PURIFICATION AND CHARACTERIZATION OF C4-BINDING PROTEIN FROM HUMAN SERUM

Marie-Bernadette VILLIERS, Angeline REBOUL, Nicole M. THIELENS and Maurice G. COLOMB
Centre d'Etudes Nucléaires de Grenoble, DRF, Laboratoire de Biologie Moléculaire et Cellulaire, 85 X, 38041 Grenoble cedex and *Université Scientifique et Médicale de Grenoble, ERA CNRS no. 695, Unité INSERM no. 238, Grenoble, France*

Received 6 July 1981

1. Introduction

Activation of the classical pathway of complement proceeds through the transient formation of a C3 convertase, a bimolecular complex due to an interaction of fragments C4b and C2a from components C4 and C2 [1,2]. This C3 convertase is highly labile and retains only shortly its capacity for proteolyzing C3. In [3,4] control factors were described which could destabilize the C3 convertase. A protein has been purified from human serum, which is able to bind to native C4b and termed C4-binding protein (C4bp); this protein is also able to inhibit the formation of the C3 convertase and to accelerate the decay of its catalytic activity [5,6]. This protein is probably similar to a macromolecular cofactor described in [7] and detailed in [8,9].

C4bp has been purified from mouse, guinea pig and human plasma [5,10,11]. Most of the techniques described are complex and time-consuming. This paper describes a simplified method for the purification of C4bp from human plasma, to give the major characteristics of the purified protein and to demonstrate one of its interactions with native C4b.

2. Materials and methods

Human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble). Serum was prepared as in [12].

Yeast alcohol dehydrogenase, horse spleen apoferritin, beef liver catalase were obtained from Calbiochem, di-isopropylphosphorofluoridate was purchased from Sigma and ampholine were from Pharmacia. Sheep erythrocytes were obtained from Bio-Merieux. Hemolysin and antisera to human C3, C4 and ceruloplasmin were from Behring. Antisera to human serum was purchased from ICL Scientific. Antisera to human IgM, $C\bar{1}$ Inh, C1q, $C\bar{1}r$, $C\bar{1}s$, C2, C4bp were obtained in our laboratory. Antisera to human β1H, 11βs (C4bp) and IgM were generous gifts from Dr Fountaine (Rouen), Dr Chapuis (Lausanne) and Dr Nussenzweig (New York), respectively. Other chemicals were of analytical grade.

$C\bar{1}s$ and C4 were purified as in [13,14]. Purified proteins were estimated either according to [15] or from their A_{280}, using $E^{1\%}_{1\,cm} = 9.5$ for $C\bar{1}s$ [16] and $E^{1\%}_{1\,cm} = 10$ for C4 [17]. Other A_{280} were determined by weighing purified proteins lyophylised from solution in 10 mM *N*-ethylmorpholine (pH 9.7). C4bp purification was evaluated from an arbitrary A_{280} for total proteins of $E^{1\%}_{1\,cm} = 10.0$. C4bp was selectively estimated by radial immunodiffusion [18] using 1% agarose and 1% anti-C4bp rabbit serum. Double immunodiffusion in 1% agarose according to [19] was used for identification of the proteins.

Molecular mass was taken as 200 000 for C4 and for its chains as α = 87 500, α' = 78 000, β = 70 000, γ = 31 000, respectively [14].

Sucrose gradient ultracentrifugation was as in [20]; runs were at 32 000 rev./min, at 4°C, with a TST 54 rotor in a Kontron TGA 50 ultracentrifuge; horse spleen apoferritin (17.6 S), beef liver catalase (11.4 S) and yeast alcohol dehydrogenase (7.6 S) were used as

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetraacetic acid; DFP, di-isopropylphosphorofluoridate; PEG, polyethylene glycol; STI, soybean trypsin inhibitor; EA, erythrocyte-bearing specific antibodies; IgM, immunoglobulins M

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

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

markers and proteins were estimated in each fraction as in [15].

For isoelectric focusing, proteins were directly applied on polyacrylamide gels containing ampholine (pH 3.5–10) as in [21]; focusing was carried out for 13 h at 25°C. Crossed immunoelectrophoresis was as in [22] and the 50% haemolysis time assay was according to [23].

