Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b

(protein-protein interaction/protein conformation/C3 convertase/complement regulation/blood coagulation)

BJÖRN DAHLBÄCK, CRAIG A. SMITH, AND HANS J. MÜLLER-EBERHARD

Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Contributed by Hans J. Müller-Eberhard, March 7, 1983

ABSTRACT C4b-binding protein (C4bp) participates in the regulation of the C3 convertase of the classical pathway of complement. By binding to C4b, which is one of the structural subunits of this enzyme, C4bp accelerates the decay-dissociation of the enzyme and renders C4b susceptible to degradation by factor I (C3b inactivator). C4bp is a high molecular weight plasma protein ($M_r = 570,000$) composed of apparently identical subunits ($M_r = 70,000$) linked by disulfide bonds. In plasma and in purified form C4bp also forms a bimolecular complex ($K_d = 0.9 \times 10^{-7}$ M) with protein S, a recently identified vitamin K-dependent plasma protein. The binding sites on C4bp for protein S and C4b are distinct and noncompetitive and protein S does not influence the function of C4bp as a regulator of the C3 convertase. C4bp, C4b, and protein S were visualized by electron microscopy by negative staining. C4bp was found to have an unusual spider-like structure. It is composed of seven thin (30 Å), elongated (330 Å), and flexible subunits that are linked to a small central body. Protein S exhibited two globular domains of equal size with a center-to-center distance of ≈ 50 Å. Protein S was found to bind to the C4bp through only one of its domains by attaching to a short subunit that is distinct from the other seven subunits. C4b imaged as an irregular, relatively compact molecule. It was found to interact with the peripheral ends of the elongated subunits, suggesting seven C4b-binding sites per molecule of C4bp.

C4b-binding protein (C4bp) is a regulator of the classical complement pathway (1–4). It is required as cofactor for the degradation of fluid phase C4b by the enzyme factor I (C3b inactivator) (1–4). It accelerates the decay of the C3 convertase of the classical pathway by dissociating C2a from the C4b,2a complex (3). It has been shown that in mice the gene for C4bp is linked to the major histocompatibility complex (5).

C4bp is a large protein with a M_r of $\approx 570,000$, as estimated by sedimentation equilibrium centrifugation (6). The protein has a high frictional ratio, 2.1, indicating that the molecule is highly asymmetrical (6). It was suggested that the protein is composed of six to eight subunits possessing an apparent M_r of 70,000 and an identical amino-terminal sequence (7, 8). The subunits of the human protein are linked by disulfide bridges. C4bp is multivalent for C4b and it has been suggested that at least five or six molecules of C4b can bind to a molecule of C4bp (1, 9).

Recently it was demonstrated that C4bp in human plasma forms a complex with a vitamin K-dependent protein called protein S (7). In contrast to the vitamin K-dependent coagulation proteins, it has not yet been possible to demonstrate that protein S is a zymogen of a serine protease (10–13). No definitive function in the coagulation system has been assigned to protein S, although it has been reported to enhance the deg-

radation of coagulation factor Va by activated protein C (14, 15). Studies on the protein S–C4bp interaction using purified proteins have shown the formation of a 1:1 stoichiometric complex with a K_d of 0.9×10^{-7} M (6). The binding site for protein S on the C4bp molecule was found to be distinct from the binding sites for C4b and protein S did not influence the function of C4bp as a cofactor for factor I in cleaving C4b (6, 9). These studies also indicated that C4bp, in addition to the six to eight identical subunits, contains a different disulfide-linked subunit bearing the protein S-binding site (6, 9).

The high molecular weight of C4bp, its subunit composition, hydrodynamic properties, and its ability to form complexes with C4b and protein S prompted its examination by electron microscopy. This technique has proved useful in the elucidation of protein structure and of protein-protein interactions (e.g., see refs. 16–22). We now report that C4bp has a spider-like structure, that C4b binds to the peripheral ends of the seven subunits resembling tentacles, and that protein S has a two-domain substructure and binds to an eighth, distinct subunit of the C4bp.

MATERIALS AND METHODS

Protein Purification. Protein S and C4bp were purified from human plasma as described (6, 13). The purified protein S was a gift from Daryl Fair. Purified human C4 (23) was converted to C4b by the isolated activated form of C1s, C1s, supplied by Robert Ziccardi. The C4 (10 mg/ml) was incubated with C1s (20 μ g/ml) in 50 mM Tris-HCl/0.15 M NaCl, pH 7.5, containing 2 mM EDTA for 2 hr at 37°C. At the high substrate concentration used, nascent C4b forms covalent dimers and polymers which were separated from monomeric C4b by gel filtration chromatography on a column (1 $\times$ 80 cm) of S-300 (Pharmacia) in 0.1 M $\text{NH}_4\text{OAc}/0.05$ M NH_4HCO_3 , pH 7.35. Approximately 30% of the applied material was found to be C4b polymers.

