Structural Characterization of Factor I Mediated Cleavage of the Third Component of Complement†

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ABSTRACT: Inactivation of C3b by factor I involves proteolysis of the C3b α'-polypeptide chain and results in two large fragments of M_r 68 000 and 43 000. Both of these peptides remain covalently bonded to the β chain in iC3b. Analysis of these cleavage products on sodium dodecyl sulfate–polyacrylamide gels led Harrison and Lachmann [Harrison, R. A., & Lachmann, P. J. (1980) *Mol. Immunol. 17*, 9] to suggest that an M_r 46 000 intermediate was first formed and that it was further cleaved to yield the M_r 43 000 peptide. The work presented here confirms, by amino-terminal sequence analysis, that there are two factor I mediated cleavage sites. In addition, these sites have been positioned within the α chain of C3. C3b was cleaved to iC3b by incubation with factors I and H. Following reduction and alkylation, the M_r 43 000 peptide was resolved from the M_r 68 000/β-chain mixture by gel filtration on Sepharose CL6B. The β chain was then isolated from the mixture by chromatography on DEAE-Sephacel in 8 M urea. The β chain, the M_r 43 000 peptide, and the M_r 68 000/β-chain mixture were each subjected to automated Edman degradation. Comparison of these amino-terminal sequences with the amino-terminal sequences of the α' and β chains [Tack, B. F., Morris, S. C., & Prahl, J. W. (1979) *Biochemistry 18*, 1497] showed the M_r 68 000 peptide to be amino terminal in the C3b α' polypeptide. When methylamine-inactivated C3 was digested with factors I and H, equimolar amounts of the M_r 46 000 and 43 000 peptides were generated. These peptides were resolved from the β-chain/M_r 78 000 peptide by gel filtration, and the mixture was subjected to automated Edman degradation. After subtraction of the known M_r 43 000 peptide amino-terminal sequence, a second distinct sequence was observed. This provides direct evidence for two factor I mediated cleavage sites in the α' chain of C3b. The first results in peptides of M_r 68 000 (amino terminal) and 46 000. A further cleavage releases a small amino-terminal fragment from the M_r 46 000 peptide to produce the M_r 43 000 peptide seen in the final product of factor I action on C3b, iC3b.

The third component of complement (C3) consists of two disulfide-linked polypeptide chains (Nilsson & Mapes, 1973), α (M_r 125 000) and β (M_r 75 000). Activation of C3, mediated by either the classical pathway C3 convertase, C4b2b (Müller-Eberhard et al., 1966), or the alternative pathway C3 convertase, C3bBb (Müller-Eberhard & Götze, 1972; Vogt et al., 1974), involves proteolytic cleavage and produces the activation peptide, C3a (M_r 9000), and the major fragment, C3b (M_r 190 000) (Dias da Silva et al., 1967; Bokisch et al., 1969; Budzko et al., 1971; Tack et al., 1979). On activation, C3b acquires the transient ability to form a covalent bond with a variety of acceptor molecules and surfaces (Law & Levine, 1977).

