STRUCTURAL CHANGES IN C4 PRODUCED BY CLEAVAGE WITH $\overline{C1}$s

A. REBOUL, N. THIELENS, M. B. VILLIERS and M. G. COLOMB

DRF/Biologie Moléculaire et Cellulaire, Centre d'Etudes Nucléaires de Grenoble, 85 X, 38041 – Grenoble Cedex, and *Université Scientifique et Médicale de Grenoble, France*

Received 18 March 1980

1. Introduction

The cleavage of C4 catalysed by $\overline{C1}$s is a necessary step in the formation of the C3 convertase of the classical pathway.

When iodination is catalysed by lactoperoxidase the ^{125}I-labelling patterns of C4 and $\overline{C1}$s-cleaved C4 are different [1]. As only a small percentage of $\overline{C1}$s-cleaved C4 takes part effectively in the formation of the C3 convertase, it appears logical to look for a structural basis of explanation of this selectivity. To analyse the basic structural modifications of the isolated and soluble C4 and $\overline{C1}$s-cleaved C4 we have used two experimental approaches: sulfhydryl group titrations and circular dichroism (CD) spectroscopy. The results confirm [1]; CD spectra exclude any significant contribution of C4a to an ordered organisation of C4.

Using ^{125}I-labelling procedure in [1] and taking into account that calcium enhances the structural modifications induced in C4 by $\overline{C1}$s cleavage we have studied the binding of calcium to C4 and $\overline{C1}$s-cleaved C4. The results confirm that these molecules bind calcium.

2. Materials and methods

C4 and $\overline{C1}$s were purified as in [1,2]. The proteolysis of C4 by $\overline{C1}$s was carried out by incubation for 30 min at 37°C of the two proteins in a 20:1 (w/w) ratio. Detection of C4a was as in [1]. C4a was separated from C4b by 2% trichloroacetic acid precipitation of $\overline{C1}$s-cleaved C4. These conditions were found optimal for the maximal selective recovery of C4a in the supernatant.

^{125}I-labelling of proteins by the method of Bolton and Hunter and with LPO were done as in [1]. ^{125}I and Bolton and Hunter reagent were purchased from Amersham, France.

SDS–PAGE of proteins was as in [3]. Protein estimation was done using the Coomassie blue staining method in [4], using C4 as a reference and assuming an $E^{1\%}_{1\,cm} = 10$ at 280 nm and mol. wt 200 000 for C4. As the C4a UV absorption spectrum does not show a significant peak at 280 nm, C4a concentration was estimated by A_{230} using C4 as a reference.

Calcium-binding was measured as in [5] using a homemade flow dialysis apparatus and ^{45}Ca from CEA, France.

^{14}C-Labelled sulfhydryl reagents (NEM, *p*-CMB, iodoacetamide, DTNB, CPDS) were purchased from CEA, France. Their binding was measured in 0.1 M phosphate, 0.12 M NaCl (pH 6.8) (conductivity, 25 mS at 20°C) for NEM and *p*-CMB, in 0.1 M Tris–HCl, 0.15 NaCl, 5 mM EDTA (pH 8.2) (conductivity, 25 mS at 20°C) for iodoacetamide, DTNB, CPDS. Protein, at ~1 mg/ml, was incubated with the reagent at 37°C for 30 min in a 2:1 (v/v) ratio in 0.15 ml total vol. Then free reagent was separated from protein-bound reagent by chromatography on a G-50 fine Sephadex 1 ml column made in the reaction buffer as in [6]. C4a was retained on the column. Protein was measured in the eluate as in [4] and ^{14}C radioactivity was counted in 10 ml scintillation fluid [7]. For each reagent several measurements were made using different concentrations until a saturation

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetracetic acid; LPO, lactoperoxidase; *p*-CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); CPDS, 6,6'-dithiodinicotinic acid

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

plateau was obtained. For sulfhydryl reagents fixation measurements the C4 used was prepared as in [1] but without using iodoacetamide during the preparation.