Electrophoretic and Immunochemical Techniques. Gradient (2.5–10% or 7.5–12.5%) polyacrylamide slab gel electrophoresis in the presence of NaDodSO₄ was performed as described (24) by using the buffer system of Laemmli (25). The gels were stained with Coomassie blue and scanned with a soft laser scanning densitometer (Biomed). Double-immunodiffusion was performed as described by Ouchterlony (26). The antisera to C4bp and protein S were prepared as described (6, 13).

Electron Microscopy. C4bp (10 μ g/ml), protein S (3 μ g/ml), or C4b (5 μ g/ml) in 0.1 M $\text{NH}_4\text{OAc}/0.05$ M NH_4HCO_3 , pH 7.35, were adsorbed to thin carbon films as described (27) and then were negatively stained with 1.5% uranyl formate (Eastman Kodak) by using the pleated sheet technique (19, 28). To visualize the complexes of C4bp and C4b, the following pro-

cedure was used: C4bp (0.1 mg/ml) was incubated with C4b (0.1 mg/ml) (molar ratio of C4bp to C4b, $\approx 1:3$) in 0.1 M $\text{NH}_4\text{OAc}/0.05$ M NH_4HCO_3 , pH 7.35, at 37°C for 1 hr and then was diluted 1:20–1:40 either in the same buffer or with buffer diluted 1:2 or 1:5 in distilled H_2O . Within 5 min after dilution the proteins were adsorbed to the carbon film and were negatively stained as described above. The photographs were taken at a primary magnification of 60,000 $\times$ in a Hitachi 12A transmission electron microscope, operating at 75 kV with a 200- μm C2 aperture and a 50- μm objective aperture.

RESULTS

Characterization of the C4b-Binding Protein. A complete separation of protein S from C4bp is not achieved by the purification procedure used, even after gel filtration in 1 M NaCl (6). By measuring the content of γ -carboxyglutamic acid residues in different C4bp preparations, it was estimated that the purified C4bp contained ≈ 3 –5% protein S, indicating one molecule of protein S per every third to fifth C4bp molecule (6). NaDodSO₄/polyacrylamide gel electrophoresis of the C4bp used in this study revealed a protein band (indicated by an arrow in Fig. 1A) that migrated like isolated protein S. This protein band, which was only observed when relatively large amounts of C4bp were examined, represented 2–5% of the material analyzed, as judged by densitometric scanning of the stained gels. The presence of protein S–C4bp complexes in the C4bp preparation was demonstrated by immunochemical techniques. Fig. 1B shows that the purified C4bp was precipitated by a monospecific antiserum to protein S and that this precipitin line fused with that produced by an antiserum to C4bp. The spurring of the latter precipitin line indicates the presence of C4bp molecules that are devoid of protein S. A pattern of immunological identity was obtained when purified C4bp and protein S were analyzed with an antiserum to protein S (Fig. 1C).

Visualization of C4b-Binding Protein. The purified C4bp was examined in the electron microscope and a typical field view

FIG. 1. Electrophoretic and immunochemical analysis of purified C4bp. (A) NaDodSO₄/polyacrylamide gel electrophoresis of C4bp under nonreducing (lane 1) or reducing (lane 2) conditions. The gel in lane 1 was a 2.5–10% polyacrylamide gradient slab gel, whereas a 7.5–12% gel was used for lane 2. Approximately 25–30 μg of protein was applied per gel. (B and C) Double-immunodiffusion analysis of purified C4bp and protein S by using antisera against C4bp and protein S. The concentration of C4bp in B was 0.5 mg/ml and in C, 2 mg/ml. After washing, staining, and destaining the gels, the wells proved to be difficult to visualize on photographs.

is shown in Fig. 2A. Consistently, the molecules exhibited a morphology that, in several respects, resembles that of a spider. Multiple elongated, thin tentacles are linked to a small central body, each tentacle presumably representing one C4bp subunit. Fifty different C4bp images were examined and the number of visible tentacles was counted. The number of tentacles per C4bp molecule distributed as follows: 2% of the molecules had four, 8% had five, 52% had six, and 38% had seven tentacles. In no image could eight tentacles be observed. These elongated structures appear to be highly flexible, many of them showing one or two sharp bends. The angle formed by two adjacent tentacles was found to vary greatly. A slight, club-like enlargement of the peripheral part of the tentacles was observed in some images. The length and the diameter of 28 visible tentacles were measured. The length (mean $\pm$ SD), measured from the center of the molecule, was found to be 327 ± 23 Å and the diameter (mean $\pm$ SD) was 30 ± 2 Å.