C3b generated by either of these enzymes has several important biological activities. It is an essential subunit of both the classical and alternative pathway C5 cleaving enzymes (C4b2b3b and $C3b_2Bb$) (Müller-Eberhard et al., 1969; Medicus et al., 1976; Daha et al., 1976; Vogt et al., 1978) and is thus required for complement-mediated lysis via either pathway. In addition, surface-bound C3b has opsonic and immune adherence activity, mediated via binding to specific cell membrane receptors (Nelson, 1953; Gigli & Nelson, 1968; Johnston et al., 1969; Lay & Nussenzweig, 1968). However, the ability of C3b to amplify its own production, by participation in the alternative pathway C3 convertase (C3bBb), has the most immediate biological consequences. In the absence of any regulatory mechanism, activation of C3 would rapidly lead to exhaustion of the alternative pathway component, factor B, and of C3 (Abramson et al., 1971; Nicol & Lachmann, 1973). Regulation of such activation is provided by factors I (C3b inactivator) and H (β1H globulin). In the presence of factor H, factor I cleaves the α' chain of both surface-bound and fluid-phase C3b. This proteolytic action produces two large peptides of M_r 68 000 and 43 000, each of which remains covalently bonded to the β chain (Nagasawa & Stroud, 1977; Pangburn et al., 1977; Whaley & Ruddy, 1976). Such cleaved C3b (iC3b) is unable to participate in C3 or C5 convertase formation and has altered opsonic and immune adherence activities (Daha et al., 1976; Tamura & Nelson, 1967; Ruddy & Austen, 1969; Alper et al., 1972). Initial reports described a single factor I mediated cleavage of the α' chain of C3b (Nagasawa & Stroud, 1977; Pangburn et al., 1977). However, Harrison and Lachmann, by analysis of C3 cleavage products using sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis, found that the α' chain was first split into fragments with molecular weights of 68 000 and 46 000. The M_r 46 000 fragment was then further cleaved to yield the M_r 43 000 peptide (Harrison & Lachmann, 1980a). These studies, which recently have been confirmed (Sim et al., 1981), suggested that there were at least two factor I mediated cleavage sites in C3b.

The studies reported here were undertaken to clarify, by sequence analysis, the number of factor I mediated cleavage sites, to position these sites definitively within the α chain, and to investigate the substrate specificity of factor I. A preliminary report of this work has been made (Davis & Harrison, 1982).

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

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Materials and Methods

TPCK-trypsin was purchased from Worthington. Sepharose CL6B, DEAE-Sephacel, and Sephadex G50 were obtained from Pharmacia. Dithiothreitol was from Calbiochem, and iodoacetamide was from Kodak. All sequencing reagents were obtained from Pierce, as was methanol. Acetyl chloride was from Sequemat. Sodium dodecyl sulfate ($NaDodSO_4$), acrylamide, *N*,*N*′-methylenebis(acrylamide), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from Bio-Rad. Acetonitrile and dichloroethane were purchased from Burdick and Jackson. Methylamine and diisopropyl phosphorofluoridate were from Sigma.

C3, factor H, and factor I were isolated by previously described methods (Harrison & Lachmann, 1979; Davis, 1981). C3b was prepared from C3 by limited digestion with trypsin [0.2% trypsin/C3 (w/w) for 5 min at 37 °C]. The trypsin was then inactivated with diisopropyl phosphorofluoridate (1 mM, 60 min, 37 °C). iC3b was produced by digestion of C3b with 1% (w/w) factor I and 2% (w/w) factor H for 1 h at 37 °C. For some experiments, C3 was inactivated by incubation with 100 mM methylamine in 0.02 M sodium/potassium phosphate containing 0.15 M NaCl, pH 7.2, for 2 h at 37 °C. All digestions of C3 (both with trypsin and with factors I and H) were done in the above buffer, except where otherwise noted.

For reduction and alkylation, lyophilized protein was dissolved in 0.05 M Tris-HCl containing 6.0 M guanidine, pH 8.0. Dithiothreitol was added to 20 mM, and the tube was flushed with nitrogen, sealed, and incubated at 37 °C for 1 h. Iodoacetamide (50 mM) was then added, and the sample was incubated for 15 min in the dark at room temperature. Alkylation was terminated by the addition of excess mercaptoethanol, and the reduced and alkylated proteins were dialyzed against water and lyophilized. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Samples were incubated in sample buffer containing 0.1 M 2-mercaptoethanol at 100 °C for 2 min. Following electrophoresis, gels were stained with Coomassie brilliant blue R250.

Automated Edman degradations were performed with a Beckman 890C sequencer modified with a cold trap and fitted with a P-6 autoconverter (Sequemat, Inc.). A 0.1 M Quadrol program was used (Brauer et al., 1975), and, in most instances, two coupling cycles were performed before the initial cleavage reaction. Conversion was performed with methanolic HCl (1 part acetyl chloride and 7 parts methanol, 65 °C, 10 min). Phenylthiohydantoin derivatives were identified by high-performance liquid chromatography by using a Zorbax ODS column (Du Pont Instruments) equilibrated in 0.01 M sodium acetate, pH 5.5, containing 20% acetonitrile and developed with an acetonitrile gradient (Zalut et al., 1980).

