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Human complement component C4

Structural studies on the fragments derived from C4b by cleavage with C3b inactivator

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1. One of the activation products of C4, C4b, was prepared, and the reactive thiol group on the α′-chain was radioactively labelled with iodo[2-^{14}C]acetic acid. The α′-chain was isolated and the *N*-terminal amino acid sequence of the first 13 residues was determined. 2. C4b was cleaved by C3bINA in the presence of C4b-binding protein and C4d and C4c isolated. The radioactive label and therefore the reactive thiol group were located to C4d. 3. C4c was reduced and alkylated and the two α′-chain fragments of C4c were separated. 3. The molecular weights, amino acid analyses and carbohydrate content of the three α′-chain fragments were determined. C4d has a mol.wt. of 44500 and a carbohydrate content of 6%. The two α′-chain fragments of C4c have mol.wts. of 25000 (α3) and 12000 (α4) and carbohydrate contents of 10 and 22% respectively. 4. The *N*-terminal amino acid sequences of C4d, the α3 and the α4 fragments were determined for 18, 24 and 11 residues respectively and, by comparison with the *N*-terminal sequence of the C4b α′-chain, the 25000-mol.wt. fragment (α3) was shown to be derived from the *N*-terminal part of the α′-chain. 5. *C*-Terminal analyses were done on the α′-chain and its three fragments. Arginine was found to be the *C*-terminal residue of C4d and of the α3 fragment. The *C*-terminal residue of the α′-chain and of the α4 fragment could not be identified. The order of the three fragments of the α′-chain is therefore: α3(25000) – C4d(44500) – α4(12000). The specificity of C3bINA is for an Arg–Xaa peptide bond.

The activation of the classical pathway of complement is initiated by the binding of the first component (C1) to immune complexes, followed by the activation of the fourth component (C4) by the bound $C\bar{1}$. Activated C4 binds to acceptor sites on cell surfaces and to antibody–antigen aggregates. However, the amount of C4b bound to antibody-coated erythrocytes was found to be 10–13 times more than that bound to the aggregates, although the C3 convertase activity in the presence of C2 was the same. It was therefore postulated that only the C4b bound to antibody–antigen aggregates was effective in forming C3 convertase, $C\bar{4}\bar{2}$ (Goers & Porter, 1978). Campbell *et al.* (1980) showed that C4b binds directly to the antibody in the immune complexes. This binding is through the α′-chain of C4b and the Fd fragment of the heavy chain of IgG and is probably covalent in nature. It is similar to that described for C3 binding to cell surfaces, which involves an ester-like covalent bond with surface structures (Law & Levine, 1977). Law *et al.* (1979*a*,*b*) have shown that the covalent bond involves an acyl group on C3d, which is a fragment of the α′-chain of C3b.

Janatova *et al.* (1979) reported the appearance of a reactive thiol group in both C3b and C4b that is not present in haemolytically active C3 and C4, although it is present in haemolytically inactive C3 and C4, and they located the reactive groups to the C3d fragment and to the α′-chain of C4b. Tack *et al.* (1980) radioactively labelled the acyl group of C3 and the reactive thiol group and found both radioactively labelled groups in the same peptide.

Abbreviations used: The nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968); activated components are indicated by an overbar, e.g. $C\bar{1}$, or by the activation product, e.g. C4b; C3b inactivator is C3bINA; iPr_2P-F, di-isopropyl phosphorofluoridate; IgG and IgM, immunoglobulins G and M; Fd, *N*-terminal half of the heavy chain; SDS, sodium dodecyl sulphate; Quadrol, 1,1′,1″,1‴-(ethane-1,2-diyldinitrilo)tetrakis(propan-2-ol); Polybrene, *NNN′N′*-tetramethylhexa-1,6-diamine polymer with 1,3-dibromopropane.