Complex Between C4b-Binding Protein and Protein S. In $\approx 20\%$ of the C4bp images a structure with a morphology different from the tentacles was detected (indicated by an arrow in Fig. 1B). It appeared to have at least two distinct domains of equal size (center-to-center distance, ≈ 50 Å). One of the domains was linked to the central portion of the C4bp molecule via a short subunit, distinct from the other seven, which is visualized in the second and fourth images from the left in Fig. 1B. The two-domain structure could also be observed dissociated from the C4bp. Because other methods had indicated that ≈ 20 –30% of the C4bp molecules were in complex with protein S, identification of the two-domain structure was attempted by comparing it with isolated protein S. As shown in Fig. 1C, isolated protein S is composed of two similar-sized domains, with the average distance ($\pm$ SD) between the centers of the two domains being 53 ± 3.7 Å. The image of isolated protein S was essentially identical to that of the structure found linked to the central portion of some of the C4bp molecules. When protein S (50 $\mu\text{g}/\text{ml}$) was added to C4bp (200 $\mu\text{g}/\text{ml}$) (2 hr at 37°C), the two-domain structure was visible in $\approx 80\%$ of the C4bp images (not shown) and consistently only one of the domains was linked to the central portion of C4bp through a small linking arm. The distance (mean $\pm$ SD) between the center of the C4bp molecule and the center of the bound domain was very constant and was estimated in 14 images to be 119 ± 10 Å. The diameter of the linking arm was between 15 and 25 Å. These results strongly suggest that protein S binds to the center of the C4bp molecule via a short spacer and that the two-domain structure observed in some C4bp images actually was protein S.

Complex Between C4b-Binding Protein and C4b. The structure of isolated C4b is shown in Fig. 3A and is easily distinguishable from the structure of C4bp and protein S. The molecules appear to contain at least four or five domains in a relatively compact arrangement. Mixtures of C4bp and C4b were subjected to electron microscopy and selected images are shown in Fig. 3C and D. When C4b alone was examined, the molecules were evenly distributed throughout the field. In mixture with C4bp they were arranged in groups of four to six molecules. In many instances it was difficult to visualize the C4bp molecules in these mixtures. However, all images were consistent with the interpretation that a C4b-binding site is located at the peripheral end of each C4bp subunit (tentacle). In the first three images of Fig. 2C, only one C4b molecule appears to be bound per C4bp molecule. The interaction between the end of a single C4bp subunit and C4b is visible. In the latter three images of Fig. 3C and the first three of Fig. 3D, two or three molecules of C4b appear bound to one molecule of C4bp, whereas four to six molecules are bound to the C4bp in the three images on the right of Fig. 3D. It was difficult to saturate the

FIG. 2. Electron micrographs of C4bp, C4bp-protein S complexes, and protein S. (A) Field view of C4bp. (B) Selected images demonstrating the C4bp structure and C4bp-protein S complexes. Arrow indicates the two-domain protein S that was bound to the center portion of C4bp. (C) Purified protein S. (A–C, $\times 300,000$.) The scale bar in A represents 300 Å.

binding sites on C4bp with C4b and still visualize both structures in the complex. Therefore, the binding capacity of the saturated C4bp could not be estimated.

DISCUSSION

The M_r of human C4bp has been estimated by sedimentation equilibrium ultracentrifugation to be 570,000 (6). Because the M_r of the subunits is 70,000, it was suggested that C4bp probably contains eight subunits (6). In the human protein the subunits are disulfide-bonded and have an identical amino-terminal sequence (1, 7, 8). C4bp exists in plasma in two forms that have slightly different M_r s (540,000 and 590,000) and net charges (1, 29). The higher molecular weight form represents $\approx 80\%$ of C4bp in plasma (6, 30) and it was recently found that only this form was capable of binding the vitamin K-dependent protein S (6). However, both forms can bind C4b (9, 29) and serve as a cofactor in the degradation of C4b by factor I (29). These observations and the fact that protein S and C4b bind noncom-

petitively to C4bp led to the suggestion that the higher molecular weight form contains an additional distinct subunit that bears the protein S-binding site (6, 9). As to the valency of C4bp for C4b, it was reported previously that at least four to six C4b molecules could bind to one molecule of C4bp (1, 9). However, reliable estimates of the stoichiometry and the affinity of this interaction are not available.

