and methods section. Two peptides were found to contain methionine, namely Bb,T-1e,SP-5 and Bb,T-1e,SP-14.

The entire sequence of nine residues of peptide Bb,T-1e,SP-5, corresponding to positions 204–212 of fragment Bb, was determined by automated sequence analysis (Fig. 2 and Table 1). The *C*-terminal sequence of this peptide provided an overlap of three residues with the *N*-terminal sequence of peptide CB-VII-1. This provided the alignment of peptides CB-VII-2 and CB-VII-1 in the order:

CB-VII-2–CB-VII-1

The entire sequence of 12 residues of peptide Bb,T-1e,SP-14, corresponding to positions 213–224 of fragment Bb, were determined by automated sequence analysis (Fig. 2 and Table 1). The amino acid composition of this peptide indicates that it is contaminated, although only a single sequence was obtained. The *C*-terminal sequence of this peptide overlapped by three residues with the *N*-terminal sequence of peptide CB-VIa, giving the alignment of CNBr-cleavage peptides CB-VII-1 and CB-VIa in the order:

CB-VII-1–CB-VIa

A third peptide containing methionine, which would

Fig. 2. Alignment of the CNBr-cleavage peptides from the Bb fragment

The alignment of peptides CB-VII-3 and CB-IV at the N-terminus and peptide CB-II at the C-terminus of the Bb fragment respectively and the N-terminal sequences of the CNBr-cleavage peptides have been presented previously (Christie *et al.*, 1980; Christie & Gagnon, 1982). Residues identified after automated Edman degradation are shown by $\rightarrow$, and the sequence is numbered from the N-terminus of the Bb molecule. The following peptide nomenclature is used: CB, CNBr-cleavage peptides; T, tryptic-digest peptide; Cl, clostripain-digest peptide; SP, V8-proteinase-digest peptide. Amino acids are in the single-letter notation: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (N) indicates sites that are likely to be occupied by asparagine residues with carbohydrate residues attached.

have provided an overlap between the C-terminal sequence of CB-V and CB-VII-2, was not recovered from the V8-proteinase digest of peptide Bb,T-1e.

The sequence of a peptide Bb,T-1e,SP-21 confirmed the sequence of residues 161–190 (Fig. 2) of the Bb fragment (results not shown).

Peptide Bb,T-1d. A major sequence Ala-Thr-Tyr-Pro-Lys- and a minor sequence corresponding to Tyr-Gly-Leu-Val-Thr-Tyr-Ala-Thr-Tyr- was obtained when peptide Bb,T-1d was subjected to automated sequence analysis. This heterogeneity indicates that most of the peptide was derived from a chymotryptic-like cleavage of a Tyr-Ala bond at position 55 of the Bb fragment. The minor sequence represents the expected peptide from cleavage of the Arg-Tyr bond at position 49 of the Bb fragment. Peptide Bb,T-1d was subjected to digestion with V8 proteinase, and the peptides were isolated by h.p.l.c. as described for peptide Bb,T-1e. A peptide containing methionine was isolated, although the sequence could not be extended beyond Tyr-104 from the previously established sequence of CB-IV (results not shown).

Isolation and sequence of peptides Bb,Cl-3-Cl-1 and Bb,Cl-3,Cl-2 generated from the Bb fragment by proteolysis with clostripain

Proteolysis of the Bb fragment by clostripain results in an N-terminal fragment Bb,Cl-3 with a molecular weight of 30000 (Christie & Gagnon, 1982). This peptide can be isolated by gel filtration on Sephadex G-75 (Fig. 3a), and from its amino acid composition it contains approx. 8 mol of methionine and 7 mol of arginine/mol. The peptide Bb,Cl-3 was thus a convenient starting material from which to isolate the remaining peptides required to complete the alignment of the CNBr-cleavage peptides of the Bb fragment. The strategy was still dependent on cleavage at arginine residues, although in this case clostripain was used to digest the Bb,Cl-3 fragment further with avoidance of difficulties encountered with trypsin owing to some chymotryptic-like activity (i.e. peptide Bb,T-1d). The Bb,Cl-3 fragment was fully reduced and alkylated and succinoylated to minimize the possibility of cleavage at lysine residues and render the fragment susceptible to