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The enzyme C3bINA was shown to digest C3b and C4b in the presence of cofactors (Pangburn *et al.*, 1977; Nagasawa *et al.*, 1980). Fujita *et al.* (1978) showed that the high-molecular-weight protein C4b-binding protein acted as cofactor in the digestion of C4b by C3bINA and that cleavage occurred at two positions in the α′-chain of C4b to give rise to a fragment of apparent mol.wt. 47000, α2 or C4d, which is not covalently bound through disulphide linkage to the other cleavage product, C4c. C4c consisted of two fragments of the α′-chain, α3 and α4, of apparent mol.wts. 25000 and 17000 and the β- and γ-chains of C4, all in disulphide-bond linkage. This was also suggested by Nagasawa *et al.* (1980).

The present work reports the radioactive labelling of the reactive thiol group in C4b and the isolation of the fragments C4d and C4c from the digestion mixture of C4b-binding protein, C4b and C3bINA. The two α′-chain fragments of C4c have also been isolated, and structural studies are reported on the α′-chain of C4b that give the alignment of all three fragments in the α′-chain.

Materials and methods

Materials

Chemicals. These were obtained as follows: iPr_2P-F, Calbiochem, San Diego, CA, U.S.A.; SDS, H_2O_2 (Aristar) and guanidine hydrochloride, BDH Chemicals, Poole, Dorset, U.K.; acrylamide, Fisons Scientific Apparatus, Loughborough, Leics., U.K.; *NN′*-methylenebisacrylamide, Coomassie Blue R-250 and hydroxyapatite (DNA-grade Bio-Gel HTP), BioRad Laboratories, Bromley, Kent, U.K.; PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene], Koch–Light Laboratories, Colnbrook, Bucks., U.K.; NCS (Nuclear Chicago Solubilizer), Hopkins and Williams, Romford, Essex, U.K.; Schiff reagent, Difco Laboratories, West Moseley, Surrey, U.K.; iodo[2-^{14}C]-acetic acid (54Ci/mol), The Radiochemical Centre, Amersham, Bucks., U.K.; putrescine dihydrochloride, bovine serum albumin, ovalbumin, lysozyme and cytochrome *c*, Sigma Chemical Co., Poole, Dorset, U.K.; Quadrol trifluoroacetate, Fluorchem (Fluka) Ltd., Glossop, Derbyshire, U.K.; Polybrene, Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals used in the sequencer were obtained from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. Sephacryl S-200 (superfine grade), Sepharose CL-6B, Sepharose 6B and Sephadex G-25 (fine grade), Pharmacia Fine Chemicals, Uppsala, Sweden; carboxypeptidase A and carboxypeptidase B (Worthington), Millipore (U.K.) Ltd., London N.W.10, U.K.

Outdated human plasma was obtained from the Churchill Hospital, Oxford, U.K. It was made 20mM with $CaCl_2$ and left to clot overnight at 4°C. The clot was removed by centrifugation and the serum stored at −20°C.

Complement components. Human C4 was prepared as described by Campbell *et al.* (1980), $C\bar{1}s$ was prepared from $C\bar{1}$ (Gigli *et al.*, 1976) and C3bINA was prepared from human plasma (Crossley & Porter, 1980). Human C4b-binding protein was prepared by fractionation of a euglobulin precipitate of human serum on a column (90cm × 5cm) of Sepharose 6B in 0.2M-NaCl/0.05M-sodium acetate buffer (pH5.5)/5mM-$CaCl_2$ (R. B. Sim, unpublished work), C4b-binding protein was eluted ahead of C1 and the fraction that was pooled also contained IgM, but it was not further purified. The pooled fraction was concentrated by ultrafiltration to 1.5–2mg/ml and was dialysed against 0.13M-NaCl/0.02M-Tris/HCl/0.02% NaN_3, pH7.1.

Methods

Polyacrylamide-gel electrophoresis. SDS/polyacrylamide slab gels of 8.5 and 15% acrylamide-gel concentrations were prepared by the method of Laemmli (1970). Preparation of samples for electrophoresis was as described by Dodds *et al.* (1978). Gels were stained for protein with Coomassie Blue and for carbohydrate by the periodic acid/Schiff procedure described by Kapitany & Zebrowski (1973).