The CD spectra were recorded on a Jobin-Yvon Dichrograph III. Iosoandrosterone was used to standardize the dichrograph. Measurements were made at 22°C. Cuvettes of 1 mm and 5 mm pathlength were used between 200–250 nm and 250–400 nm, respectively. Samples were preparated by dialysis against a 0.1 M phosphate buffer (pH 7.4) and the buffer served as reference solvent. CD of C4a was made in water. The final spectra were obtained by substracting the reference scans from the protein solution scans. CD magnitudes are given as mean residue ellipticities (mol. wt_{MR} = 115).

The UV spectra were recorded on a Cary 219 double beam spectrophotometer. Neutron diffusion measurements were made on samples equilibrated in D_2O by dialysis.

3. Results

3.1. *Binding of calcium. Effects of calcium binding on C4 and $\overline{C1}$s-cleaved C4 structures*

Table 1 shows the radioactivity distribution between the three chains of C4 and $\overline{C1}$s-cleaved C4 labelled by the LPO technique in presence of EDTA of calcium. In C4 calcium increases the labelling of the α and β chains as compared with the γ chain. The effect of calcium on γ chain labelling is enhanced by $\overline{C1}$s cleavage. These results indicate that calcium

Table 1
Influence of calcium on the distribution of ^{125}I radioactivity between the three chains of C4 and $\overline{C1}$s-cleaved C4 labelled by LPO technique

	EDTA			Ca^{2+}		
Protein \ Chain	α	β	γ	α	β	γ
C4	28	17	55	37	23	40
$\overline{C1}$s-cleaved C4	7	12	81	19	23	58

Proteins were iodinated as described in (1) in 0.1 M Tris–HCl, 0.15 M NaCl, pH 7.4 containing either 5 mM EDTA or 5 mM $CaCl_2$. Samples were reduced, alkylated and analyzed by SDS–PAGE. Electrophoresis and counting were as described in (1). Results are expressed as the radioactivity measured in each chain divided by the molecular weight of the chain and given as a percentage of the total

Table 2
Parameters of calcium binding on C4 and $\overline{C1}$s-cleaved C4

Parameter	C4	$\overline{C1}$s-cleaved C4
mol calcium bound/mol protein	2–2.3	0.9
K_d (μM)	80	45

Calcium fixation was measured as in section 2, at 25°C, in 20 mM Tris–HCl, 0.145 M NaCl (pH 7.4) at ~1 mg protein/ml. For the titration calcium was varied between 12 and 680 μM

affects the whole structure of C4 and $\overline{C1}$s-cleaved C4 molecules. The increased accessibility of the N-terminal portion of the α chain of C4 and of C4a in $\overline{C1}$s-cleaved C4 that we have observed in presence of calcium [1] is another manifestation of the influence of calcium on the structure of these molecules.

We found that the effects of calcium on C4 and $\overline{C1}$s-cleaved C4 were associated with calcium binding properties of these molecules. The binding parameters that we have calculated from results are reported in table 2.

3.2. *Sulfhydryl reagent binding*

No significant binding of any of the 5 sulfhydryl reagents used was detectable on C4. In contrast C4 could bind sulfhydryl reagents after $\overline{C1}$s cleavage (table 3). Urea (6 M) treatment, which we have shown to induce a C4b-like structure [1], also allowed the fixation of sulfhydryl reagents on C4 (table 3). No influence of calcium on the binding was observed.

The results obtained with CPDS, NEM and DTNB show the fixation of 1 mol sulfhydryl reagent/molecule of protein. *p*-CMB and iodoacetamide indicate a number of titratable sulfhydryl groups of the same order.

Analysis of the competition for binding between

Table 3
Binding of sulfhydryl reagents to $\overline{C1}$s-cleaved C4 and 6 M urea treated C4

Protein \ Reagent	*p*-CMB	iodoacetamide	CPDS	NEM	DTNB
$\overline{C1}$s-cleaved C4	0.4	0.7	1.06	0.95	0.9
Urea-treated C4	n.d.	0.4	0.9	n.d.	n.d.