Samples reduced with dithiothreitol or unreduced were prepared for SDS–PAGE as in [13]. Gels containing 4.5% (reduced samples) or 3.5% (unreduced samples) acrylamide were prepared after [24] and run at an intensity of 5 mA/gel during 5 h. Proteins were stained with Coomassie blue and sugars were visualized according to [25]. α, β and γ chains of reduced C4 and a mixture of multimeric IgG [5] were used as reference for molecular mass estimation of reduced and unreduced proteins, respectively.

Antibodies to C4bp contaminants were raised in rabbits according to [26] with a fraction of contaminants prepared from the DEAE–Sephadex A 25 eluate (chromatography step described in the C4bp purification) as follows: after concentration by ultrafiltration on PM 10, the eluate was applied to a column of Sepharose-C4b equilibrated in 10 mM Tris–HCl, 25 mM NaCl, 5 mM EDTA (pH 7.4) at 4°C. The fraction not retained by the column and corresponding to C4bp contaminants was concentrated by ultrafiltration on PM 10.

Other antisera were raised in rabbit also according to [26]. Total immunoglobulins were purified from pooled rabbit serum by sodium sulfate precipitation and proteins were coupled to Sepharose 4B as in [27].

3. Results

3.1. *Purification of C4bp*

Unless otherwise stated, all operations were done at 4°C.

3.1.1. Treatment of plasma by inhibitors

Frozen citrated plasma (500 ml) was thawed, centrifuged at 19 000 × *g* for 15 min and incubated for 40 min at 20°C with 11 mM EDTA, 5 mM DFP, 3 mM iodoacetamide and 25 μg STI/ml.

3.1.2. PEG extract

PEG 4000 15% (w/v) in 50 mM phosphate buffer (pH 7.55) containing 50 mM NaCl, 15 mM EDTA and 5 mM DFP was added drop-wise to the plasma under continuous stirring to a final concentration of 5%; after 20 min the medium was centrifuged for 15 min at 9000 × *g* and the resulting pellet redissolved in 92 ml 10 mM Tris–HCl buffer (pH 8.6) with 75 mM NaCl, 5 mM EDTA, 5 mM DFP for an incubation of 1 h under stirring.

3.1.3. DEAE-Sephadex A 25

After centrifugation of the PEG extract at 27 000 × *g* for 15 min, the supernatant was applied to a column of DEAE-Sephadex A 25 (3 × 25 cm) equilibrated in 10 mM Tris–HCl buffer (pH 8.6) with 75 mM NaCl, 5 mM EDTA, at a flow rate of 50 ml/h. The column was washed with the same buffer until the eluate A_{280} was <0.05. Protein elution was carried out with a linear gradient of 75–300 mM NaCl in 5 mM EDTA, 10 mM Tris HCl (pH 8.6) (total vol. 700 ml), 4 ml fractions were collected. C4bp and C4 were detected in the eluate by double immunodiffusion and fractions containing C4bp free of C4 were pooled, corresponding to a conductivity of 6.7 mS measured at 0°C.

3.1.4. Affinity chromatography on Sepharose-bound anticontaminants

The DEAE-Sephadex A 25 pool contained several proteins. The major contaminants, identified as IgM, were not separated from C4bp by molecular sieve chromatography (even at high ionic strength, acid pH or in 4 M urea), ion-exchange chromatography, Sepharose–con A or Sepharose–heparin chromatography. The only successful method was a reverse immune-affinity chromatography on a column of Sepharose 4B-linked antibodies to C4bp contaminants.

