Identification of the peptide bond cleaved during activation of human C1r

Gerard J. Arlaud and Jean Gagnon+

Departement de Recherches Fondamentales (UA CNRS no. 556, Unite INSERM no. 238), Laboratoire de Biologie Moleculaire et Cellulaire, CEN-Grenoble 85X, 38041 Grenoble Cedex, France and *+MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, England*

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CNBr cleavage of unreduced proenzyme C1r yielded fragment CP2b, isolated by gel filtration and high-pressure gel permeation chromatography. This fragment (~ M_r 55000) comprised at least 4 disulphide-linked peptides, which were separated by gel filtration after reduction and alkylation. Peptide CP2bRA4, overlapping the A- and B-chain regions in proenzyme C1r was digested by V8 staphylococcal protease, and the digest separated by reversed-phase HPLC. N-terminal sequence analysis of peptide CP2bRA4SP9 established that C1r activation involves the cleavage of a single Arg-Ile bond, located in the sequence: ... Gln-Arg-Gln-Arg-Ile-Ile-Gly-Gly ... .

Human C1r *Sequence* *Serine protease activation* *Limited proteolysis*

1. INTRODUCTION

C1, the first component of the classical pathway of complement, comprises a recognition unit, C1q, and two zymogens of serine proteases, C1r and C1s, which are sequentially activated through limited proteolysis during C1 activation [1,2]. In human, proenzyme C1r is a dimer consisting of two apparently identical single-chain glycoproteins (~M_r 90000), and during activation each monomer undergoes an autocatalytic cleavage generating two disulphide-linked polypeptide chains A (M_r 56000–57000) and B (M_r 30000–35000) [3–5]. The B chain, which contains the active site serine residue, is derived from the C-terminal part of proenzyme C1r, and its complete sequence has been determined [5]. Partial sequence of the N-terminal A chain has also recently been obtained, including the complete sequence of the C-terminal 95 residues [6]. Thus, the primary structure of regions on both sides of the cleavage site is known, although it is not established whether C1r activation results from cleavage of a one or more peptide bond(s). The isolation from proenzyme C1r of a peptide covering the cleavage site, and the sequence of this peptide, allow a definitive answer to this question.

2. MATERIALS AND METHODS

2.1. *Materials*

Iodo[2-^{3}H]acetic acid (106 mCi/mmol) was from the Radiochemical Centre, Amersham. Spectrapor 6 dialysis tubing (M_r cut-off = 1000) was purchased from Spectrum Medical Industries. V8 proteinase from *Staphylococcus aureus* was from Miles Laboratories. Reagents used for HPLC were purchased from Merck, and those used for automated amino acid sequence analysis were obtained as described in [7].

2.2. *Isolation of human proenzyme C1r*

Human C1r was isolated in its proenzyme form from outdated plasma by a procedure involving affinity for insoluble IgG-ovalbumin aggregates, as described in [4].

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2.3. *CNBr cleavage of proenzyme C1r*

Unreduced proenzyme C1r (200 nmol) was dissolved in 70% (v/v) formic acid (1.3 ml) containing CNBr (6.4 mmol) and kept in the dark for 24 h at 4°C. The mixture was diluted 1:10 with water, freeze-dried, then re-dissolved in 0.1 M NH_4HCO_3 (4.5 ml) and applied to a column (2.5 × 110 cm) of Sephadex G-100, equilibrated with 0.1 M NH_4HCO_3 and run at a flow rate of 14 ml/h. Seven pools were collected and freeze-dried. Pools 1 and 2 were re-dissolved in 6 M urea/0.2 M formic acid (1.0 ml) and submitted to high-pressure gel-permeation chromatography on a column (7.5 × 600 mm) of TSK-G3000 SW (LKB) as described previously [8]. In both cases, three pools were collected, which were dialysed against 0.5% (v/v) acetic acid and freeze-dried.

2.4. *Reduction and alkylation of CNBr fragment CP2b*

Fragment CP2b was dissolved in 6 M guanidine–HCl/0.4 M Tris–HCl/2 mM EDTA, pH 8.0 (3 ml) and reduced with 20 mM dithiothreitol, then alkylated by iodo[2-^{3}H]acetic acid (200 μCi) as described by Johnson et al. [9]. After exhaustive dialysis against 0.1 M NH_4HCO_3, peptides generated by reduction of fragment CP2b were applied to a column (2.5 × 110 cm) of Sephadex G-75 equilibrated with 0.1 M NH_4HCO_3 and run at a flow rate of 12 ml/h. Six pools were collected and freeze-dried.