Results

Amino-Terminal Sequence Analysis of the M_r 68 000 and 43 000 α-Chain Fragments Generated by Factor I and Factor H Action on C3b. C3b (35 mg) was incubated at 37 °C for 1 h in 0.02 M sodium/potassium phosphate containing 0.15 M sodium chloride, pH 7.2, with factors I (0.35 mg) and H (0.71 mg) (Figure 1). iC3b thus generated was reduced and alkylated and applied to Sepharose CL6B (Figure 2). Peak A contained both the β-chain and the M_r 68 000 peptide, while peak B consisted of the M_r 43 000 peptide. Fractions from the chromatogram were pooled as indicated, dialyzed exhaustively into water, and lyophilized. $NaDodSO_4$–polyacrylamide gel electrophoresis of each pool is shown in Figure 3.

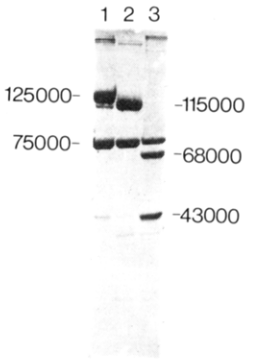

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of C3, C3b, and iC3b. Proteins were prepared as described under Materials and Methods and under Results. A 10% polyacrylamide gel was used. Track 1, C3; track 2, C3b; track 3, iC3b.

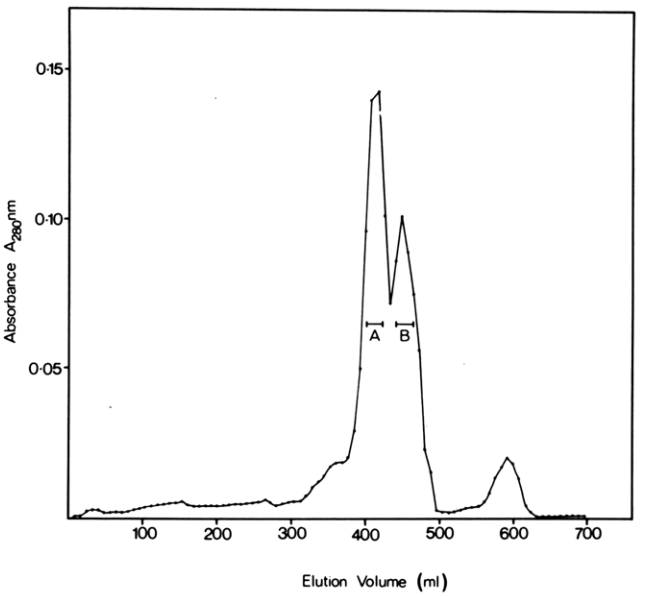

FIGURE 2: Elution profile of reduced and alkylated iC3b chromatographed on Sepharose CL6B. The column (3 × 170 cm) was equilibrated in 0.05 M Tris-HCl containing 0.2% $NaDodSO_4$, pH 8.0, at a flow rate of 30 mL/h. The β-chain/M_r 68 000 peptide peak (A) and the M_r 43 000 peptide peak (B) were pooled as indicated.

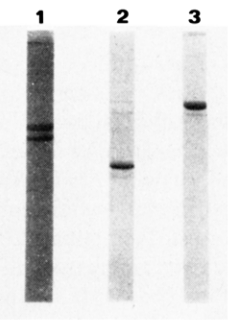

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of the isolated iC3b-derived peptides. The peptides were prepared as described under Results. Track 1, β-chain/M_r 68 000 peptide mixture (7.5% polyacrylamide gel); track 2, M_r 43 000 peptide (10% polyacrylamide gel); track 3, β chain (10% polyacrylamide gel).