After addition of DFP (5 mM final con.), the DEAE-Sephadex pool was dialyzed against 20 mM NaH_2PO_4, 0.5 M NaCl, 0.1 M glycine, 5 mM DFP (pH 9.0) and applied at a flow rate of 20 ml/h to a column of Sepharose 4B-bound antibodies to C4bp contaminants equilibrated in the same buffer. Fractions of 4 ml were collected. This step led to the specific fixation of a majority of contaminants by the immune-affinity antibodies and the non-specific fixation of C4bp on the activated Sepharose. Only one protein perfused with an app. $M_r \sim 70\,000$ determined by SDS–PAGE in reduced and unreduced conditions; this unidentified protein was called '70 k protein'. The elution was stopped when the A_{280} eluate was <0.02 and the column was then put at 20°C for 2 h before elution

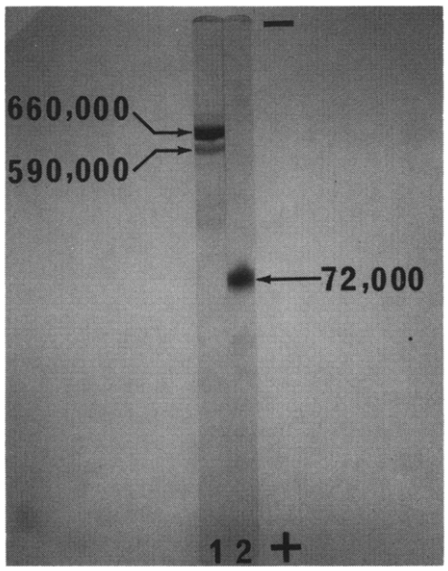

Fig.1. SDS–PAGE analysis of C4bp. Electrophoresis was done as in section 2: 15 μg protein was applied to each gel; (1) unreduced C4bp on 3.5% acrylamide gel; (2) reduced and alkylated C4bp on 4.5% acrylamide gel.

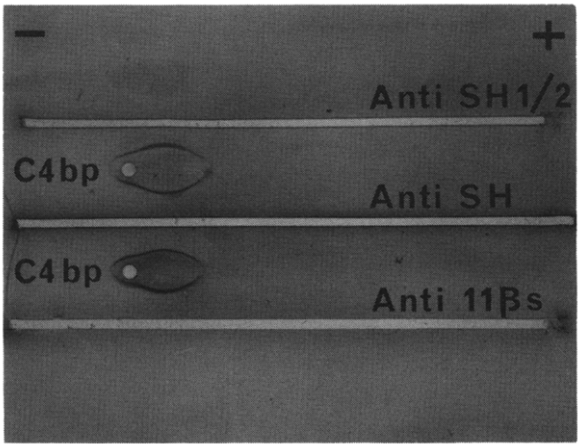

Fig.2. Immunoelectrophoresis of purified C4bp. Diffusion against anti human serum diluted to 1/2 (anti SH 1/2) or undiluted (anti SH) and against anti-human 11βs (anti-11βs). Immunoelectrophoresis was done as in section 2.

of C4bp by the same buffer. After concentration of the eluate by ultrafiltration on PM 10, ~10 mg C4bp were obtained: its purity was ⩾98%, from evaluation by SDS–PAGE. Controls by double immunodiffusion in agarose were negative with the following antisera to human IgM, β1H, $C\bar{1}$ Inh, C1q, $C\bar{1}$r, $C\bar{1}$s, C2, C3, C4. Immunoelectrophoresis in agarose showed only one precipitation line with undiluted antisera to human serum and a second fine line with the same antisera diluted to 1/2 (fig.2).

A typical purification run is presented in table 1.

3.2. *Characteristics of purified C4bp*

C4bp was found to have app. M_r ~ 800 000 as determined by gel filtration; the purified protein exhibited two bands on SDS–PAGE under non-reducing conditions (fig.1) of app. M_r 660 000 and 590 000. The proportion of the two bands, calculated from gel scanning, was 83% for the high form and 17% for the low form; except for the molecular mass, no difference between the two forms of C4bp could be observed: their ratio was not modified by heating (14 h at 37°C) or freezing and thawing or during the purification. Both proteins gave a positive periodic acid–Schiff reaction, with an intensity corresponding to a similar proportion of carbohydrates. After reduction, a single band of app. M_r 72 000 was observed. These results

Table 1
C4bp Purification

	Volume (ml)	Protein[a] (mg/ml)	Total protein (mg)	C4bp[a] (mg/ml)	Total C4bp (mg)	Yield (C4bp %)	Purification (–factor)
Citrated plasma	500.0	58.00	29 000.0	0.226	113.0	100.0	1.0
PEG-precipitate	98.0	29.20	2862.0	0.850	83.3	73.7	7.5
DEAE-eluate	120.0	0.57	68.4	0.246	29.5	26.1	110.7
Affinity column eluate after concentration	10.8	0.87	9.4	0.850	9.2	8.1	251.2