2.5. *V8 digest of the CNBr peptide overlapping the A and B chains*

Peptide CP2bRA4 from pool 4 of Sephadex G-75 (33 nmol) was dissolved in 0.1 M NH_4HCO_3/2 mM EDTA, pH 7.8 (0.5 ml) and digested by V8 staphylococcal protease as described in [5]. The digest was freeze-dried, redissolved in 0.1% (v/v) trifluoroacetic acid (1 ml) and fractionated by reversed-phase HPLC on a μBondapak C18 column (3.9 × 300 mm) using solvent system 2 as described previously [5].

2.6. *Amino acid analysis and sequence determination*

Amino acid analysis was carried out as in [7]. Samples were analysed on a Durrum D-500 or an LKB 4400 amino acid analyser. Automated Edman degradation was performed on an upgraded Beckman 890C sequencer with program number 345802. Phenylthiohydantoin derivatives were identified and quantified by reversed-phase HPLC, with an ODS-Hypersil 5μ column (5 × 100 mm), equilibrated at 1 ml/min with a mixture of solution A (0.01 M sodium acetate (pH 4.2)/methanol, 9:1, v/v) and solution B (acetonitrile/ethanol, 1:1, v/v) in the ratio 95:5 (v/v), then eluted with a linear gradient (27 min) to give a final ratio of 55:45 (v/v).

3. RESULTS

3.1. *Isolation of the CNBr-cleavage peptide overlapping the A and B chain moieties in proenzyme C1r*

Unreduced proenzyme C1r was submitted to

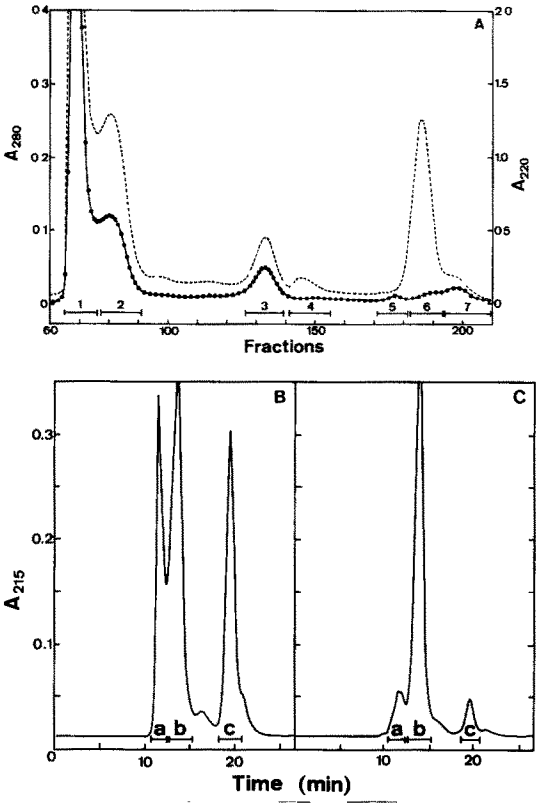

Fig.1. Isolation of CP2b, the CNBr-cleavage peptide overlapping the A and B chains regions in proenzyme C1r. (A) Initial fractionation of the CNBr digest from unreduced proenzyme C1r (200 nmol) by gel filtration on Sephadex G-100. (B,C) Further purification of CP2b by fractionation of pools 1 (B) and 2 (C) from Sephadex G-100 by high-pressure gel permeation on TSK-G3000 SW.

CNBr cleavage, and the CNBr-cleavage peptides were first fractionated by gel-filtration on Sephadex G-100 (fig.1A). Pools 1 and 2 from Sephadex G-100 were further analysed by high-pressure gel-permeation on TSK G3000 SW, and both resolved in three peaks denoted a,b and c (fig.1B,C). Peak 1b, originating from pool 1 (fig.1B) and peak 2b, originating from pool 2 (fig.1C) both contained a single fragment of apparent M_r approx. 55000 as determined by SDS–polyacrylamide gel electrophoresis, and were pooled together. This fragment, named CP2B, was reduced with dithiothreitol and alkylated by iodo[2-^{3}H]acetic acid, and the resulting peptides, generated by reduction of the disulphide bridges, were separated by gel filtration on Sephadex G-75 (fig.2).

Amino acid analysis of the pools from Sephadex G-75 indicated that pools 1 and 2 contained the same peptide, identified as CNBr-cleavage peptide CN1a from $C\bar{1}r$ A chain [6]. By the same technique, pools 3 and 5 were found to contain CNBr-cleavage peptides CB1a from $C\bar{1}r$ B chain [5,8] and CN3 from $C\bar{1}r$ A chain [6], respectively. Pool 4 contained a single peptide, CP2bRA4, with an amino acid composition (table 1) corresponding to the sum of individual amino acid compositions of CN2b, the C-terminal CNBr-cleavage peptide from the N-terminal A chain of $C\bar{1}r$ [6], and CB4, the N-terminal CNBr-cleavage peptide from the C-terminal B chain of $C\bar{1}r$ [8]. CP2bRA4 was thus identified as the CNBr cleavage peptide overlapping the A and B chain moieties in proenzyme C1r.