The β-chain/M_r 68 000 peptide mixture was applied to a column of DEAE-Sephacel (3 × 19 cm) equilibrated in 0.05 M Tris-HCl containing 8.0 M urea, pH 8.0. The column was washed with 300 mL of starting buffer and developed with a NaCl concentration gradient consisting of 250 mL of starting buffer and 250 mL of starting buffer containing 2.0 M NaCl. While the bulk of the β chain eluted prior to the M_r 68 000

Table I: Amino-Terminal Sequences of the Isolated β Chain and of the β/M_r 68 000 Peptide Mixture[a]

cycle	$\beta + M_r$ 68 000	yield (nmol) β chain[b]	yield (nmol) M_r 68 000[c]	β	yield (nmol)[d]	deduced M_r 68 000
1	Ser	6.0		Ser	0.8	Ser
2	Pro, Asn	10.9	5.0	Pro	1.0	Asn
3	Met, Leu	12.1	12.2	Met	3.8	Leu
4	Tyr, Asp	30.4		Tyr	6.2	Asp
5	Ser, Glu	3.8	5.7	Ser	0.9	Glu
6	Ile, Asp	12.8	6.0	Ile	2.4	Asp
7	Ile	19.5		Ile	2.5	Ile
8	Thr, Ile	5.5	8.6	Thr	1.5	Ile
9	Pro, Ala	8.1	6.3	Pro	1.9	Ala
10	Asn, Glu	9.1	3.6	Asn	1.1	Glu
11	Ile, Glu	10.6	5.0	Ile	1.8	Glu
12	Leu, Asn	12.1	2.6	Leu	2.1	Asn
13	Arg, Ile		3.3	Arg		Ile
14	Leu, Val	10.6	5.5	Leu	1.5	Val

[a] The repetitive yield for the β/M_r 68 000 peptide mixture was 96% (based on the prolines at positions 2 and 9 and the isoleucines at positions 6 and 11 for the β chain and on the glutamic acid residues at positions 5, 10, and 11 for the M_r 68 000 peptide). The initial yield (calculated by extrapolation of the isoleucine recoveries for each chain) was 42%. These values were 96% and 31% (calculated from isoleucine and leucine recoveries), respectively, for the isolated β chain. No residues other than those indicated were detected at levels above background. PTH-aspartic acid methyl ester and PTH-tyrosine were poorly resolved under the HPLC conditions used, and quantitation of these residues was therefore unreliable, as was quantitation of PTH-arginine. [b] Yield of β-chain residues from sequence analysis of the β/M_r 68 000 peptide mixture. [c] Yield of M_r 68 000 peptide residues from sequence analysis of the β/M_r 68 000 peptide mixture. [d] Yield from sequence analysis of the isolated β chain.

peptide, the latter could not be resolved from the residual β chain.

The β-chain/M_r 68 000 peptide mixture (65 nmol), the isolated β chain (10 nmol), and the isolated M_r 43 000 peptide (35 nmol) were then each subjected to automated amino-terminal sequence analysis. Samples were dissolved in anhydrous trifluoroacetic acid or in water before being placed in the cup. The amino-terminal sequences of the β-chain/M_r 68 000 peptide mixture and of the isolated β chain and the deduced sequence of the M_r 68 000 peptide are given in Table I. As shown, the mixture sequence revealed the release of two amino acids at each cycle except at cycle 1, where only serine was detected, and at cycle 7, where only isoleucine was detected.

The β-chain amino-terminal sequence determined here differs from that reported by Tack et al. (1979) at residue 7, where they found a glycine rather than an isoleucine residue. The amino-terminal sequence of the M_r 68 000 peptide was deduced from the amino-terminal sequences determined for the β chain and for the β-chain/M_r 68 000 peptide mixture. This deduced amino-terminal sequence (Table I) is identical with the reported amino-terminal sequence of the α chain of C3b, except at residue 12, where Tack et al. (1979) identified aspartic acid, rather than asparagine. The amino-terminal 27 residues of the M_r 43 000 peptide are shown in Chart I. This, like the α′ and β chains, also has an amino-terminal serine.

