FORMATION AND FUNCTION OF A COMPLEX OF THE C3 PROACTIVATOR WITH A PROTEIN FROM COBRA VENOM*‡

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(Received for publication 16 October 1972)

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Recently a second, alternate pathway of complement activation has been described which bypasses C1, C2, and C4 and enters the classical pathway with the reaction of C3 (1–3).[1] One of the proteins of this pathway, a heat-labile β-globulin, is cleaved on triggering of the alternate pathway in serum; since the major cleavage product of this protein activates C3, the precursor has been termed the C3 proactivator (C3PA) (1).[2] C3PA is antigenically related to glycine-rich β-globulin (GBG) isolated by Boenisch and Alper (4) and β_2-glycoprotein type II isolated by Haupt and Heide (1, 5). C3PA is also felt to be identical with factor B of the properdin system (6, 7), since it participates in the bactericidal activity of serum (1, 8) and in the lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (9).

C3PA was originally identified as the protein in normal serum which combines with a protein isolated from cobra (*Naja naja*) venom to form a complex capable of activating C3 and initiating complement action at this step (10–17). Recently Alper et al. (18) and Hunsicker et al. (19), on the basis of studies with isolated GBG and β_2-glycoprotein II, have independently challenged the concept that C3PA forms a complex with cobra venom factor (CoF), and that it is this complex which acts on C3.

These reports necessitated a reinvestigation of the role of C3PA and CoF in the formation of the principle which is capable of initiating complement action at the C3 step. The results of these studies clearly demonstrate the occurrence of two distinct modes of interaction of C3PA with CoF.

Materials and Methods

C3PA was isolated from human serum as described by Götze and Müller-Eberhard (1). CoF was obtained by the method described by Müller-Eberhard and Fjellström (16), except

* This is publication no. 658 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037.

‡ This work was supported by United States Public Health Service Grant no. AI-07007.

§ Dr. Cooper is supported by United States Public Health Service Research Career Development Award no. 5-K4-AI-33,630-02.

[1] Terminology conforms to the recommendations of the World Health Organization Committee on Complement Nomenclature (1968. *Bull. W. H. O.* **39**:939). Terminology employed for the proteins of the alternate pathway is as in reference 24.

[2] *Abbreviations used in this paper*: C3PA, C3 proactivator; C3PAse, C3PA convertase; CoF, cobra venom factor; GBG, glycine-rich β-globulin; HSFa, hydrazine-sensitive factor.

that G-200 was used in place of G-100 Sephadex. C3 and other complement components were isolated by published methods (20). Preparations of C3PA and CoF were trace labeled with ^{125}iodine and ^{131}iodine by the chloramine-T method (21) without loss of functional activity. Rabbits were immunized with isolated C3PA to prepare antisera to the protein. Anti-β_2-glycoprotein type II was purchased from Behringwerke AG., Marburg, W. Germany, while antiserum to GBG was kindly provided by Dr. C. A. Alper. Serum was depleted of C3PA by absorption with insolubilized antiserum to C3PA (1); the author wishes to thank Dr. O. Götze for his gift of serum depleted of C3PA.

7–31% linear sucrose gradients in 0.05 M Tris-HCl, pH 7.5, containing 0.0002 M Ca^{++} and 0.001 M Mg^{++}, were centrifuged at 39,000 rpm for 16 h in an SW-50L rotor in a Beckman L2-50 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Markers were C1q (11S) and human serum albumin (4.5S), both of which were localized by immunochemical analysis, equine cytochrome *c* (1.7S), which was identified spectrophotometrically, and the meniscus. Polyacrylamide gel electrophoresis was performed in 6% gels in Tris-HCl buffer, pH 8.7 (22).

C3 hemolytic activity was determined by molecular titrations utilizing EAC1, 4, 2, C5, and an EDTA serum reagent deficient in C3 and C5 (23). C3 inactivating activity was quantitated by incubating gradient fractions or mixtures of C3PA and CoF with 5 μg of C3 for 30 min at 37°C in a total volume of 50–100 μl. The mixtures were then diluted for hemolytic assay of residual C3.

C3PA was measured as follows. Gradient fractions were added to 0.2 ml of a 1:15 dilution of serum previously heated at 50°C for 20 min. Serum heated in this manner is deficient in C3PA (9). Subsequently, 20 μg of inulin was added and the mixtures incubated for 30 min at 30°C. Then the serum was diluted and residual C3 quantitated by effective molecule titration.