Fig. 3. Separation of the peptides from the clostripain digest of the Bb fragment

(a) The freeze-dried clostripain digest of the Bb fragment (10mg) was dissolved in 0.1M-NH₄HCO₃ (2.0ml) and applied to a column (2 cm × 90 cm) of Sephadex G-75 equilibrated with 0.1M-NH₄HCO₃. The flow rate was 10 ml/h, and 2.5 ml fractions were collected. The peptide Bb,Cl-3 was pooled as indicated and freeze-dried. —, A_{280} . (b) Reduced and S-carboxymethylated succinoylated peptide Bb,Cl-3 was further digested with clostripain and subjected to gel filtration as described for (a). Portions (15 μ l) of column fractions (2 ml) were taken for counting of radioactivity. Peptide Bb,Cl-3,Cl-1 and peptide Bb,Cl-3,Cl-2 were pooled as indicated. —, A_{280} ; - - -, radioactivity.

further cleavage by clostripain. Two main fragments were isolated by gel filtration of the clostripain digest of Bb,C1-3 fragment on Sephadex G-75 (Fig. 3b). The peptides Bb,C1-3,C1-1 and Bb,C1-3,C1-2 were pooled as indicated. Both peptides were found to contain methionine and each was about 70 residues long. The amino acid analysis of peptide Bb,C1-3,C1-1 showed it to contain carbohydrate, although the amount of glucosamine was not quantified. The presence of carbohydrate accounts for the separation of the two peptides on Sephadex G-75.

A small amount (approx. 5 nmol) of each of the peptides Bb,C1-3,C1-1 and Bb,C1-3,C1-2 were subjec-

camine, and automated Edman degradation was resumed. This enabled the identification of the *N*-terminal 47 residues of peptide Bb,C1-3,C1-2 (Fig. 2 and Table 3). This completes the sequence of peptide CB-V and provides an overlap with the *N*-terminal sequence of peptide CB-VII-2, corresponding to residues 161–207 of the Bb fragment. The sequence of residues 191–199 of the Bb fragment (Fig. 2) was confirmed by the sequence of a peptide CB-V,SP-14 isolated from a V8-proteinase digest of peptide CB-V (results not shown). The sequence of peptide Bb,C1-3,C1-2 completes the alignment of all of the CNBr-cleavage peptides of the Bb fragment in the order:

CB-VII-3–CB-IV–CB-VIb–CB-V–CB-VII-2–CB-VII-1–CB-VIa–CB-II

ted to *N*-terminal sequence analysis, which showed the peptides to be derived from cleavage of the arginine residues at positions 49 and 156 of the Bb fragment respectively.

Peptide Bb,C1-3,C1-1. This peptide was digested with V8 proteinase, and the peptides were isolated as described in the Materials and methods section. Fractions SP-8 and SP-9 contained peptides of identical amino acid composition, and these were pooled before sequence analysis. From the amino acid composition, peptide Bb,C1-3,C1-1,SP-8/9 contains 2 mol of methionine/mol (Table 1). The *N*-terminal 30 residues of this peptide showed it to result from cleavage of a Glu–Asp bond at position 86 of the Bb fragment (Fig. 2 and Table 1). This sequence extended the sequence of peptide CB-IV and provided an overlap with the sequence of peptide CB-VIb, aligning these two peptides in the order:

CB-IV–CB-VIb

The sequence of peptide Bb,C1-3,C1-1,SP-8/9 revealed a -Ser-Met-Met-Ser- sequence after position 104, and thus an additional methionine residue between the CNBr-cleavage peptide CB-IV and CB-VIb. The sequence of peptide Bb,C1-3,C1-1,SP-8/9 does not totally agree with the results of amino acid analysis, which are notably high in valine and low in methionine. Despite this, a single sequence was obtained during automated Edman degradation.

Peptide Bb,C1-3,C1-2. Similar difficulties to those described for peptide Bb,T-1e were encountered during sequence analysis of peptide Bb,C1-3,C1-2 owing to the inefficient coupling of the tyrosine at position 163 of the sequence of fragment Bb. In this case, however, the sequencer was stopped after 16 cycles of Edman degradation, when proline was the *N*-terminal residue of the main sequence. The other sequences were blocked by the addition of fluores-

This work also provides the complete sequence of all but the large *C*-terminal CNBr-cleavage peptide CB-II, which comprises residues 1–253 of the Bb fragment (Fig. 2).