Amino-Terminal Sequence Analysis of the M_r 46 000 α-Chain Fragment Seen Transiently during the Generation of iC3b. Preliminary experiments were performed in attempts to find conditions under which the quantity of the M_r 46 000 peptide would be maximized. Under varying conditions of pH, time, and temperature, varying concentrations of C3b (or methylamine-inactivated C3), and varying concentrations of factors I and H, no more than approximately equal quantities

Chart I: Amino-Terminal Sequence of the M_r 43 000 Peptide

Ser[1]-Glu-Glu-Thr-Lys-Glu-Asn-Glu-Gly-Phe-Thr-Val-Thr-Ala-Glu-
Gly[16]-Lys-Gly-Gln-Gly-Thr-Leu-Ser-Val-Val-Thr-Met-

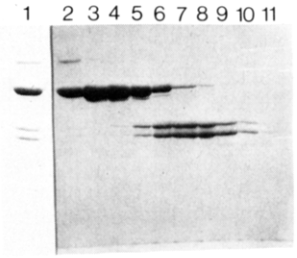

FIGURE 4: Isolation of the M_r 43 000/46 000 peptide mixture. Methylamine-inactivated C3 was digested with factor I in the presence of factor H, reduced and alkylated, and chromatographed on a 3 × 170 cm column of Sepharose CL6B in 0.05 M Tris-HCl containing 0.2% $NaDodSO_4$, pH 8.0. The flow rate was 30 mL/h. The starting material and the fractions through the chromatogram were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Track 1, methylamine-inactivated C3 after digestion with factors I and H; tracks 2–11, chromatographic fractions from the Sepharose CL6B column. Fractions containing the M_r 43 000/46 000 peptides (tracks 6–11) were pooled and concentrated, and chromatography on Sepharose CL6B was repeated. The fractions shown in tracks 2–5 contained both the β-chain and the M_r 78 000 peptide. These are not resolved on the 10% polyacrylamide gel used.

of the M_r 46 000 and 43 000 peptides could be generated (data not shown). Therefore, the two peptides were isolated together and sequenced as a mixture, rather than attempting to generate the M_r 46 000 peptide alone. Approximately equal quantities of the two peptides (as assessed by visual inspection of $NaDodSO_4$–polyacrylamide gels) were produced by incubation of methylamine-inactivated C3 (31.5 mg) at 37 °C for 19 h with factors I (0.32 mg) and H (0.32 mg) in 0.5% NH_4HCO_3 (Figure 4, lane 1). This digest was lyophilized and then reduced and alkylated. The reduced and alkylated material was dialyzed into water, lyophilized, and dissolved in 10 mL of 0.05 M Tris-HCl containing 0.2% $NaDodSO_4$, pH 8.0. It was then applied to Sepharose CL6B equilibrated in the above buffer. A $NaDodSO_4$–polyacrylamide gel of the fractions through the chromatogram is shown in Figure 4, lanes 2–11. The fractions from lanes 6–11 were pooled and chromatographed again on the same column. Thirty-five nanomoles of this M_r 43 000/46 000 peptide mixture, following dialysis and lyophilization, was dissolved in water and subjected to automated Edman degradation.

Table II shows sequence analysis of the mixture. Two amino acids were unequivocally detected at each position except at cycle 1, where only serine was found, and at cycle 6, where only glutamic acid was detected. As indicated, the previously determined sequence of the M_r 43 000 peptide is contained within this mixture sequence. The deduced sequence for the M_r 46 000 peptide is also shown. The quantity of glutamic acid recovered at cycle 6 was that expected for release at a single site only. Since this position is occupied by a glutamic acid residue in the M_r 43 000 peptide, and since no other amino acid was detected, no assignment has been made at this position for the M_r 46 000 peptide.

