PURIFICATION OF HUMAN COMPLEMENT SUBCOMPONENT C4

C4 cleavage by $C\bar{1}s$

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1. Introduction

The formation of the C3 convertase of the classical pathway in the complement cascade involves peptides C2a and C4b generated by the proteolysis of C2 and C4 by $C\bar{1}$ [1].

The purification and the structural characteristics of C4 [2–4] and the binding properties of C4b to membranes and immune complexes have been described [5,6]. The binding of C4b to immunoglobulins has also been demonstrated [7]. Since most, if not all, C4 purification protocols lead to C4 containing contaminating traces of C4-binding protein (C4bp) [8], we have worked out a technique which allows the purification of C4 free from any trace of C4bp.

With the view of studying the cleavage of C4 by $C\bar{1}s$ we labelled C4 with ^{125}I. The distribution of the label in C4 depends on the labelling conditions used; it reflects a clear-cut difference between C4 and C4b structures and points to a relatively masked position of the N-terminal portion of the α chain in C4 and of C4a in $C\bar{1}s$ cleaved C4. The interaction between C4 and $C\bar{1}s$ in the presence and in the absence of calcium is discussed.

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetracetic acid; DFP, diisopropylphosphorofluoridate; PEG, polyethylene glycol; STI, soybean trypsin inhibitor

The nomenclature of the components of complement is that recommended by World Health Organization (1968). A bar indicates the activated state of a component

2. Materials and methods

Human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble). $C\bar{1}s$ was purified as in [9].

Protein estimation during C4 purification was by a modified Folin reaction, using bovine serum albumin as a reference [12]. C4 was selectively estimated by radial immunodiffusion. Double immunodiffusion in agarose was used for qualitative identification of protein.

Sucrose gradient centrifugation was done as in [13]. SDS–PAGE of proteins was as in [9].

C4-deficient human serum was kindly provided by Dr G. Hauptmann (Strasbourg). Antibodies against this serum were raised in rabbits using 0.5 ml serum with complete Freund adjuvant and 0.125 ml for the boost injection, essentially according to [10]. The total immunoglobulin fraction of the antisera was precipitated with sodium sulphate and the resulting protein coupled to Sepharose 6B as in [9].

C4 functional assay was by a one-step method using C4-deficient guinea pig serum, according to [11]; the strain of C4-deficient guinea pigs was a generous gift from Dr P. Lachmann (Cambridge).

The proteolysis of C4 by $C\bar{1}s$ was carried out by incubation for 30 min at 37°C of the two proteins in a 20:1 w/w ratio. To detect C4a, the total incubation medium was submitted to SDS–PAGE and Coomassie blue staining.

^{125}I labelling of proteins was by two methods, that of Bolton and Hunter [14] and with lactoperoxidase (LPO), which was a modification of the technique of

Heusser et al. [15]: to 1 mg of protein dissolved in 1 ml 100 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA and 5 mM DFP (pH 7.4) the following were added successively: 50 μl 20 μM KI, 50 μl $Na^{125}I$ (spec. act. 1 μCi/μl), 50 μl 1 mg/ml lactoperoxidase (Calbiochem grade A) and finally 40 μl H_2O_2 (dilution 1/6000) in 4 equal aliquots at times 0, 3, 6 and 9 min of incubation at 37°C. After a further 16 min incubation the reaction was stopped with 20 μl NaN_3 (20% solution, w/v). Free iodine was eliminated by dialysis against 100 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, (pH 7.4). ^{125}I was measured with a CG 2000 Intertechnique Counter on the different solutions or on 2 mm gel slices after SDS–PAGE of the labelled proteins. The average radioactive iodine binding was between 0.03 and 0.1 mol ^{125}I/mol C4.

Fluorescence measurements were made with a MPF2A Perkin Elmer Spectrofluorimeter using the classical tryptophan excitation and fluorescence spectra.

3. Results

3.1. *Purification of C4*

3.1.1. Treatment of plasma by inhibitors

Frozen plasma was thawed and incubated for 40 min at 20°C in 5 mM DFP, 5 mM EDTA, 2.7 mM iodoacetamide, 10 μg/ml heparin and 25 μg/ml STI. At the end of the incubation the pH was adjusted to 7.4 with NaOH.