[a] Total protein and C4bp were determined as in section 2

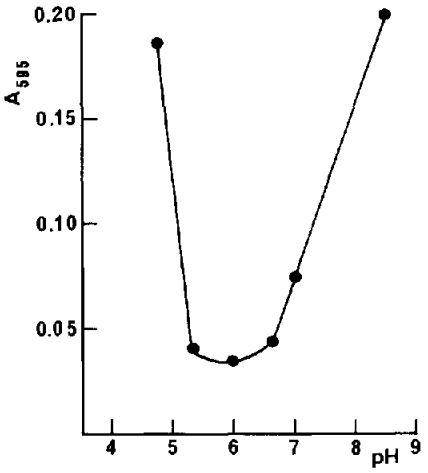

Fig.3. Solubility of C4bp at different pH-values: 100 μl 50 mM Tris–HCl, 0.1 M glycine buffer, adjusted at different pH-values were added to 50 μl C4bp (50 μg) in 10 mM Tris–HCl, 25 mM NaCl and 5 mM EDTA (pH 7.4). The resulting pH-value was measured on each mixture; conductivity was 6 mS at 0°C. After incubation of 2 h at 20°C followed by centrifugation (6 min at 9000 × g), proteins were estimated in the supernatant according to [15].

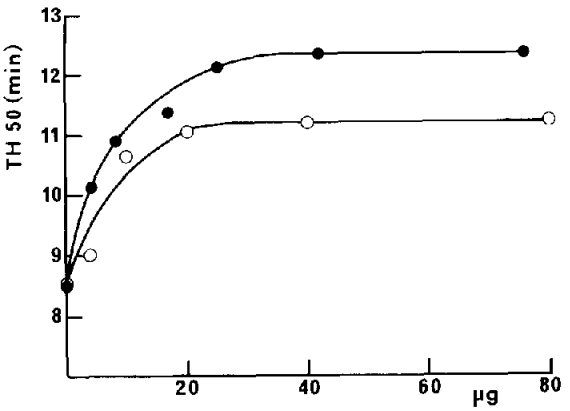

Fig.4. 50% haemolysis time assay. Sheep erythrocytes (10^9 cells/ml) in 2.5% glucose, 5 mM veronal, 142 mM NaCl, 0.05% gelatine, 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (pH 7.5) were sensitized with rabbit antiserum to sheep red cells: 3 ml EA diluted to 4 × 10^6 cells/ml were pre-incubated 5 min in a spectrophotometer cell thermostatted at 30°C; 20 μl serum were injected in the cell suspension, immediately followed by addition of 100 μl C4bp or 70 k protein containing from 0 80 μg protein in 20 mM NaH_2PO_4, 0.5 M NaCl, 0.1 M glycine (pH 9.0). After rapid mixing, the absorbance was monitored at 660 nm.

suggest that C4bp is a polymer of identical polypeptide chains linked by several disulfide bonds.

Sedimentation coefficient measured by sucrose density gradient ultracentrifugation was 11.2 S for purified C4bp and was not modified by freezing and thawing.

Two isoelectric points were observed by electrofocusing of C4bp on polyacrylamide gels: pH 5.6 and pH 5.9. Although the pH 5.9 form was predominant, it is not yet possible to assimilate the two isoelectric values to the high and the low fraction of C4bp described above. These data were confirmed by two other experiments:

- The solubility of C4bp at different pH (fig.3) showed a minimum at pH 5.9, which reflected a probable isoelectric precipitation.
- Migration of purified C4bp on immunoelectrophoresis was anodal at pH 8.7 and cathodal at pH 5.2, there was no appreciable migration at pH 6.0.

A_{280} of $E^{1\%}_{1\,cm} = 9.3$ and $E^{1\%}_{1\,cm} = 5.1$ were obtained as in section 2 for C4bp and the 70 k protein, respectively, in favour of a sharp difference of structure between the two proteins. By radial immunodiffusion and using the above A_{280}, a level of ~250 mg/l was calculated for human plasma C4bp.