Table 1

Amino acid compositions of selected peptides covering the cleavage site of proenzyme C1r[a]

Amino acid	Residues/mol				
	CN2b[b]	CB4[c]	CN2b + CB4	CP2bRA4	CP2bRA4SP9
Asx	2.0 (2)	–	(2)	2.4	1.1 (1)
Thr	2.7 (3)	–	(3)	2.7	0.4 (0)
Ser	1.8 (2)	–	(2)	1.9	–
HSe[d]	–	0.9 (1)	(1)	0.6	0.7 (1)
Glx	11.3 (11)	1.1 (1)	(12)	12.9	4.4 (4)
Pro	3.9 (4)	–	(4)	3.4	3.6 (4)
Gly	4.7 (5)	2.1 (2)	(7)	7.0	3.1 (3)
Ala	1.9 (2)	1.0 (1)	(3)	2.5	1.1 (1)
½Cys[e]	3.0 (3)	–	(3)	3.0	1.7 (2)
Val	3.6 (4)	–	(4)	3.5	2.7 (3)
Ile[f]	1.8 (2)	1.5 (2)	(4)	2.9	2.0 (3)
Leu	1.0 (1)	–	(1)	1.2	0.9 (1)
Tyr	1.0 (1)	–	(1)	1.4	0.4 (0)
Phe	–	–		–	–
His	–	–		–	–
Lys	3.8 (4)	1.9 (2)	(6)	5.5	3.6 (4)
Arg	4.7 (5)	–	(5)	5.0	3.0 (3)
Trp	(1)		(1)		

[a] Figures in brackets are obtained from sequence
[b] Values taken from [6]
[c] Values taken from [8]
[d] Quantified as the sum of homoserine and homoserine lactone values, except for peptide CP2bRA4 (homoserine value)
[e] Estimated as *S*-(carboxymethyl)cysteine
[f] The low value for isoleucine in CB4, CP2bRA4 and CP2bRA4SP9 is due to the presence of an Ile-Ile bond partially resistant under standard hydrolysis conditions

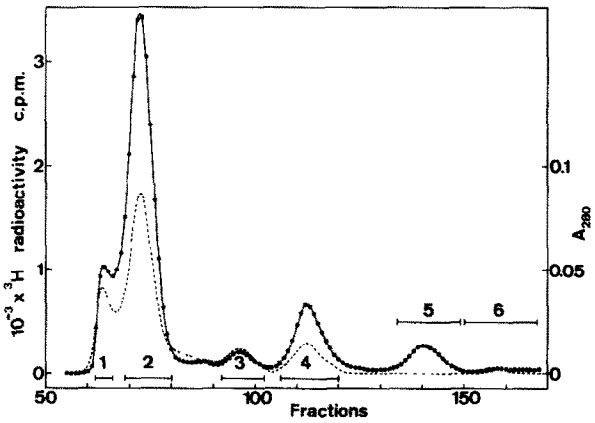

Fig.2. Separation of the peptides resulting from reduction and *S*-[^{3}H]carboxymethylation of fragment CP2b. Fragment CP2b was reduced with dithiothreitol and alkylated by iodo[2-^{3}H]acetic acid as described in section 2. Peptides were separated by gel filtration on Sephadex G-75. ---, A_{280}; ●, radioactivity of *S*-[^{3}H]carboxymethylcysteine.

3.2. *Subdigestion of peptide CP2bRA4 and sequence of the peptide covering the site cleaved during activation of C1r*

Peptide CP2bRA4 was submitted to subdigestion by V8 staphylococcal protease, and the digest was separated by reversed-phase HPLC (fig.3). Eleven pools were collected, containing eight different peptides identified by amino acid analysis. As summarized in fig.4, these peptides resulted from expected cleavage of glutamyl bonds at positions 8,10,25 and 29 of CP2bRA4. Cleavage at positions 8,10 and 29 was incomplete, whereas the Glu-Gln bond at position 46 proved entirely resistant to enzymatic cleavage.