Preparation of C4b from C4 and labelling of the reactive thiol group with iodo[2-^{14}C]acetic acid. C4 in 0.01M-Tris/HCl/0.2M-NaCl/2mM-$CaCl_2$, pH7.4, was incubated at 37°C with $C\bar{1}s$ (1:40, w/w) in the presence of 10mM-putrescine for 40min. iPr_2P-F was then added at 4°C to a concentration of 2.5mM. The C4b was incubated at 37°C for 40min with a 10-fold molar excess (to achieve complete derivatization) of iodo[2-^{14}C]acetic acid (54Ci/mol), followed by a further incubation for 30min at 37°C with a 1000-fold molar excess of iodoacetic acid. The radioactively labelled C4b was exhaustively dialysed against 0.1M-NaCl/0.02M-Tris/HCl/0.02% NaN_3, pH7.1, at 4°C. The radioactivity of the dialysed C4b was determined by adding a 50μl sample to 5ml of 1,4-dioxan containing 2% (w/v) naphthalene and 0.5% (w/v) PPO. Samples were counted for radioactivity with an LKB–Wallac 1210 Ultrobeta counter.

Preparation of C4d and C4c from C4b. C4b (30mg) in 15ml of 0.1M-NaCl/0.02M-Tris/HCl/0.02% NaN_3 was incubated for 3h at 37°C, pH7.1, with C4b-binding protein (30mg) in 15ml of 0.13M-NaCl/0.02M-Tris/HCl/0.02% NaN_3 and C3bINA (0.3mg) in 0.5ml of 0.15M-NaCl/0.025M-Tris/HCl/0.02% NaN_3. iPr_2P-F was added at 4°C to 2.5mM and, after addition of NaCl (1.8g), the digestion mixture was incubated at 4°C for 1h and fractionated on a column (60cm × 3.7cm) of Seph-

acryl S-200 (superfine grade) in 0.5 M-NaCl/0.02 M-sodium phosphate/0.02% NaN_3, pH 7.0, at 4°C. The A_{280} and the radioactivity of the column eluate were determined. SDS/8.5%-polyacrylamide-slab-gel electrophoresis was also performed on the column eluate.

Isolation of the α'-chain fragments from C4c. Reduction and alkylation of C4c was as described by Gigli *et al.* (1977) for C4. The reduced C4c (20mg) was fractionated on a column (90cm × 2.6cm) of Sepharose CL-6B in 6M-guanidine hydrochloride, pH 8.0. The fractions containing the α'-chain fragments were detected by SDS/15%-polyacrylamide-slab-gel electrophoresis of the column eluate desalted on a column (0.9cm × 60cm) of Sephadex G-25 in 0.05 M-NH_3. After total reduction in 6M-guanidine hydrochloride/0.3 M-Tris/HCl/50mM-dithiothreitol, pH 8.6, for 3h at 37°C and alkylation with 120mM-iodo[2-^{14}C]acetic acid (67mCi/mol) for 1h at 4°C, the α'-chain fragments were separated on a column (93cm × 1.6cm) of Sephacryl S-200 (superfine grade) in 6M-guanidine hydrochloride, pH 8.0. The A_{280} of the column eluate was determined and also the radioactivity determined by the addition of 25μl samples to 0.75ml of 90% (v/v) NCS (a 0.6M solution of a quaternary ammonium base in toluene) in water and 7.5ml of toluene containing 0.5% PPO and 0.03% POPOP. The fractions containing the α'-fragments were desalted by chromatography on a column (1.9cm × 60cm) of Sephadex G-25 in 0.05 M-NH_3 and freeze-dried.

Amino acid analysis. Samples were hydrolysed under vacuum at 110°C for 24h in constant-boiling HCl containing 4mM-phenol. No corrections were made for destruction of threonine and serine, and tryptophan was not measured. Cystine and methionine in C4d were measured as cysteic acid and methionine sulphone respectively, after oxidation with performic acid (Hirs, 1956) and acid hydrolysis. Cystine in the α'-fragments of C4c was measured as *S*-carboxymethylcysteine after acid hydrolysis, under vacuum, of the reduced and alkylated samples in the presence of 0.05% 2-mercaptoethanol. Analyses were performed on a Durrum 500 analyser.