Discussion

Initial efforts in this study were directed toward determination of the amino-terminal amino acid sequences of the M_r 68 000 and 43 000 peptides derived by factor I from the α′ chain of C3b. This provided the amino acid sequence on the carboxy-terminal side of the cleavage and, by comparison with

Table II: Amino-Terminal Sequences of the M_r 43 000/46 000 Peptide Mixture[a]

cycle	M_r 43 000 + 46 000	yield (nmol) M_r 43 000[b]	yield (nmol) M_r 46 000[c]	M_r 43 000	yield (nmol)[d]	deduced M_r 46 000
1	Ser	1.2		Ser	0.5	Ser
2	Glu, Val	13.7	0.9	Glu	5.9	Val
3	Glu, Lys	11.7	3.2	Glu	5.4	Lys
4	Thr, Ile	2.3	9.5	Thr	1.0	Ile
5	Lys, Thr	9.0	2.6	Lys	4.2	Thr
6	Glu	11.5		Glu	4.7	X
7	Asn, Arg	9.6		Asn	3.6	Arg
8	Glu, Ile	10.5	7.6	Glu	3.5	Ile
9	Gly, Thr	13.4	1.5	Gly	5.7	Thr
10	Phe, Trp	14.9	4.4	Phe	6.0	Trp
11	Thr, Glu	1.7	3.3	Thr	0.6	Glu

[a] The repetitive yield for the mixture, based on the recovery of glutamic acid at cycles 2, 3, and 6 (M_r 43 000 peptide) and on the recovery of isoleucine at cycles 4 and 8 (M_r 46 000 peptide), was 95–96%. Calculation of the initial yield, as described in Table I, using the recovery of glutamic acid in each peptide gave a value of 55%. The repetitive yield for the isolated M_r 43 000 peptide, determined from the recoveries of glutamic acid at cycles 2, 3, 6, 8, and 15 and from the recoveries of glycine at cycles 9, 16, 18, and 20, was 96%. The initial yield was only 17%. [b] Yield of M_r 43 000 peptide residues from sequence analysis of the M_r 46 000/43 000 peptide mixture. [c] Yield of M_r 46 000 peptide residues from sequence analysis of the M_r 46 000/43 000 peptide mixture. [d] Yield from sequence analysis of the isolated M_r 43 000 peptide.

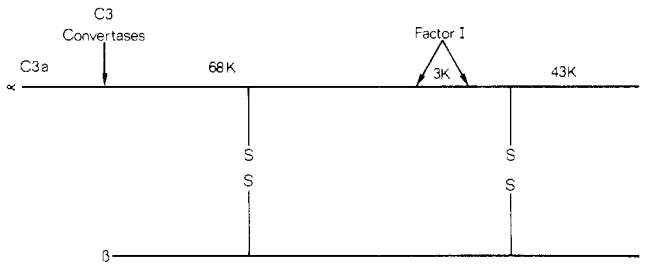

FIGURE 5: Schematic representation of the polypeptide chains of C3. The sites of C3 convertase and factor I action are indicated.

the known amino-terminal sequence of the α' chain, definitively orders the two peptides within it (Figure 5). Harrison & Lachmann (1980b) earlier reported that under certain conditions C3 was susceptible to cleavage by factor I at a position 78 000 daltons from the amino terminus of the intact α chain. It is now known that the C3 used to derive this information was C3 in which the internal thio ester had been hydrolyzed (Pangburn & Müller-Eberhard, 1980). A similar deduction as to the position of the factor I cleavage site was made by Crossley & Porter (1980). The sequence data reported here thus confirm earlier deductions regarding the position of this site.