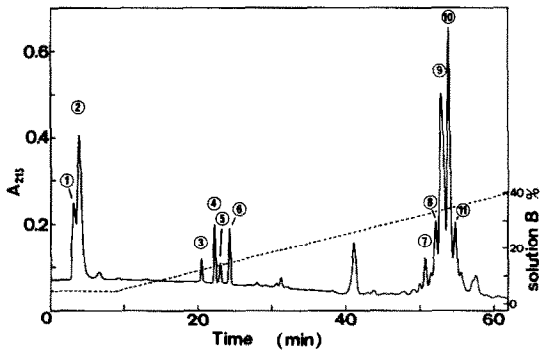

Fig.3. Separation by reversed-phase HPLC of peptides from subdigest of CP2bRA4 by V8 staphylococcal protease. The digest was dissolved in 0.1% (v/v) trifluoroacetic acid and separated on μBondapak C18 using solvent system 2 as described previously [5].

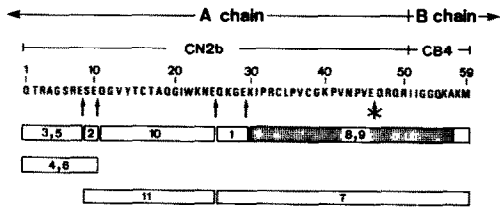

Fig.4. Diagrammatic representation of fragments generated by subdigestion of peptide CP2bRA4 by V8 staphylococcal protease. Fragments are numbered according to their elution position on HPLC (see fig.3). Lack of cleavage of the glutamyl bond at position 46 is indicated by a crossed-out arrow. Stippling denotes the portion of peptide CP2bRA4SP9 that was sequenced.

Pools 8 and 9 both contained the C-terminal peptide from CP2bRA4, CP2bRA4SP9, originating from cleavage of the Glu-Lys bond at position 29, and overlapping the A and B chain moieties in proenzyme C1r (fig.4). Peptide CP2bRA4SP9 (7 nmol) was submitted to N-terminal sequence analysis, which allowed identification of a continuous stretch of 28 amino acid residues, corresponding to positions 30–57 of CP2bRA4 (fig.4). The sequence data, in agreement with amino acid composition of peptide CP2bRA4SP9 (table 1), indicate unequivocally that, in the sequence of proenzyme C1r, the arginine residue that is C-terminal in $\overline{C1r}$ A chain forms a peptide bond with the isoleucine residue that is N-terminal in $\overline{C1r}$ B chain. This demonstrates that activation of proenzyme C1r proceeds through the cleavage of a single Arg-Ile bond, in the sequence ...Gln-Arg-Gln-Arg-Ile-Ile-Gly-Gly...

4. DISCUSSION

Peptide CP2b, resulting from CNBr-cleavage of unreduced proenzyme C1r, comprises at least 4 peptides, which are disulphide-linked. These peptides originate from both the N-terminal A-chain moiety of proenzyme C1r (CN1a and CN3), and the C-terminal B-chain moiety (CB1a), peptide CP2bRA4 overlapping both regions. As peptide CB1a contains a single half-cystine residue, located

at position 114 of C1r B chain [5,8], it can be concluded that this residue is involved in the single disulphide bridge that links the A and B chains of C1r. This conclusion is in agreement with previous observation [5] that half-cystine 114 of C1r B chain is homologous to that implicated in the interchain disulphide bond in chymotrypsinogen, plasminogen, factor X and prothrombin [10].

Subdigestion of peptide CP2bRA4 by V8 staphylococcal protease under conditions designed for specific cleavage of glutamyl bonds [11] indicate incomplete cleavage of some of these bonds, a result which could be explained by the presence of another glutamic acid residue in the vicinity of the cleavage sites [12]. More surprising is the lack of cleavage of the Glu-Gln bond at position 46 of peptide CP2bRA4. Given that identification of glutamic acid at this position was unequivocally established from both sequences of peptide CP2bRA4SP9 and of peptide CN2b from C1r A chain [6], and that Glu-Gln bonds are not reported to be resistant to cleavage by V8 staphylococcal protease [11,13], it is likely that the lack of cleavage of this bond arises from a lack of accessibility, due to the persistence of secondary structure in peptide CP2bRA4.

The sequence of peptide CP2bRA4SP9 unambiguously establishes that activation of proenzyme C1r proceeds through the cleavage of a single Arg-Ile bond in the sequence Gln-Arg-Gln-Arg-Ile-Ile-Gly-Gly. It is noteworthy that the vicinal Arg-Gln bond is not cleaved during activation of C1r, as shown by the C-terminal sequence of CN2b, the C-terminal CNBr-cleavage peptide of C1r A chain [6]. Given that, once activated, C1r undergoes two further autolytic cleavages, both occurring at Arg-Gly bonds [6], this protease clearly appears specific for arginyl bonds, in agreement with its known esterolytic specificity [14] and its 'trypsin-like' nature, apparent from the amino acid sequence of its catalytic B chain [5].

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