Molecular-weight determinations. Totally reduced and alkylated α'-chain fragments were chromatographed on a column (93cm × 1.6cm) of Sephacryl S-200 (superfine grade) in 6M-guanidine hydrochloride, pH 8.0. The column was calibrated with reduced and alkylated samples of bovine serum albumin, ovalbumin, lysozyme and cytochrome *c* and the molecular weights of the fragments calculated.

Automated amino-acid-sequence determination. The *N*-terminal amino acid sequences of the α'-chain of C4b and of its fragments were determined by automated Edman degradation in a Beckman 890c sequencer and the amino acid phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography as described previously (Johnson *et al.*, 1980).

C-Terminal amino acid analysis. The α'-chain fragments of C4b (3nmol) in 0.2ml of 0.02M-sodium phosphate/0.1% SDS, pH 7.2, were incubated with either carboxypeptidase A or B [substrate/enzyme ratio 10:1 (w/w)] at 37°C for 1 and 2h respectively. The digests were adjusted to pH 2.2 by the addition of 0.1 M-HCl and the samples freeze-dried and analysed for amino acids on the Durrum amino acid analyser. Samples of the fragments alone and the carboxypeptidases alone were also incubated and treated as the digestion mixtures and analysed.

Results

Preparation of α'-chain of C4b and N-terminal amino-acid-sequence determination

C4b was prepared as described under 'Methods', but the reactive thiol group was not labelled with iodo[2-^{14}C]acetic acid. The C4b was partially reduced and alkylated with iodo[2-^{14}C]acetic acid and the α'-chain separated by fractionation on a column of hydroxyapatite (Gigli *et al.*, 1977); it was eluted after the β-chain, but before any α-chain present in the preparation. The α'-chain fraction was then totally reduced and alkylated with iodo[2-^{14}C]acetic acid as described by Gigli *et al.* (1977). The reduced and alkylated protein was dialysed exhaustively against 0.05 M-NH_3 and freeze-dried. The *N*-terminal amino acid sequence of residues 1–13 of the α'-chain is shown in Table 1. In Fig. 1 the *N*-terminal amino acid sequences of the α'-chains of C3b (Tack *et al.*, 1979) and C4b are compared; the sequences have been aligned to show maximum homology and, in addition to the six identical residues, the predominance of acidic and hydrophobic residues is a feature of both sequences.

Preparation of C4b, C4d and C4c

Digestion of C4 with $C\bar{1}s$ was in the presence of 10mM-putrescine, as it had been reported by Campbell *et al.* (1980) that putrescine inhibits the formation of C4b dimers and therefore ensures maximum yield of C4b and of C4d. It had been reported by Janatova *et al.* (1979) that a reactive thiol group, not present in haemolytically active C4, became available after digestion with $C\bar{1}s$ and also that it was present in inactive C4. In order to locate this reactive thiol group, the C4b was incubated with a 10-fold molar excess of iodo[2-^{14}C]acetic acid (54Ci/mol), followed by a 1000-fold molar excess of iodoacetic acid. After exhaustive dialysis the radioactivity of C4b ranged from 5000 to 50000c.p.m./

	1		5		10		15		20
C4bα′	Ala-Leu-	-Glu-	-Ile-Leu-Gln-Glu-Glu-Asp-Leu-Ile-Asp-Glu						
C3bα′	Ser-Asn-Leu-Asp-Glu-Asp-Ile-Ile-Ala-Glu-Glu-Asp-Ile-Val-Xaa-Arg-Ser-Glx-Phe-Ile								
α3 Fragment	Ala-Leu-	-Glu-	-Ile-Leu-Gln-Glu-Glu-Asp-Leu-Ile-Asp-Glu-Asp-Asp-Ile-Pro-Val Met -Arg-Ser-Phe-Phe-Pro-Glu						