3.1.2. PEG precipitation

PEG 4000 was dissolved in 100 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 15 mM EDTA, 5 mM DFP, 500 μg/ml iodoacetamide, 10 μg/ml heparin and 25 μg/ml STI. All subsequent operations were performed at 4°C. PEG 15% (w/v) was added to 300 ml plasma to 5% (w/v) final conc., after 30 min the medium was centrifuged for 20 min at 5000 × *g*. PEG 26% (w/v) was added to the supernatant to 12% final conc.; after 30 min incubation, the resulting precipitate was collected by centrifugation for 20 min at 7000 × *g*. The pellet was redissolved in 80 ml 20 mM phosphate buffer (pH 7.4) with 5 mM EDTA, 5 μ/ml STI, 5 mM DFP and incubated for 16 h.

3.1.3. DEAE cellulose steps

The PEG extract was diluted to a conductivity of 2.9 mS and applied to a column of DE-52 cellulose (1.6 × 6 cm) equilibrated in 20 mM phosphate buffer (pH 7.0) containing 10 mM NaCl, 5 mM EDTA and 5 μg/ml STI. The column was eluted with the same buffer until the eluate A_{280} was < 0.4. C4 was detected in the eluate by double immunodiffusion and pooled for the next step. A second DE-52 cellulose column (2.8 × 5.5 cm) was prepared in 20 mM phosphate buffer (pH 7.0) with 30 mM NaCl, 5 mM EDTA and 5 μg/ml STI. DFP (5 mM) was added to the pooled eluate from the first column before the second chromatography. After application of the fraction the column was washed with the equilibration buffer until the eluate A_{280} was < 0.1. Elution was carried out with a linear gradient of 30–200 mM NaCl in 5 mM EDTA, 20 mM phosphate (pH 7.0) (total vol. 360 ml). C4 was detected in the eluate by double immunodiffusion and pooled in the presence of 5 mM DFP. After concentration by ultrafiltration on PM10

Table 1
C4 purification

Step	Total protein (mg)	C4 antigen[a] (mg)	Yield in C4 antigen %	Spec. act.[a] (CH 50/mg prot. units C4)		Purification factor	
						Protein	Spec. act.
Plasma	16 500	96.0	100	0.98	10^4	1.0	1.0
5–12%, PEG precipitate	7178	79.2	82.5	0.86	10^4	1.9	0.9
1st DEAE eluate	3330	33.3	34.7	1.29	10^4	1.7	1.3
2nd DEAE eluate after concentration	33	19.7	20.5	1.04	10^6	103.0	106.0
Affinity column eluate after concentration	15	13.8	14.0	4.70	10^6	158.0	479.5

[a] Total protein, antigenic and functional C4 were determined as in section 2

(Amicon) the pool was dialyzed against 100 mM Tris–HCl, 150 mM NaCl and 5 mM EDTA (pH 7.4).

3.1.4. Affinity chromatography on Sepharose-bound antibodies to C4-deficient serum

Immunoadsorbent (30 ml) was equilibrated in the buffer used for the preceding dialysis step. The dialyzed fraction was put onto the column which was then washed with the equilibration buffer. C4 was eluted slightly after the void volume due to its non-specific interaction with the activated Sepharose matrix. The fractions containing C4 were pooled and concentrated on PM10 in the presence of 5 mM DFP.

A typical purification run is presented in table 1. The final yield of C4 approaches 15% with a purification factor of ~ 160 on a protein basis and 480 on a specific activity basis. The difference between these two values becomes apparent at the last purification step, which underlines the efficiency of the affinity column.

The purity of the purified C4 was checked by SDS–PAGE. As shown in fig.1, C4 appears as a monoband protein prior to reduction and exhibits the three characteristic α, β and γ chains after reduction and alkylation, in a 0.56 : 0.82 : 1 molar ratio, calculated from scanning at 550 nm and app. mol. wt 31 000, 70 000 and 87 500.

The control by double immunodiffusion in agarose was negative with the following antisera to: C4-deficient serum, C4-binding protein, prepared in the laboratory; β 1 1 S protein (gift from Dr R. M. Chapuis, Dr Isliker, Lausanne); C3, IgG, IgM, transferrin, albumin, ceruloplasmin, plasminogen, $C\bar{1}r$, C1t, $C\bar{1}In$, $C\bar{1}s$ and C5; with antiserum to whole serum only 1 precitin line was detected.