A value of 200 μg/ml was used as the serum concentration of C3PA for the purpose of determining molar ratios. Molecular weights of 80,000, 150,000, and 185,000 were used for C3PA, CoF, and C3, respectively.

RESULTS

Characterization of C3PA and CoF Preparations.—Several approaches were employed to determine molecular homogeneity and functional activity of the C3PA preparations employed in these studies. C3 proactivator preparations gave a single stained band after electrophoresis in 6% polyacrylamide gels. Similarly, radiolabeled C3PA preparations gave one discrete peak of radioactivity during electrophoresis in polyacrylamide gels or during ultracentrifugation in sucrose density gradients. Rabbits immunized with C3PA produced antibody which gave a single line on Ouchterlony analysis with human serum. In order to determine if the preparations contained additional proteins masked by the position of the C3PA band in polyacrylamide gels, 50 μl of antiserum to C3 proactivator was added to 5 μg of [^{125}I]C3PA before electrophoresis in polyacrylamide gels. Subsequently the gels were sectioned and radioactivity present in the segments was determined. As shown in Fig. 1, previous incubation of [^{125}I]C3PA with anti-C3PA prevented all radioactivity from entering the gel. Identical results were obtained from incubation of [^{125}I]C3PA with either 50 μl of antiserum to β_2-glycoprotein type II, or of antiserum to glycine-rich β-glycoprotein. While a given antiserum might contain antibody to proteins possibly contaminating the C3PA preparations, it is unlikely that all of the antisera employed would contain antibody to the same contaminant.

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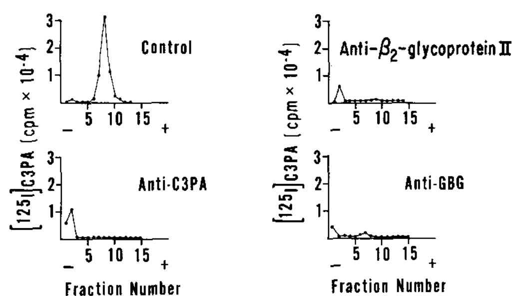

FIG. 1. Polyacrylamide gel electrophoresis of 5 μg of [^{125}I]C3PA previously incubated with various antisera. After electrophoresis the gels were sectioned and the amount of radioactivity present in the segments determined.

Three tests were employed to determine the functional activity of isolated preparations of C3PA. First, unlabeled and radiolabeled C3PA preparations were examined for their ability to be cleaved by incubation with C3PA convertase (C3PAse) and activated hydrazine-sensitive factor (HSFa) (24). Secondly, C3PA preparations were tested for their ability to restore the C3 cleaving activity in heated serum (50°C, 20 min). In this test inulin was used as the triggering substance. Thirdly, C3PA preparations were analyzed for their capacity to cleave C3 upon addition of an equimolar concentration of CoF and factor D (see below). The preparations used in this study were found to be fully active in all three tests.

CoF preparations were also examined for molecular homogeneity. Only one stained band, or, in the case of radiolabeled preparations, only a single symmetrical peak of radioactivity, was evident after electrophoresis in polyacrylamide gels. Antisera reactive only with CoF were produced in rabbits. Incubation of [^{131}I]CoF preparations with the γG fraction of anti-CoF serum before electrophoresis in 6% polyacrylamide gels completely prevented the entry of radioactivity into the running gel. CoF preparations had the functional activity characteristic of this protein, i.e., C3 cleaving activity appeared upon addition of CoF to serum.

The above studies indicate that contaminating proteins represent a very minor proportion, less than 5%, of the total protein present in the C3PA and CoF preparations. The preparations have the functional activities characteristic for these proteins.