Discussion

In this paper we have presented details of the isolation and amino acid sequences of peptides derived from cleavage of the Bb fragment at arginine residues. This has enabled the alignment of all the CNBr-cleavage peptides from the Bb fragment, and completes the sequence of all but one of these peptides, accounting for the *N*-terminal 253 residues of the Bb fragment. The sequence of the *C*-terminal CNBr-cleavage peptide CB-II and the complete sequence of the Bb fragment is presented in the following paper (Christie & Gagnon, 1983).

A total of 18 peptides were isolated from a tryptic digest of succinoylated fragment Bb, by using a combination of gel filtration, ion-exchange and h.p.l.c., in yields ranging from 12 to 88% (Tables 1 and 2). It should be noted that fraction T-6–1a/2a contains two peptides of sequence Ser-Arg and Thr-Arg. The peptide corresponding to residues 1–49 of the Bb fragment was not isolated from the digest, although it is probable that that peptide was eluted from the Sephadex G-50 column immediately before the T-1 pool (Fig. 1a). No attempt was made to recover this peptide, which would have a blocked *N*-terminal residue. From the complete sequence of the Bb fragment in the following paper (Christie & Gagnon, 1983) it can be seen that fragment Bb contains 20 arginine residues/molecule. Allowing for the presence of two resistant Arg–Pro bonds at positions 244 and 334 of the sequence, a total of 19 tryptic peptides would have been expected, which is in complete agreement with our results.

Three of the four tryptic peptides containing methionine are present in the T-1 pool (mol.wt.

approx 6000). The similar size and charge of these peptides required a more complicated purification procedure than would normally be expected. Ion-exchange chromatography (Fig. 1b) provided a partial separation, although h.p.l.c. was required as a final step. H.p.l.c. alone was not sufficient to purify these peptides.

In addition to difficulties in isolating the methionine-containing peptides from the T-1 pool, problems were also encountered during *N*-terminal sequence analysis. Extended sequence analysis of peptide Bb,T-1e was not possible owing to the inefficient coupling of the tyrosine residue corresponding to position 163 of the sequence of fragment Bb. We have not experienced such difficulties with other succinoylated tryptic peptides, nor have we seen such troubles reported in the literature. The poor reactivity of the tyrosine residue may well result from the conformation of this peptide, particularly after the introduction of negatively charged groups on the lysine residues.

The other difficulty resulted from the unexpected chymotryptic-like cleavage of some bonds by trypsin. This prevented useful information being obtained for peptide Bb,T-1d, which would have provided an overlap between peptide CB-IV and peptide CB-VIb. *N*-Terminal sequence analysis revealed a major sequence consistent with cleavage of the Tyr-Ala bond at position 55 and a minor sequence from cleavage of an Arg-Tyr bond at position 49 of the Bb fragment. It also appears that peptide Bb,T-1d was shortened at the C-terminus as a result of chymotryptic-like activity; the methionine-containing peptide isolated from a V-8 proteinase digest of peptide Bb,T-1d was shorter than would have been predicted from the amino acid sequence; also, an additional peptide (T-2) was isolated from the tryptic digest of fragment Bb with an amino acid composition corresponding to residues 108–120 of the Bb fragment, suggesting cleavage at the methionine residue at position 107.

Despite these problems the tryptic digest at the Bb fragment enabled the alignment of most of the CNBr-cleavage peptides (Fig. 2).

We adopted a slightly different approach to isolate the peptides required to align the CNBr-cleavage peptides CB-IV and CB-VIb and to confirm the alignment of peptides CB-V and CB-VII-2. Clostripain cleaves the Bb fragment to generate an *N*-terminal fragment Bb,Cl-3 (mol.wt. approx. 30000) that is resistant to further proteolysis (Christie *et al.*, 1980). This fragment contains 8 methionine and 7 arginine residues, and this is good starting material from which to isolate the remaining peptides. We once again chose to cleave at arginine residues, although in this instance we used clostripain to cleave peptide Bb,Cl-3 after rendering it susceptible to further proteolysis by complete reduc-