3.2. Iodination of C4 and of $C\bar{1}s$-treated C4

Iodination by ^{125}I was used to evaluate the structural changes resulting from splitting, of C4 by $C\bar{1}s$. The two different techniques described in section 2 were used concurrently and led to different labelling of C4. In C4 the LPO technique as shown in fig.2 preferentially labelled the γ chain; the α chain was also efficiently labelled. In contrast, the Bolton and Hunter technique (fig.3) led to the predominant labelling of the β chain, and very low radioactivity in the α chain.

With C4 incubated with $C\bar{1}s$ in EDTA, the Bolton and Hunter technique gave approximately the same

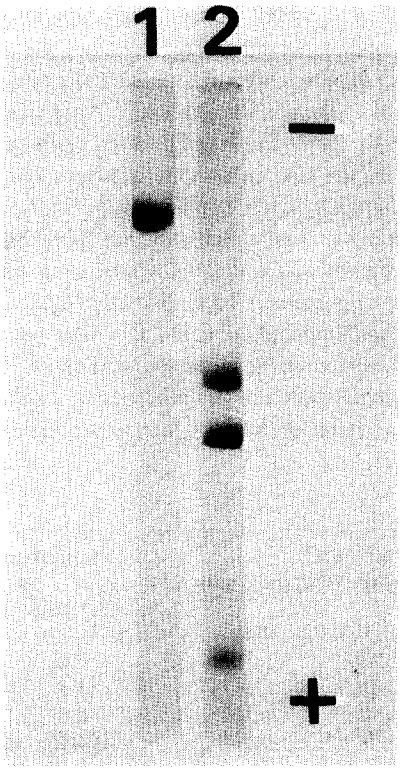

Fig.1. SDS–PAGE of C4. Electrophoresis was carried out as described in section 2. ~ 35 μg protein were applied to each gel. (1) Unreduced C4; (2) Reduced and alkylated C4.

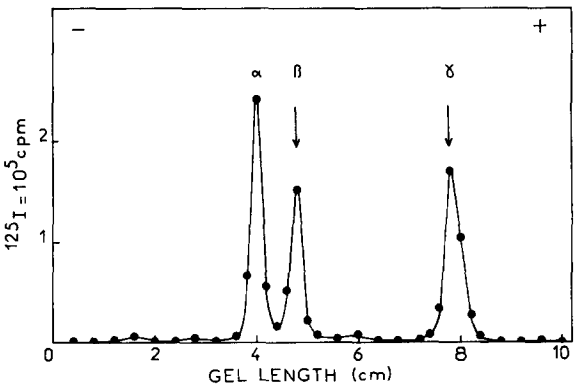

Fig. 2. Distribution of radioactivity in C4 chains (LPO technique). Reduced and alkylated sample. Electrophoresis and counting were as described in section 2.

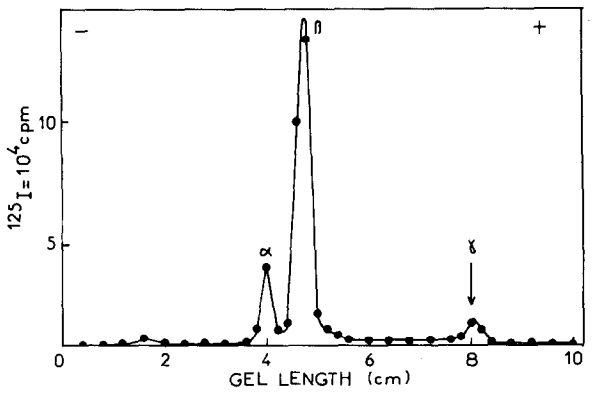

Fig. 3. Distribution of radioactivity in C4 chains (Bolton and Hunter technique). Reduced and alkylated sample was loaded onto the gel. Electrophoresis and counting were as described in section 2.

pattern of radioactivity for C4b chains as for C4 chains.