The present results together with previous observations permit the formulation of the following structural hypothesis. The C4bp is composed of eight subunits, seven of which are identical (for schematic model see Fig. 4). The chains are linked by disulfide bonds only at one end. The seven identical chains form 330-Å long tentacular structures extending from a small central body, thus evoking the image of a spider. This image is enhanced by the apparent flexibility of the tentacles. They can assume different positions relative to each other—that is, the angle formed by the two tentacles is extremely variable. In addition, there appear to be joints within the tentacles themselves, as suggested by one or two sharp bends seen in a num-

FIG. 3. Electron micrographs of C4b, C4bp, and complexes between C4bp and C4b. (A) Purified C4b. (B) Selected images of C4bp. (C and D) Complexes between C4bp and C4b; note the binding of C4b to the peripheral end of the C4bp subunit and also the variable number of C4b bound per C4bp. (A–D, $\times 300,000$.) The scale bar represents 300 Å.

ber of these subunits. The binding sites of C4b are located at the peripheral end of the tentacles, suggesting seven sites per C4bp molecule. In contrast, the binding site for protein S is located near the central body of the molecule on a short arm.

It was recently reported that limited chymotryptic cleavage of C4bp resulted in the liberation of a M_r 48,000 fragment containing the cofactor activity of C4bp (31). Remaining after the digestion was a carbohydrate-containing core composed of disulfide-linked polypeptide chains with an apparent M_r of 25,000. These results agree with the C4bp structure reported here, including the localization of the C4b-binding site to the end of each subunit.

The morphology of the C4bp is quite different from that of the two C3b binding proteins, factor H and properdin. Factor

FIG. 4. Schematic model of C4bp and its complexes with C4b and protein S. The dimensions indicated are mean values, with the number of determinations and the SD given in the *Results*. The C4b molecule is simplified to an ellipsoid and the dimensions are only approximate.

H is an elongated molecule measuring 280×30 Å and properdin is a cyclic polymer formed from a 260×30 Å flexible protomer (unpublished data). Another binding protein, C1q, resembles in the electron microscope a bunch of tulips in which six terminal subunits that contain the immunoglobulin binding sites are connected to a central portion by connecting strands (16–18). These strands consist of collagen triple helix and, unlike the tentacles of the C4bp, are very rigid. The only flexibility is provided by a semi-flexible joint located where the connecting strands join the central portion and where the collagen amino acid sequence is interrupted. Whereas there is a correlation between unusual morphology and chemical structure in the case of C1q, no unusual repeating unit of amino acids has been found in sequence analysis of properdin, although 40% of its amino acid residues represent glutamic acid, glycine, and proline (32). The amino acid composition of C4bp reveals no unusual predominance of any amino acid (4, 6, 8).

Protein S is composed of two major domains of similar size. Presumably the phospholipid binding part of protein S, containing the γ -carboxyglutamic acid residues, is not involved in the C4bp–protein S interaction (6). Because the electron micrographs demonstrate that only one of the protein S domains is involved in the binding to C4bp, it is probable that the phospholipid-binding site and the C4bp-binding site are localized in different domains. The electron microscopic appearance of protein S resembles that of Bb and C2a, which are serine proteases derived from the zymogens factor B and C2 (20, 33). These two-domain enzymes also bind to their respective cofactors C3b and C4b only through one of the two domains.

The functional significance of the complex between protein S and C4bp is still unknown. It has been proposed that protein S, through its affinity for negatively charged phospholipids, may

be instrumental in binding the C4bp to injured membranes, thereby suppressing the local activation of the classical pathway of complement (6, 7, 9). This control would be exerted by non-covalent binding and inactivation of C4b (1–4), inhibition of C2 binding to C4b (3), and covalent trapping of nascent C4b by C4bp (34).

It may be deduced from the reported morphology that the C4bp is an efficient regulator of bound or fluid-phase C4b. The multiplicity of its binding sites and the unusual flexibility of the molecule should enable the C4bp to interact with C4b molecules in many different spatial distributions at the cell surface and with C4b molecules randomly moving in the fluid phase. Because of the wide span of its tentacles (≈ 660 Å), a single molecule of C4bp should be able to bind simultaneously to C4b molecules that are relatively distant from each other and thus to control a rather large area of a cell surface. The knowledge of its versatile morphology gained in this study may stimulate further exploration of the functional properties of C4bp and guide future structural studies.