Fig. 1. *N-terminal amino acid sequences of the α′-chains of C4b and C3b and of the α3 fragment of the α′-chain of C4b*
The sequence of the α′-chain of C3b (Tack *et al.*, 1979) has been aligned for maximum homology with C4b α′-chain. Identical residues have been underlined and Xaa is an unidentified residue.

mg of C4b. The C4b was concentrated and incubated with C4b-binding protein and C3bINA as described under 'Methods'. The digestion mixture was fractionated on a column of Sephacryl S-200 (superfine grade) and three fractions were separated, as shown in Fig. 2. SDS/polyacrylamide-slab-gel electrophoresis was performed to identify the fractions; the first contained C4b-binding protein, the second C4c and the third was C4d. About 75% of the radioactivity of the C4b was in the C4d fraction and the small amount of radioactivity in the second fraction was identified from the SDS/polyacrylamide-slab-gel electrophoresis as being due to residual C4b, which was partially separated from the C4c. Janatova *et al.* (1979) had reported that the reactive thiol group was in the α′-chain of C4b, and the location of the thiol group to the C4d fragment is a further example of the structural homology that exists between C3b and C4b, since Janatova *et al.* (1979) had also located the reactive thiol group of C3b to the C3d fragment.

Fig. 2. *Separation of C4d and C4c after digestion of C4b with C3bINA in the presence of C4b-binding protein*
The digestion mixture was chromatographed on a column (60 cm × 3.7 cm) of Sephacryl S-200 (superfine grade) in 0.5 M-NaCl/0.02 M-sodium phosphate/0.02% NaN_3, pH 7.0. ●, A_{280}; ○, radioactivity of *S*-[^{14}C]carboxymethylcysteine.

Table 1. *Automated sequence analysis of α'-chain of C4b and of its fragments*

Residue no.	Amino acid identified in: α'-Chain	25000-mol.wt. fragment	C4d	12000-mol.wt. fragment
1	Ala	Ala	Thr	Gly
2	Leu	Leu	Leu	Gly
3	Glu	Glu	Glu	Phe
4	Ile	Ile	Ile	Lys
5	Leu	Leu	Pro	—
6	Gln	Gln	Gly	Leu
7	Glu	Glu	Asn	Ala
8	Glu	Glu	Ser	Leu
9	Asp	Asp	Asp	—
10	Leu	Leu	Pro	Leu
11	Ile	Ile	Asn	Gly
12	Asp	Asp	Met	
13	Glu	Glu	Ile	
14		Asp	Pro	
15		Asp	Asp	
16		Ile	Gly	
17		Pro	Asp	
18		Val/Met	Phe	
19		Arg		
20		Ser		
21		Phe		
22		Phe		
23		Pro		
24		Glu		
Amount used (nmol) ...	110	14	10	40
Recovery at first step (nmol) ...	32	4	6	5
Stepwise yield (%) ...	93	96	93	90

The C4d was dialysed against 0.05 M-NH_3 and freeze-dried. The C4c was concentrated by ultrafiltration and used to prepare the α'-chain fragments.

Determination of carbohydrate on the α'-chain fragments of C4c and on C4d

Samples of equimolar mixtures of C4d and C4c were reduced and electrophoresed in SDS/15%-polyacrylamide gels. The gels were stained for protein with Coomassie Blue to locate the chains and also stained for carbohydrate by the periodic acid/Schiff procedure. C4d and the other two α'-chain fragments stained for carbohydrate with equal intensity, but the intensity of staining with Coomassie Blue was approximately in proportion to their molecular weights. It was deduced, therefore, that the carbohydrate on the α'-chain of C4b (9.4%) (Gigli *et al.*, 1977) is distributed approximately equally between the three fragments and this estimate of carbohydrate content was used in the calculations of the amino acid compositions of the fragments (Table 2).

Structure of C4d

The molecular weight of this fragment, determined by gel filtration in 6 M-guanidine hydrochloride, is 44500, of which 6% is estimated to be carbohydrate from the staining intensity for carbohydrate of the fragment in SDS/polyacrylamide slab gels. The amino acid analysis of C4d is shown in Table 2 and the *N*-terminal amino acid sequence in Table 1. From the latter Table it can be seen that C4d is not derived from the *N*-terminal part of the α'-chain of C4b.