In earlier studies, Harrison & Lachmann (1980a) deduced, by analysis of C3 cleavage products on $NaDodSO_4$–polyacrylamide gels, that fluid-phase C3b was cleaved at two points by factor I. Similar data have recently been reported by Sim et al. (1981). In order to confirm that there is a second peptide bond cleaved by factor I, and to position it within the α chain of C3, it was necessary either to obtain the amino-terminal sequence of the M_r 46 000 fragment or to isolate and sequence the M_r 3000 fragment presumed to be generated during factor I action. Attempts to limit factor I action to the generation of the M_r 46 000 fragment only were unsuccessful. However, conditions were found in which scission of the M_r 46 000 fragment to the M_r 43 000 fragment was slow, and, using these, it was possible to generate and isolate an approximately equimolar mixture of the two polypeptides.

Table III: Comparison of the Amino-Terminal Sequences Resulting from Factor I Cleavages of C3b and C4b

	M_r	sequence
C3	46 000	Ser-Val-Lys-Ile- Thr- X -Arg-Ile- Thr-Trp-Glu
C3	43 000	Ser-Glu-Glu-Thr-Lys-Glu-Asn-Glu-Gly-Phe-Thr
C4d[a]		Thr-Leu-Glu-Ile- Pro-Gly-Asn-Ser- Asp-Pro-Asn
C4α4[a]		Gly-Gly-Phe-Lys- X -Leu-Ala-Leu- X -Leu-Gly

[a] Press & Gagnon (1981).

If a small peptide is released by a second factor I mediated cleavage, it could lie at either the amino-terminal or the carboxy-terminal end of the M_r 46 000 peptide. If the second cleavage site is near the carboxy-terminal end of the α chain, the new amino-terminal sequence revealed by this cleavage would be within the small released peptide and would be separated from the M_r 43 000/46 000 peptide mixture during gel filtration. Thus, sequence analysis of the mixture would reveal only one amino-terminal sequence, and this sequence would be identical with that of the M_r 43 000 peptide. If, however, the second cleavage site is near the amino terminus of the M_r 46 000 peptide, two sequences would be detected, one of which would be a new sequence and one of which would be the same as that of the M_r 43 000 peptide (Table II).

Sequence analysis of the M_r 46 000/43 000 peptide mixture showed two residues at most positions, and, after subtraction of the known amino-terminal sequence of the M_r 43 000 fragment, a unique sequence for the M_r 46 000 fragment could be deduced. This confirms that there are in fact two sites of factor I action. In addition, the second site can now be positioned, close to that producing the M_r 43 000 peptide, 46 000 daltons from the carboxy terminus of the α chain. This is shown schematically in Figure 5.

Factor I is a protease with a limited substrate range, its only known substrates being C3b (Nagasawa & Stroud, 1977; Pangburn et al., 1977), C4b (Cooper, 1975; Shiraishi & Stroud, 1975; Fujita et al., 1978; Nagasawa et al., 1980), and the "C3b or C4b-like" thio ester cleaved intact molecules (Pangburn & Müller-Eberhard, 1980; von Zabern & Gigli, 1982). In Table III, the amino-terminal sequences of the M_r 46 000 and 43 000 peptides are compared with those recently reported for the products of factor I action on C4b (Press & Gagnon, 1981), indicating that if the proteolytic specificity of factor I is determined by primary sequence, this must lie amino terminal to the bond split. While we have not determined the carboxy-terminal sequences of either the M_r 68 000 or the M_r 3000 polypeptides, arginine is known to lie at this point in both factor I derived fragments of C4 (Press & Gagnon, 1981). This suggests that factor I has a trypsin-like specificity and is consistent with the high degree of sequence homology between it and serine proteases (Davis, 1981; Hsiung et al., 1982). Specificity of cleavage could also be imposed on factor I by its cofactor requirement (factor H or C4 binding protein) for expression of proteolytic activity.

Thus, we have confirmed that factor I cleaves C3b at two sites, approximately 3000 daltons apart, and 46 000 and 43 000 daltons from the carboxy-terminal end of the α chain. Recently, we have isolated the M_r 3000 peptide, showing that it is not covalently bound to the larger iC3b molecule and that its amino-terminal sequence is that of the M_r 46 000 peptide (data not given). We are now performing total structural determination of this fragment and investigating its possible physiological functions.

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