Complexing of CoF with C3PA in Serum.—C3PA and CoF were added to normal human serum in ^{125}I- and ^{131}I-radiolabeled forms, respectively. 5 μg of [^{125}I]C3PA was added to the approximately 8 μg of C3PA present in 40 μl of serum. In a series of 15 experiments, CoF was added to the serum mixtures to achieve various molar ratios between CoF and C3PA. After 30 min at 37°C, marker substances were added and the mixtures were analyzed by sucrose density gradient ultracentrifugation. When an equimolar ratio of CoF with C3

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was employed or when C3PA was in excess, the sedimentation rate of [^{131}I]CoF was increased from approximately 7S, the velocity in the absence of serum, to 8–9S (Fig. 2). The sedimentation rate of C3PA was also increased from 5–6S to 8–9S; furthermore, its position correlated with that of CoF (Fig. 2). At less than equimolar CoF:C3PA ratios, C3PA appeared as a 5–6S peak, with a heavier shoulder coinciding in position with the 8–9S CoF peak. Essentially the converse was seen when adding CoF in molar excess (Fig. 2): C3PA was

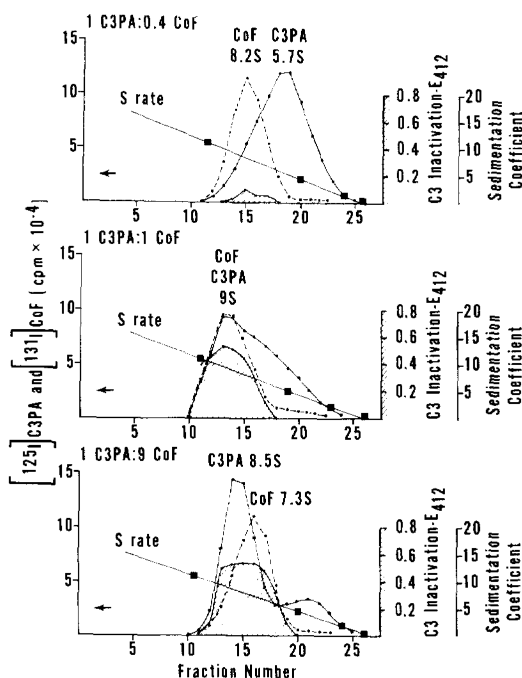

FIG. 2. Sucrose gradient ultracentrifugation of serum to which a trace of [^{125}I]C3PA and varying amounts of [^{131}I]CoF have been added. Radioactivity and C3 inactivating activity of the gradient fractions was determined.

entirely in the 8–9S region along with some of the CoF; the bulk of CoF, however, sedimented with an S rate of 7.3S. Identical results were obtained from Ouchterlony analyses of the gradient fractions with antiserum to C3PA. Fig. 3 demonstrates such a study with a partially purified C3PA preparation.

Similar studies were performed with serum previously depleted of C3PA by absorption with insolubilized anti-C3PA (Fig. 4). In five experiments with two preparations of C3PA-depleted serum, CoF added to the depleted serum remained in 7S position. A shift of CoF to 8–9S was readily produced by addition of isolated C3PA to the mixture of CoF and C3PA depleted serum.

C3 cleaving activity was regularly demonstrable in the 8–9S region of the

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normal serum density gradients. Its distribution correlated exactly with that of CoF when C3PA was in molar excess over CoF, with the peak of both CoF and C3PA when these proteins were present in equimolar concentrations, and with the C3PA peak when CoF was in molar excess. C3 cleaving activity was

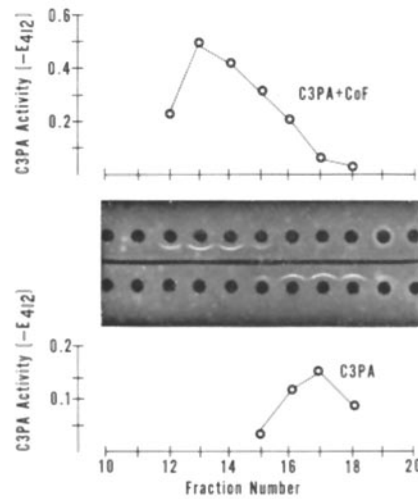

FIG. 3. Sucrose gradient ultracentrifugal analyses of partially purified C3PA (lower panel) and C3PA previously incubated with CoF (upper panel). The control panel shows the reactivity of the gradient fractions with anti-C3PA. The direction of sedimentation is to the left.