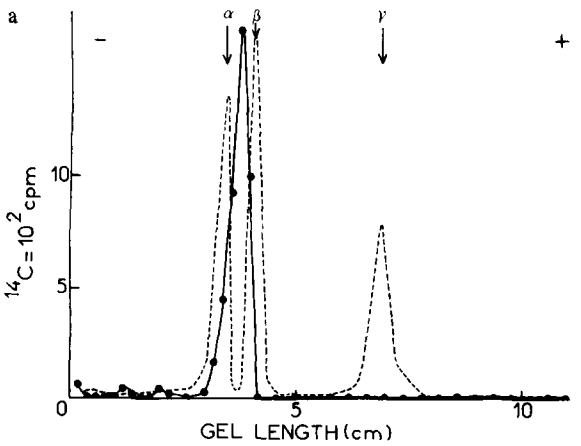
Binding was measured as in section 2. Results are given as no. mol reagent bound/mol protein. n.d., not determined

Table 4
Competition for binding on $\overline{C1}$s-cleaved C4 between iodoacetamide and CPDS or *p*-CMB

CPDS (M)	0.6×10^{-4}	0.6×10^{-4}	0.6×10^{-4}	0.6×10^{-4}	0.6×10^{-4}
Iodoacetamide (M)	0.22×10^{-3}	0.22×10^{-4}	0.22×10^{-5}	0.22×10^{-6}	0
mol CPDS bound/mol $\overline{C1}$s-cleaved C4	0.03	0.28	0.75	0.81	0.98
p-CMB (M)	0.48×10^{-4}	0.48×10^{-4}	0.48×10^{-4}	0.48×10^{-4}	0.48×10^{-4}
Iodoacetamide (M)	0.22×10^{-3}	0.22×10^{-4}	0.22×10^{-5}	0.22×10^{-6}	0
mol *p*-CMP bound/mol $\overline{C1}$s-cleaved C4	0.02	0.28	0.39	0.36	0.4

Binding was measured as in section 2 using unlabelled iodoacetamide and ^{14}C-labelled CPDS or *p*-CMB

iodoacetamide and CPDS or *p*-CMB (table 4) clearly shows that the different reagents bind to the same site and points to the presence of one single free sulfhydryl group per $\overline{C1}$s-cleaved C4 molecule. Labelled samples were analyzed by SDS–PAGE. Two gels were run in parallel: one was stained with Coomassie blue to determine the position of the protein bands, the other was cut in 2 mm slices and counted for ^{14}C radioactivity. Scanning of a stained gel with C4 is given in fig.1a to indicate the position of the α, β and γ chains. For iodo[^{14}C]acetamide (fig.1a) and [^{14}C]NEM (fig.1b) labelled $\overline{C1}$s-cleaved C4 the radioactive peak coincided with the position of the α′ chain, which lies between the α and the β chains. In the case of C4 which has been labelled by iodo[^{14}C]acetamide after 6 M urea treatment (fig.1c) the major part of the radioactivity was found at the level of the α chain.