tion, alkylation and succinoylation. Succinoylation, as well as disrupting the structure of the Bb,Cl-3 fragment, was a precaution against any 'non-specific' cleavage of lysine residues. From results obtained with clostripain it is clear that this enzyme provides a useful alternative to trypsin for arginine-specific cleavage of proteins and peptides. It is noteworthy, however, that not all arginine bonds were cleaved: the arginine residue at position 156, which is adjacent to a succinoylated lysine residue, was cleaved in preference to the Arg-Glu bond at position 160 of the Bb sequence (Fig. 2). The sequence of two clostripain peptides Bb,Cl-3,Cl-1 and Bb,Cl-3,Cl-2 completed the alignment of the CNBr-cleavage peptides of the Bb fragment.

As was the case for peptide Bb,T-1e, it was necessary to sub-digest peptide Bb,Cl-3,Cl-1 with V8 proteinase to isolate methionine-containing peptides. We have relied heavily on the use of h.p.l.c. for peptide separation, and the solvent systems employed in the present work are suitable for the vast majority of peptides containing 3–50 residues.

The sequence of peptide Bb,Cl-3,Cl-1,SP-8/9 revealed the presence of an additional methionine residue and a sequence -Ser-Met-Met-Ser- corresponding to positions 105–108 of the sequence of fragment Bb. The presence of free homoserine as a product of CNBr cleavage would have been missed during separation of the small peptides by h.p.l.c. (Christie & Gagnon, 1982). Thus the Bb fragment contains a total of 9 methionine residues. This value is one residue more than that deduced from the amino acid composition of the Bb fragment, although the result is in agreement with the amino acid composition of Factor B, assuming that the Ba fragment contains only one methionine residue (Christie & Gagnon, 1982). The *N*-terminal sequences of the Ba fragment also shows the presence of a Met-Thr bond at position 128 and the sequence of a peptide not isolated from CNBr digests of fragment Bb or Factor B.

Met-Thr bonds and to a smaller extent Met-Ser bonds are resistant to CNBr cleavage, and in some cases can result in the formation of an internal homoserine residue without chain cleavage (Schroeder *et al.*, 1969). The presence of two such bonds in close proximity probably results in the presence of the partial CNBr-cleavage product CB-III (mol.wt. approx. 20000) from CNBr digests of fragment Bb and Factor B, and may account for peptide CB-IV containing 1.3 mol of homoserine/mol (Christie & Gagnon, 1982). However no peptide with the *N*-terminal sequence Hse-Ser-Trp was detected.

The likely sites of carbohydrate attachment through asparagine residues in the *N*-terminal sequence of fragment Bb are at positions 26 and 119. In both cases the sequence is consistent with the

Asn-Xaa-Ser or Asn-Xaa-Thr sequence requirement for the *N*-linkage of carbohydrate (Neuberger *et al.*, 1972). Moreover, the glucosamine contents of peptides CB-IV and CB-VIb account for all the glucosamine in the Bb fragment (Christie & Gagnon, 1982). With peptide CB-VIb additional evidence is provided from amino acid analysis (Christie & Gagnon, 1982), which shows the presence of one aspartic acid/asparagine residue additional to those accounted for by sequence analysis. Peptide CB-IV is too large for meaningful conclusions to be drawn from the amino acid composition. It is probable that positions 26 and 119 contain the only carbohydrate present in the Bb fragment, as the Bb fragment does not contain *O*-linked carbohydrate, as indicated by absence of galactosamine from the Bb fragment (Christie & Gagnon, 1982). The presence of carbohydrate at positions 26 and 119 probably results in the resistance of peptide Bb, Cl-3 to further degradation by clostripain. It may be noted that one potential site for *N*-linked carbohydrate, the asparagine residue at position 36 of fragment Bb, is unglycosylated.

A free thiol group at position 33 has previously been localized in the Bb fragment (Christie & Gagnon, 1982). This residue is located in a portion of the molecule containing carbohydrate-attachment sites and lacking disulphide bonds. It was shown that the cysteine residues at positions 219 and 252 were disulphide-linked to cysteine residues within the C-terminal CB-II portion of the Bb fragment.

The present work, along with the sequence of the major CNBr-cleavage fragment of Bb in the following paper (Christie & Gagnon, 1983), provides the complete sequence of the catalytically active Bb fragment from human complement Factor B.

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