Structure of the α'-chain fragments of C4c

The molecular weights of the two α'-chain fragments, determined by gel filtration in 6 M-guanidine hydrochloride after reduction and alkylation, are 25000 for the larger fragment and 12000 for the smaller. The carbohydrate contents are estimated to be 10 and 22% respectively. The amino acid compositions of the fragments based on these molecular weights are shown in Table 2. The *N*-terminal amino acid sequence of the α3 fragment (Table 1) shows that this fragment is derived from the *N*-terminal part of the α'-chain of C4b.

Table 2. *Amino acid compositions of fragments of the α'-chain of C4b*

The amino acid compositions are given as mol of residue/mol of fragment to the nearest integer. The samples were hydrolysed at 110°C for 24h. No corrections were made for serine and threonine destruction, and tryptophan was not determined. The composition of C4d is the average of four analyses based on a mol.wt. of 44500 and 6% carbohydrate. The 25000-mol.wt. fragment has an estimated carbohydrate content of 10% and the 12000-mol.wt. fragment an estimated carbohydrate content of 22% and both amino acid compositions were the average of two analyses.

	Amino acid composition (mol/mol)		
Amino acid	C4d	25000-mol.wt. fragment	12000-mol.wt. fragment
Asp	35	17	11
Thr	26	11	5
Ser	32	14	4
Glu	45	26	13
Pro	23	13	2
Gly	47	15	9
Ala	45	14	4
Val	24	21	7
Cys	4*	3‡	1‡
Met	6†	1	2
Ile	13	7	3
Leu	42	25	8
Tyr	10	5	4
Phe	12	9	2
His	11	5	2
Lys	15	8	6
Arg	15	12	3

* Measured as cysteic acid.
† Measured as methionine sulphone.
‡ Measured as *S*-carboxymethylcysteine.

C-terminal amino acid analysis of the α'-chain and of the three fragments of the α' chain of C4b

Carboxypeptidase A digestion did not release any amino acids from the α'-chain of C4b nor from the three fragments. Carboxypeptidase B digestion released arginine (0.6mol/mol) from the α3 fragment and arginine (0.3mol/mol) from the C4d fragment. No amino acids were released by digestion with carboxypeptidase B from either the α'-chain nor from the α4 fragment. These results, in conjunction with the *N*-terminal amino acid analyses (Table 1) establish the order of the three fragments of the α' chain of C4b as:

α3 — C4d — α4

Discussion

Change in conformation of C4 on activation

The appearance of a reactive thiol group on the α' chain of C4b, first reported by Janatova *et al.* (1979), has been confirmed in the present work. Reboul *et al.* (1980) have reported the radioactive labelling of the α'-chain of C4b with thiol-specific reagents and of the α-chain of C4, denatured by urea, as was also reported by Janatova *et al.* (1979) for haemolytically inactive C4. However, native C4 (haemolytically active) does not have a reactive thiol group and this has been interpreted as indicating a change in conformation of C4 on proteolytic cleavage by $C\bar{1}s$ and also on activation of C4. Von Zabern *et al.* (1980) reported that C4b and C4 treated with NH_3 or N_2H_4 (hydrazine) had the same antigenic determinants and lacked one of the antigenic determinants present on C4, a further indication of conformational change.

Cleavage of C4b by C3bINA occurs in the presence of C4b-binding protein. Native C4 is not digested by C3bINA, but we have observed that inactive C4 is digested by C3bINA, which is further evidence for a difference in conformation between C4 and inactive C4. It should be pointed out, however, that the reactive thiol group is not required for cleavage by C3bINA, since in the work reported here, the thiol group was alkylated with iodoacetic acid before incubation with C3bINA. SDS/polyacrylamide-gel electrophoresis of a mixture of C4b and inactive C4 that had been incubated with C3bINA and C4b-binding protein showed that both the α- and the α'-chains were digested, and that, in addition to the appearance of C4d and the α'-chain fragments of 25000 and 12000mol.wt., another fragment, a little larger than the γ-chain, was seen.