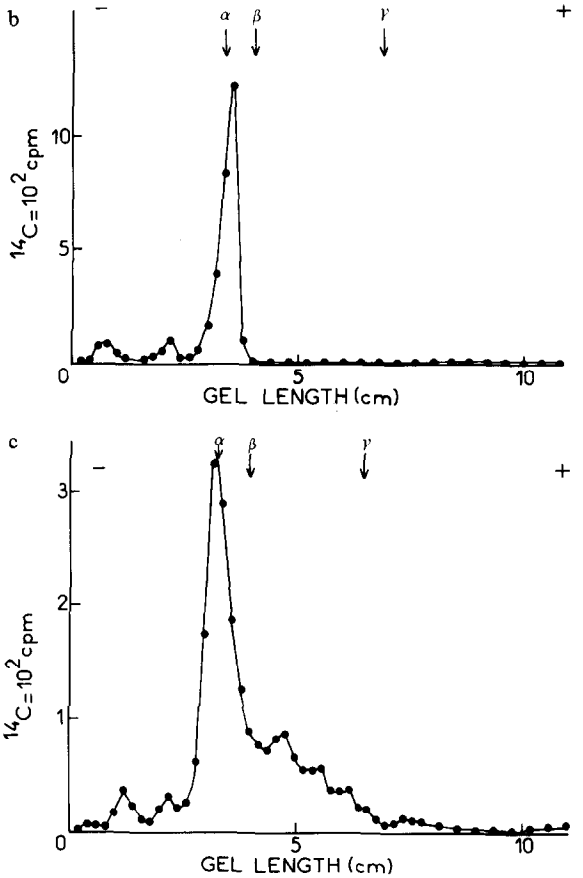


Fig.1. Distribution of radioactivity in $\overline{C1}$s-cleaved C4 and 6 M urea-treated chains after ^{14}C-labelled sulfhydryl reagent fixation. Reduced and alkylated samples. Sulfhydryl reagent fixation and electrophoresis on 5% acrylamide gels were performed as in section 2. Gels were cut in 2 mm slices and counted in 10 ml scintillation fluid [7]. (– – –) Scanning at 550 nm of a reduced and alkylated sample of C4 stained by Coomassie blue after electrophoresis. (a) Iodo[^{14}C]acetamide-labelled $\overline{C1}$s-cleaved C4; (b) [^{14}C]NEM-labelled $\overline{C1}$s-cleaved C4; (c) iodo[^{14}C]acetamide-labelled 6 M urea-treated C4.

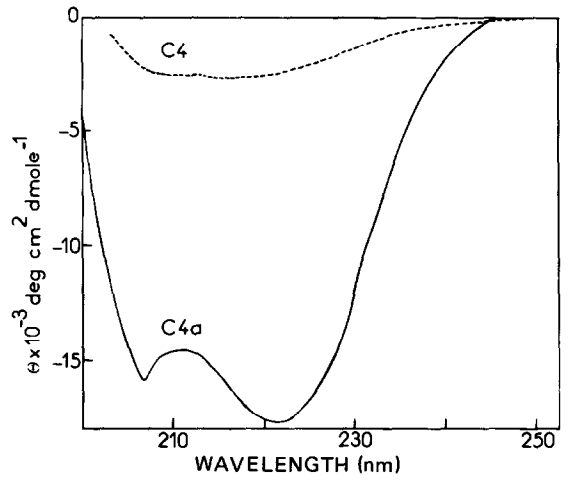


Fig.2. CD spectra of C4 and C4a. The measurements were made as in section 2.

3.3. *CD measurements*

Fig.2 gives the CD spectra of C4 and C4a measured between 200 and 250 nm. The amplitude of the signals at higher wavelengths was too small to be taken into account. The CD spectrum of C4a is characterized by two extrema around 207 and 222 nm, respectively. Based on an empirical equation [8] a 41% α helix content was calculated from ellipticity at 207 nm. In contrast CD spectrum of C4 indicates a very small content of ordered conformation. $C\bar{1}s$-cleaved C4 shows the same spectrum as C4, which is not modified after the selective removal of C4a by chromatography on G 50 Sephadex as in section 2.

4. Discussion

As reported [1] C4 proteolysis by $C\bar{1}s$ leads to structural changes in C4 detectable by peripheral iodination. This paper describes an influence of calcium on C4 and $C\bar{1}s$-cleaved C4 structure. This influence is detected on isolated molecules able to bind calcium. Further experiments are necessary to assess a direct role of calcium on C4 or C4b reactivity towards $C\bar{1}$, C4bp or C3b in a fluid or particulate phase, respectively.

In the series of structural events accompanying the proteolysis of C4 in C4b and C4a it appears that a free sulfhydryl group is available for titration in C4b; the same group is also titratable in the C4b-like conformation of C4, a finding analogous to similar observations on C3b [9]. As in the case of C3b the free sulfhydryl is located on the α' fragment of the α chain and seems responsible for a partial dimerization of C4b [1,10,11].