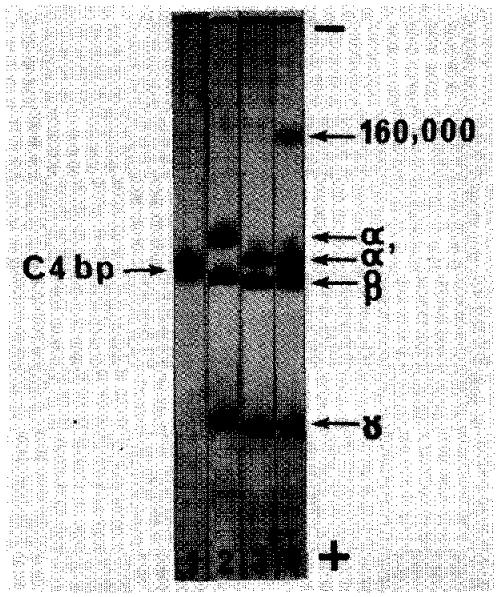

Fig.5. SDS–PAGE analysis, in reduced conditions, of a C4b-C4bp link: 7.7 μg C4bp were mixed with 82 μg C4 and 4 μg $C\bar{1}s$ in a total volume of 200 μl of 10 mM Tris–HCl, 0.15 M NaCl, 5 mM EDTA (pH 7.4). After a 30 min incubation at 37°C, an aliquot of 30 μl of the sample was reduced, alkylated and applied to a 4.5% acrylamide gel. Electrophoresis was done as in section 2. Three controls were run in parallel including: (1) C4bp + $C\bar{1}s$; (2) C4 alone; (3) C4 + $C\bar{1}s$; (4) C4bp + C4 + $C\bar{1}s$.

3.3. *Biological activity of C4bp*

C4bp biological activity was evaluated in a haemolytic assay (50% haemolysis time (TH_{50})). As shown in fig.4, EA mixed with a constant amount of serum were incubated with increasing amounts of C4bp: an increase of the TH_{50} from 8–12 min was observed, with a plateau for higher concentrations of C4bp.

A parallel assay, substituting the 70 k protein for C4bp, gave a similar decrease in haemolytic activity with a maximal TH_{50}-value plateauing at 11 min in this case.

Further experiments with purified proteins confirmed that C4bp is able to react with native C4b; SDS–PAGE analysis under reducing conditions of a mixture of C4bp, C4 and $\bar{C}1s$ showed a new protein band of app. M_r 160 000, formed by the association of one unit of C4bp (72 000) and the α'-chain of C4b (78 000) (fig.5).

4. Discussion

The purification of C4bp raises several problems due to its affinity for IgM [9,28,29] and to an apparent polymorphism represented by a high and a low M_r form [5,28]. We have consistently observed an association of a fraction of C4bp and IgM; the bimolecular complex is very coherent (section 3). IgM contaminants could be eliminated only in the last stage of reverse immune affinity. Purified C4bp shows two different forms of app. M_r 660 000 and 590 000, confirming the other results described. From the reported data one may consider them as polymers differing only by one monomer, although recent data on the binding of a vitamin K-dependent protein S to C4bp suggest that C4bp in complex with protein S is identical to C4bp-high and that C4bp-low corresponds to uncomplexed C4bp [30]. As no biological function has yet been assigned to protein S which behaves on SDS–polyacrylamide gel as a 70 k protein, more work is needed to clarify this point. An inhibitory role is detected by the haemolytic assay for the 70 k protein separated at the last stage of purification of C4bp; this result may question its analogy with protein S. Furthermore the absence of any precipitin line on double immunodiffusion analysis between anti-C4bp and the 70 k protein as well as the difference between their A_{280} leave this question open.

C4bp is able to bind native C4b to form a covalent-like association which in some aspects may recall the ester or amide bond involved in the binding of C4b to membranes or immune complexes. This binding may account for one of the reactivities of C4bp, controlling or preventing the formation of the $C\overline{42}$ convertase upon complement activation [5,31].