In contrast the LPO technique, as shown in fig. 4, led to a relative increase in the labelling of the γ chain and an inverted radioactivity α'/β in C4b compared with the α/β ratio in C4. When the LPO labelling was performed after incubation of C4 in 6 M urea, followed by dialysis to remove the urea, the ^{125}I distribution largely paralleled that of C4b. This

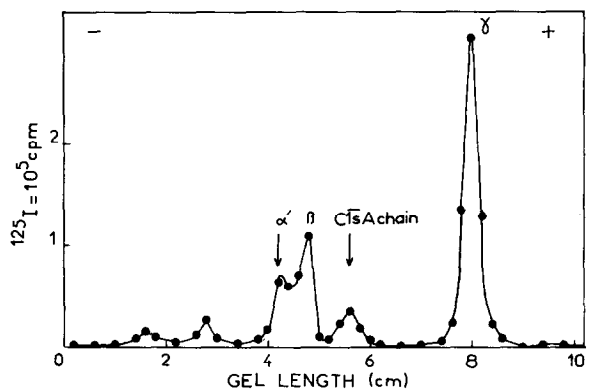

Fig. 4. Distribution of radioactivity in C1s cleaved C4 chains (LPO technique). After cleavage of C4 by C1s and direct iodination of the mixture as described in section 2 a reduced and alkylated aliquot of the incubation medium was loaded onto the gel. Electrophoresis and counting were as described in section 2.

observation, together with the above evidence for a conformational difference between C4 and C4b reflected by the ^{125}I distribution in the chains, is reinforced by the observation that this urea-treated C4 is no longer a substrate for C1s.

3.3. *^{125}I labelling of C4a*

It must be emphasized that by both techniques there is neither evidence of ^{125}I labelling of the N-terminal end of the α chain of C4 nor of the corresponding C4a peptide in C1s-treated C4. However this peptide is easily detected on SDS gels by Coomassie blue staining.

The labelling of C4a was achieved using two different sets of conditions:

(i) The incubation mixture C4 + C1s was treated with 6 M urea after the proteolysis of C4 and iodinated by the Bolton and Hunter technique: a radioactive peptide was detected on SDS–PAGE showing an app. mol. wt 10 700 and 20 500, respectively, after and before reduction, and coinciding with the C4a band revealed by Coomassie blue staining.

(ii) In more recent experiments, C4 was proteolysed by C1s in the presence of 5 mM calcium and directly labelled by the Bolton and Hunter technique without urea treatment. In this case ^{125}I-labelled C4a was detected on SDS–PAGE.

3.4. *Sucrose gradient centrifugation of C4 and C1s-cleaved C4*

When ^{125}I-labelled C4 was centrifuged in a sucrose gradient (fig.5a) a major 7.2 S peak was observed. In contrast (fig.5b) the centrifugation of ^{125}I-labelled C4 cleaved by C1s showed in addition to the 7.2 S peak a 10.4 S peak. There was no evidence for the presence of labelled C4a. There was no increase in 10.4 S proteins when different amounts of C4 were added to C4b which indicated that cleavage of C4 by C1s was necessary for dimer formation. Analysis of these peaks of C1s-cleaved C4 on SDS–PAGE confirmed the formation of dimers of C4b by the presence of high molecular weight bands in the 10.4 S peak (fig.6B) whereas a conventional pattern of C4b was found for the 7.2 S peak (fig.6A). This tendency of C4b to associate spontaneously confirms other observations on the aggregation ability of the α' chain of C4 [2] and may account for the reactivity of C4b.

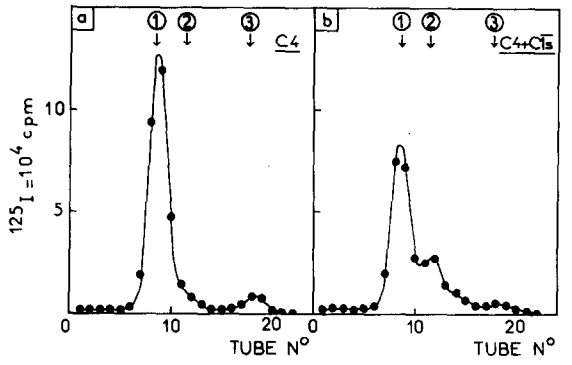

Fig.5. Sucrose gradient ultracentrifugation of C4 and $\overline{C1}$s cleaved C4. 5–20% sucrose gradients in buffer (0.1 M Tris–HCl, 0.15 M NaCl, 5 mM EDTA (pH 7.4) or 0.1 M Tris–HCl, 0.15 M NaCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$ (pH 7.4) were run at 34 000 rev./min at 3°C for 15 h in a TST 54 rotor (Kontron). Standard proteins indicated by the arrows were centrifuged in parallel; the reference values chosen were: (1) alcohol dehydrogenase, 7.6 S; (2) Catalase, 11.4 S; (3) apoferritin, 17.6 S [9]. (a) C4; (b) $\overline{C1}$s-cleaved C4.