The transient availability of a single sulfhydryl group not titratable in C4 could be accounted for by a masking of this group inside C4 under a thioester linkage. This postulate is in keeping with the observations of an ester link between membrane and C4 [12] due to the acyl group released in C4b; in this view the free sulfhydryl would be only passive evidence for the transient reactivity of native C4b. It remains to establish whether this group could play a role in the degradation of C4b by C4bp and C3b INA.

As separation of C4a from C4b is difficult under physiological conditions and requires an acidic medium [13–16] one may question the role of this apparently highly organised peptide on the C4b moiety. From the CD spectra, in spite of the trichloroacetic acid treatment, C4a appears to contain a comparable amount of α helix (40%) to that of C3a. But, in contrast with [17], it does not seem that C4a plays a net role of nucleation in C4 structure as described for C3a in C3: the CD spectra of C4 and C4a-deprived C4b appear very similar. It appears thus that C4 exhibits a higher degree of independence than C3, probably due to its 3 chains.

It is evident that most of the changes in C4 upon proteolysis are peripheral although they lead to a clear-cut change in reactivity. However a preliminary observation using neutron diffusion seems to indicate a change in the giration radius of C4 upon proteolysis into C4b from 37.5–41.7 Å; this may reflect that C4 cleavage also induces significant differences in the overall structure.

Acknowledgements

This work was supported partly by the CNRS (ERA no. 695), the INSERM (FRA no 48), the DGRST (contrat no. 78.7.0344) and the Fondation pour la Recherche Médicale. We thank Dr J.-M. Freyssinet (Grenoble) for neutron diffusion measurements and Professor R. H. Painter (Toronto) for help in preparing the manuscript.

References

[1] Reboul, A., Thielens, N., Villiers, M.-B. and Colomb, M. G. (1979) FEBS Lett. 103, 156–161.
[2] Arlaud, G. J., Sim, R. B., Duplaa, A.-M. and Colomb, M. G. (1979) Immunol. 16, 445–450.
[3] Arlaud, G. J., Reboul, A., Meyer, C. and Colomb, M. G. (1977) Biochim. Biophys. Acta 485, 215–226.
[4] Bradford, M. M. (1976) Analyt. Biochem. 77, 152–157.
[5] Colowick, S. P. and Wowack, F. C. (1969) J. Biol. Chem. 244, 774–777.
[6] Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899.
[7] Patterson, M. S. and Greene, R. C. (1965) Anal. Chem. 37, 854–857.
[8] Greenfield, N. and Fasman, G. D. (1969) Biochemistry 8, 4108–4116.
[9] Janatova, J., Prahl, J. W. and Tack, B. F. (1979) 11th Int. Congr. Biochem., Toronto, abst. 08-1-R81.
[10] Bolotin, C., Morris, S., Tack, B. and Prahl, J. (1977) Biochemistry 16, 2008–2015.
[11] Porter, R. R. (1980) personal communication.
[12] Law, S. K., Lichtenberg, N. A. and Levine, R. P. (1979) 8th Int. Complement workshop, Miami, 1979, abst.
[13] Budzko, D. B. and Müller-Eberhard, H. J. (1970) Immunochemistry 7, 227–234.
[14] Patrick, R. A., Taubman, S. B. and Lepow, I. H. (1979) Immunochemistry 7, 217–225.
[15] Gorski, J. P., Hugli, T. E. and Müller-Eberhard, H. J. (1979) Proc. Natl. Acad. Sci. USA 76, 5299–5302.
[16] Booth, N. A., Campbell, R. D., Smith, M. A. and Fothergill, J. E. (1979) Biochem. J. 183, 573–578.
[17] Hugli, T. E., Morgan, W. T. and Müller-Eberhard, H. J. (1975) J. Biol. Chem. 250, 1479–1483.