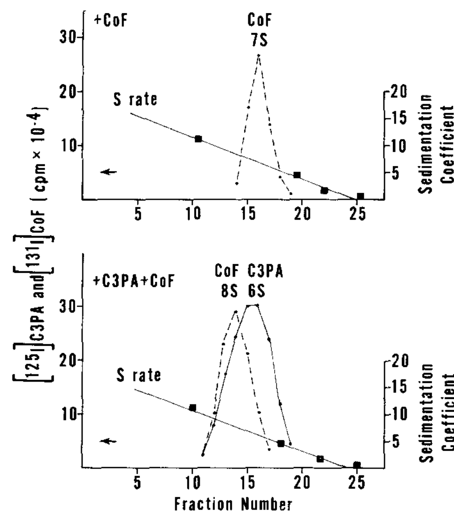

FIG. 4. Sucrose gradient ultracentrifugation of serum previously depleted of C3PA. In the upper panel CoF has been added to the serum. Complex formation is not evident. A complex is formed by addition of C3PA as well as CoF to the serum (lower panel).

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not found when adding CoF to C3PA-depleted serum. Results comparable to those given above were observed during electrophoretic analyses of [^{125}I]C3PA-[^{131}I]CoF serum mixtures in polyacrylamide gels.

Reversible Complexing of Isolated C3PA with CoF.—Mixtures of isolated radiolabeled C3PA and CoF were analyzed by ultracentrifugation in a series of 18 experiments with a number of C3PA preparations. Whenever C3PA was present in equimolar or less than equimolar ratios with CoF, C3PA sedimented as a 5–6S peak with a heavier shoulder coinciding in position with the CoF peak (Fig. 5); the sedimentation rate of CoF was also slightly increased. Reversible formation of a protein-protein complex was also demonstrable in polyacrylamide gel electrophoretic analyses of C3PA-CoF mixtures (1). Mixtures of isolated C3PA and CoF possessed C3 cleaving activity. This reaction,

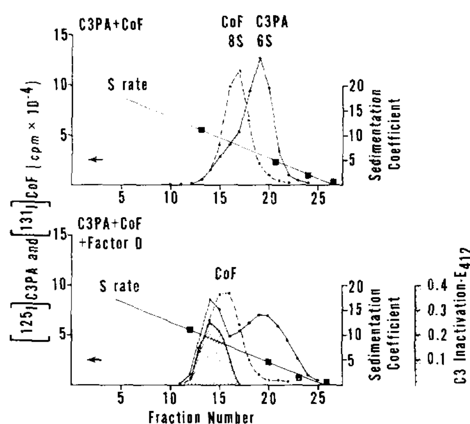

FIG. 5. Sucrose gradient ultracentrifugal analysis of an equimolar mixture of isolated CoF and C3PA (upper panel) and the same mixture in the presence of factor D.

however, was relatively inefficient and C3 cleaving activity could not be detected in gradient fractions.

Requirement for an Additional Factor for Efficient C3PA-CoF Complexing and for Generation of C3 Cleaving Activity.—Although C3PA and CoF readily formed a firm complex in serum which possessed C3 inactivating activity, isolated C3PA and CoF exhibited only a weak reversible complex with inefficient C3 cleaving activity. These observations led to a search for a serum factor which would enhance complex formation and C3 cleaving activity of C3PA-CoF mixtures. A factor with these properties was identified and isolated in partially purified form by a combination of BioRex column chromatography (elution peak 22–29 mmho/cm), DE-32 cellulose chromatography (elution peak 2–4 mmho/cm), and Pevikon block electrophoresis (slow α to fast β mobility). This substance is termed factor D because of similarity to a factor termed "D" which has been described by Hunsicker et al. (19). Factor D is

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apparently a trace constituent of serum, since less than 10 μg could be obtained per 100 ml of plasma.

The enhancing effect of factor D on the formation of a complex of C3PA with CoF is shown in Fig. 5. The reaction mixtures contained 10 μg of [^{131}I]CoF and 5 μg of [^{125}I]C3PA. C3PA incubated with CoF in the presence of factor D sedimented as a 9S peak; C3 inactivating activity also correlated in distribution with this peak. The bulk of CoF also was present in this area of the gradient, although the peak of [^{131}I]CoF sedimented at 8S. A second 3.5–4.5S peak of C3PA was often seen (Fig. 5).

Very small amounts of factor D increased the C3 cleaving activity of C3PA-CoF mixtures as shown in Table I. Enhanced C3 cleaving activity of a 1:1:2 molar ratio of C3PA:CoF:C3 was still evident upon addition of as little as 30 ng of partially purified factor D.