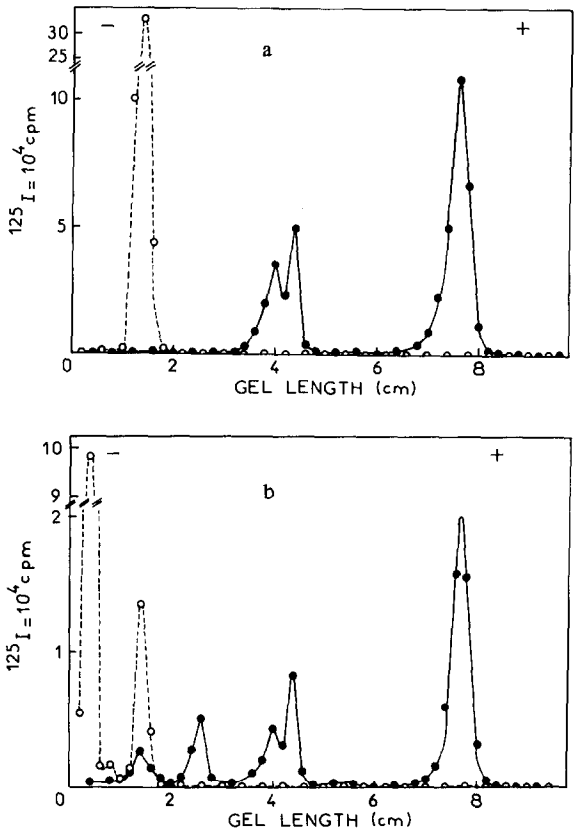

Fig.6. SDS–PAGE analysis of sedimentation peaks of $\overline{C1}$s cleaved C4. Electrophoresis and counting was as described in section 2. (–) Reduced and alkylated samples; (– – –) unreduced samples.
(a) samples corresponding to tube 8 of fig.5b.
(b) samples corresponding to tube 12 of fig.5b.

4. Discussion

It appears necessary to eliminate any trace of C4bp in C4 preparations to be used for biological activity studies, as C4bp in the fluid phase may compete with the membranes for C4b binding [16]. Reverse immune affinity with anti-C4-deficient serum immunoglobulin coupled to Sepharose appears to us the most suitable means of purification, as it combines the specific immune affinity and the less specific interaction of C4 with the activated Sepharose matrix.

A difference of conformation between C4 and C4b from differences in electrophoretic mobility and sedimentation behaviour has been discussed [17]. The different ^{125}I labelling patterns of C4 and C4b reported here point also to a difference in conformation between C4 and C4b. This has been observed only with the LPO technique and it seems that other, more drastic iodination procedures are less suitable. These results can be paralleled with a decrease in the intrinsic fluorescence of C4 after splitting by $\overline{C1}$s. The aggregation ability of C4b confirms the observation [7] and may be an important factor in the orientation of C4b in the complement cascade. From the labelling of urea-treated C4, it can be envisaged that the α, β and γ chain of C4 are likely to assume two different conformations, one corresponding to native C4 and one to a proteolysed C4 conformation. This is confirmed by observations that C4 after freezing at –20°C lacked haemolytic activity; the labelling pattern of this C4 was similar to that of the urea-treated C4. In both cases no proteolysis of the α chain was detectable in C4 by SDS–PAGE.

C4a was found to be difficult to label, which is in agreement with [17] and in contrast to [18]. This cannot be due to a deficit in its tyrosine or lysine contents as compared with the rest of the C4 molecule [17]. It appears that the N-terminal end of the α chain is relatively buried in C4, which may also explain that the separation of C4a from C4b requires

either incubation at acid pH [4,17,18] or treatment with urea and SDS at the concentration used for SDS–PAGE. The accessibility of C4a is increased by urea treatment of the EDTA $C\bar{1}s$-cleaved C4 and also by proteolysis of C4 by $C\bar{1}s$ in the presence of calcium: in the latter case $C\bar{1}s$ dimerizes and the $C\bar{1}s$ dimer–C4 interaction is sufficient to expose or release C4a available for ^{125}I binding. The role of C4a in the C3 convertase formation is presently under investigation.

Acknowledgements

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