The authors thank Dr. Chen-Ming Chang for his help and support throughout this study. This is publication no. 2951-IMM from the Research Institute of Scripps Clinic. This work was supported by National Institutes of Health Grants AI 17354, CA 27489, HL 07195, and HL 16411. B.D. is the recipient of Fogarty International Fellowship TW 03172. H.J.M.-E. is the Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic.

1. Scharfstein, J., Ferreira, A., Gigli, I. & Nussenzweig, V. (1978) *J. Exp. Med.* **148**, 207–222.
2. Fujita, T., Gigli, I. & Nussenzweig, V. (1978) *J. Exp. Med.* **148**, 1044–1051.
3. Gigli, I., Fujita, T. & Nussenzweig, V. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6596–6600.
4. Nagasawa, S., Ischihara, C. & Stroud, R. M. (1980) *J. Immunol.* **125**, 578–582.
5. Kaidoh, T., Natsuume-Sakai, S. & Takahashi, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3794–3798.
6. Dahlbäck, B. (1983) *Biochem. J.* **209**, 847–856.
7. Dahlbäck, B. & Stenflo, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2512–2516.
8. Reid, K. B. M. & Gagnon, J. (1982) *FEBS Lett.* **137**, 75–79.
9. Dahlbäck, B. & Hildebrand, B. (1983) *Biochem. J.* **209**, 857–863.
10. DiScipio, R. G., Hermanson, M. A., Yates, S. G. & Davie, E. W. (1977) *Biochemistry* **16**, 698–706.
11. DiScipio, R. G. & Davie, E. W. (1979) *Biochemistry* **18**, 899–904.
12. Stenflo, J. & Jönsson, M. (1979) *FEBS Lett.* **101**, 377–381.
13. Dahlbäck, B. (1983) *Biochem. J.* **209**, 837–846.
14. Walker, F. J. (1980) *J. Biol. Chem.* **255**, 5521–5524.
15. Walker, F. J. (1981) *J. Biol. Chem.* **256**, 11128–11131.
16. Porter, R. R. & Reid, K. B. M. (1979) *Adv. Protein Chem.* **33**, 1–71.
17. Schumaker, V., Poon, P. H., Seegan, G. W. & Smith, C. A. (1981) *J. Mol. Biol.* **148**, 191–197.
18. Strang, C. J., Siegel, R. C., Phillips, M. L., Poon, P. H. & Schumaker, V. N. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 586–590.
19. Seegan, G. W., Smith, C. A. & Schumaker, V. N. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 907–911.
20. Smith, C. A., Vogel, C.-W. & Müller-Eberhard, H. J. (1982) *J. Biol. Chem.* **257**, 9879–9882.
21. Podack, E. R. & Tschopp, J. (1982) *J. Biol. Chem.* **257**, 15204–15212.
22. Tschopp, J., Podack, E. R. & Müller-Eberhard, H. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7474–7478.
23. Lundwall, Å., Malmheden, J., Stålenheim, G. & Sjöquist, J. (1981) *Eur. J. Biochem.* **117**, 141–146.
24. Blobel, G. G. & Dobberstein, B. (1975) *J. Cell Biol.* **17**, 835–851.
25. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
26. Ouchterlony, O. (1948) *Acta Pathol. Microbiol. Scand.* **25**, 186–191.
27. Valentine, R., Shapiro, B. & Stadtman, F. (1968) *Biochemistry* **7**, 2143–2152.
28. Smith, C. A. (1979) Dissertation (Univ. of California, Los Angeles).
29. Fujita, T. & Nussenzweig, V. (1979) *J. Exp. Med.* **150**, 267–276.
30. Nussenzweig, V. & Melton, R. (1982) *Methods Enzymol.* **80**, 124–133.
31. Nagasawa, S., Mizuguchi, K., Ischihara, C. & Koyama, J. (1982) *J. Biochem.* **92**, 1329–1332.
32. Reid, K. B. M. (1981) *Methods Enzymol.* **80**, 143–150.
33. Smith, C. A., Vogel, C.-W. & Müller-Eberhard, H. J. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 1371 (abstr.).
34. Villier, M.-B., Thielens, N. M., Reboul, A. & Colomb, M. G. (1982) *Biochim. Biophys. Acta* **704**, 197–203.