DISCUSSION

An identical distribution of CoF, C3PA, and C3 cleaving activity was found on sucrose density gradient ultracentrifugation of serum containing CoF in amounts equimolar to the total C3PA present (Fig. 2). When CoF was added to serum in amounts which were less than equimolar to the C3PA content, all of the CoF sedimented as an 8–9S peak which correlated with the peak of C3 inactivating activity. Some of the C3PA also was present in this region, as shown by a shoulder on the heavy side of the 5–6S C3PA peak (Fig. 2). Conversely, C3PA sedimented with an 8–9S peak which correlated with the location of C3 cleaving activity when CoF was added to serum in a molar excess over the C3PA present. Under these conditions, CoF gave a 7S peak which had a shoulder on the heavy side which encompassed the C3PA peak. These studies document the formation in serum of a firm complex of C3PA with CoF which possesses C3 cleaving activity. The essential role of C3PA in formation of this complex is shown by the failure of CoF to form a complex by addition to serum

TABLE I

Effect of Factor D on the C3 Cleaving Activity of C3PA and CoF

C3PA*	CoF*	Factor D	E.M. of C3 consumed‡
		ng	
+	+	None	675
+	+	30	950
+	+	60	1,050
+	+	150	1,200
+	+	300	1,300
0	0	300	None

* 3.2 μg C3PA and 6 μg of CoF incubated with C3 for 30 min.

‡ 6 μg of C3 = 1,500 effective molecules (E.M.) of C3 at 37°C.

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previously depleted of C3PA by absorption with insolubilized antiserum to C3PA (Fig. 4).

Sucrose density gradient ultracentrifugal analyses of isolated C3PA and CoF revealed weak reversible interaction between the two proteins. An additional factor present in normal serum was required to induce firm complexing of isolated C3PA with CoF and to endow this complex with efficient C3 cleaving activity. This serum protein is probably identical with a substance termed factor D by Hunsicker et al. (19) which is required for mixtures of C3PA and CoF to manifest C3 cleavage activity.

The data presented here thus confirm and extend the earlier studies which indicated that CoF complexed with C3PA (1, 16). Two types of interaction were observed. In free solution the two proteins exhibited an affinity for each other and formed a reversible complex. In the presence of factor D, they gave rise to a firm complex which was able to cleave C3 enzymatically. This represents another example of a functionally relevant protein-protein interaction within the complement system. Other examples are found in the affinity of C1q for γ-globulin (25) and the formation of complexes of C1q with C1r and C1s (26), of C2 with C4 (27), of C5 with C6 and C7 (28), and of C8 with C9 (29).

The reason Hunsicker et al. (19) and Alper et al. (18) failed to observe the formation of a complex of C3PA with CoF is not clear. The present studies (Fig. 1) clearly indicate that the C3PA used in this study is antigenically related to β_2-glycoprotein type II and to GBG which Hunsicker et al. (19) and Alper et al. (18) employed in their respective studies. The data also indicate that the preparations of C3PA employed in the present study were functionally active and physicochemically homogeneous.

The identity and mode of action of factor D are uncertain. The small size, electrophoretic mobility, and elution position from anion and cation exchange columns suggest that factor D might be C3PAse, an enzyme which cleaves and activates C3PA in the presence of a C3 fragment called HSFa (24). CoF is not required for this reaction. The occasional finding of C3PA cleavage during reaction with CoF and factor D (Fig. 5) support a common identity of factor D and C3PAse. Although C3PAse activity has not been consistently detected in factor D preparations, this does not preclude identity of the two proteins because of differences in dose-response requirements. Although the exact role and identity of factor D remain to be elucidated, it is clear that it is an activator of C3PA. In its mechanism of action factor D also resembles C1s, which also induces the formation of a firm, metal-dependent, enzymatically active complex between two proteins.

SUMMARY

The role of C3 proactivator (C3PA) and a factor isolated from cobra venom (CoF) in the formation of a principle able to cleave C3 was investigated. The

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results clearly demonstrate two modes of interaction of C3PA with CoF. In isolated form, C3PA and CoF were found to form a reversible protein-protein complex in free solution. This complex had some C3 cleaving activity. In the presence of minute amounts of a partially purified normal serum substance, factor D, the C3PA-CoF complex was stabilized and its efficiency in cleaving C3 was greatly increased. Factor D is thus an activator of C3PA. A firm complex composed of C3PA and CoF, and possessing C3 cleaving activity, was also formed by addition of CoF to serum.

I thank Mrs. Cynthia Stone and Mrs. Kathleen Keogh for their very competent assistance.

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