Acknowledgements

This work was supported partly by the Délégation Générale à la Recherche Scientifique et Technique (contrat 80.7.0297) and the Fondation pour la Recherche Médicale. We thank J. Dodu for the purification of IgM.

References

[1] Müller-Eberhard, H. J. (1979) in: Modulation of Protein Function (Atkinson, D. E. and Fox, C. F. eds) vol. 13, pp. 219–232, Academic Press, New York and London.
[2] Porter, R. R. (1980) Proc. Roy. Soc. Lond. B 210, 477–498.
[3] Opferkuch, W., Loos, M. and Borsos, T. (1971) J. Immunol. 107, 313 abstr.
[4] Opferkuch, W., Loos, M. and Borsos, T. (1972) Fed. Proc. FASEB 31, 740 abstr.
[5] Scharfstein, J., Ferreira, A., Gigli, I. and Nussenzweig, V. (1978) J. Exp. Med. 148, 207–222.
[6] Daha, M. R. and Van Es, L. A. (1980) J. Immunol. 125, 2051–2054.
[7] Nagasawa, S. and Stroud, R. M. (1977) Immunochemistry 14, 749–756.
[8] Nagasawa, S., Ichihara, C. and Stroud, R. M. (1980) J. Immunol. 125, 578–582.
[9] Nagasawa, S. and Stroud, R. M. (1980) Mol. Immunol. 17, 1365–1372.
[10] Ferreira, A., Takahashi, M. and Nussenzweig, V. (1977) J. Exp. Med. 146, 1001–1018.
[11] Burge, J., Nicholson-Weller, A. and Austen, K. F. (1981) J. Immunol. 126, 232–235.
[12] Arlaud, G. J., Reboul, A., Meyer, C. M. and Colomb, M. G. (1977) Biochim. Biophys. Acta 485, 215–226.
[13] Arlaud, G. J., Sim, R. B., Duplaa, A. M. and Colomb, M. G. (1979) Mol. Immunol. 16, 445–450.
[14] Reboul, A., Thielens, N., Villiers, M. B. and Colomb, M. G. (1979) FEBS Lett. 103, 156–161.
[15] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[16] Sim, R. B., Porter, R. R., Reid, K. B. M. and Gigli, I. (1977) Biochem. J. 163, 219–227.
[17] Reboul, A., Thielens, N., Villiers, M. G. and Colomb, M. G. (1980) FEBS Lett. 115, 118–122.
[18] Mancini, G., Carbonara, A. O. and Heremans, J. F. (1965) Immunochemistry 2, 235–254.
[19] Ouchterlony, Ö. and Nillson, L. Å. (1973) in: Handbook of Experimental Immunology (Weir, D. M. ed) vol. 1, pp. 19.1–19.39, Blackwell, Oxford.

[20] Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379.
[21] John, H. A. (1980) Biochem. Biophys. Res. Commun. 92, 1223–1230.
[22] Scheidegger, J. J. (1955) Int. Arch. Allergy Appl. Immun. 7, 103.
[23] Lachmann, P. J. and Hobart, M. J. (1979) in: Handbook of Experimental Immunology (Weir, D. M. ed) vol. 1, pp. 5A.13, Blackwell, Oxford.
[24] Fairbanks, G., Stech, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[25] Kapitany, R. A. and Zebrowski, E. J. (1973) Anal. Biochem. 56, 361–369.
[26] Porter, R. R. (1955) Biochem. J. 59, 405–410.
[27] Arlaud, G. J., Reboul, A., Meyer, C. and Colomb, M. G. (1977) Biochim. Biophys. Acta 485, 215–226.
[28] Fujita, T. and Nussenzweig, V. (1979) J. Exp. Med. 150, 267–276.
[29] Kaidoh, T., Natsuume-Sakai, S. and Takahashi, M. (1981) J. Immunol. 126, 463–467.
[30] Dahlbäck, B. and Stenflo, J. (1981) Proc. Natl. Acad. Sci. USA 78, 2512–2516.
[31] Haydey, R. P., Patarroyo de Rojas, M. and Gigli, I. (1980) J. Invest. Dermatol. 74, 328–332.