This 33000-mol.wt. fragment must be derived from the α-chain and made of C4a (9000mol.wt.) plus the α3 fragment (25000mol.wt.). This observation was interpreted as indicating that the 25000-mol.wt. fragment was derived from the *N*-terminal part of the α'-chain of C4b, and this has been confirmed by the *N*-terminal sequence analyses reported in the present paper.

We incubated C4 with $C\bar{1}s$ in the presence of 2mM-$CaCl_2$, because the yield of C4b appeared to be greater in the presence of $CaCl_2$ as judged by SDS/polyacrylamide-gel electrophoresis. Reboul *et al.* (1980) reported that $CaCl_2$ effected the relative radioactive labelling of the chains of C4 and C4b by ^{125}I, a finding that they attribute to the binding of Ca^{2+} to C4 and C4b and to a consequent structural change.

Specificity of C3bINA

The specificity of C3bINA on C4b α'-chain has been shown, by *C*-terminal analyses of C4d and of the 25000-mol.wt. fragment, to be for an arginine residue. This specificity for a basic residue is a common feature of the complement enzymes $C\bar{1}r^{\bullet}$ and $C\bar{1}r$ (E. M. Press, unpublished work), $C\bar{1}s$ on

C4 (Gorski *et al.*, 1979) and $C\bar{1}s$ on C2 (Kerr, 1979), factor $\bar{D}$ (Kerr, 1979), C3 convertase (Hugli, 1975) and C5 convertase (Fernandez & Hugli, 1978). $C\bar{1}r$, $C\bar{1}s$ and factor $\bar{D}$ have been classified as serine proteinases, because they are inhibited by iPr_2P-F and can be labelled with [1,3-^{14}C]iPr_2P-F (Sim & Porter, 1976; Johnson *et al.*, 1980). Factor B and C2 are not inhibited by iPr_2P-F (Kerr, 1979) but Bb, the active component of C3 convertase, $\overline{C3bBb}$, has been shown to be a serine proteinase by the isolation of an active-site peptide from Bb that has the characteristic amino acid sequence of all serine proteinases (Christie *et al.*, 1980). In an unsuccessful attempt to classify the enzyme C3bINA, Crossley & Porter (1980) studied the effects of proteinase inhibitors, thiol-specific reagents and metal ions on C3bINA. They found no inhibition by iPr_2P-F (10mM), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') (5mM), phenylmethanesulphonyl fluoride (0.1mM) nor by benzamidine (5mM). C3bINA is a highly specific enzyme, as are all the complement enzymes, but it is unusual in cleaving at two sites in the α′-chain of C4b and in requiring the presence of the cofactor C4b-binding protein. C3b, in the presence of its cofactor β_1H, is digested at only one site by C3bINA, and there has been speculation that a second enzyme is responsible for one of the cleavages of C4b. In this connection, the work of Nagasawa *et al.* (1980) is of particular interest, as they have detected and isolated an intermediate product of the digestion of C4b by C3bINA, C4b′, in which only one peptide bond was hydrolysed to yield, on reduction, the smallest of the α′-chain fragments and a fragment consisting of C4d and the 25000-mol.wt. fragment of the α′-chain. C4b′ was digested further, only in the presence of both C4b-binding protein and C3bINA, to yield C4d and C4c. This two-stage cleavage was achieved by incubation at pH 8.5, for 20–60min, instead of at pH 7.0, which is nearer to the optimal pH of the enzyme (Crossley & Porter, 1980). It is possible that, at the higher pH, the conformation of C4b, in the C4b-binding protein–C4b complex, favours a considerably faster hydrolysis rate at one site than at the other, or, alternatively, that a necessary conformation change to allow cleavage at the second site occurs more slowly at the high pH. C4b-binding protein has been shown to be multivalent (Scharfstein *et al.*, 1978), binding four to five molecules of C4b, if the molecular weight of the cofactor is assumed to be about 600000. This fact may also have some bearing on the mechanism involved in the digestion of C4b α′-chain by C3bINA.

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