

# PROPERDIN: BINDING TO C3b AND STABILIZATION OF THE C3b-DEPENDENT C3 CONVERTASE\*

BY DOUGLAS T. FEARON‡ AND K. FRANK AUSTEN

(From the Departments of Medicine, Harvard Medical School and Robert B. Brigham Hospital,  
Boston, Massachusetts 02120)

Activated properdin ( $\bar{P}$ ),<sup>1</sup> a gamma globulin with an estimated mol wt of 223,000 daltons and an isoelectric point of greater than 9.5, has been purified free of other serum proteins and observed to consist of four apparently identical subunits of 45,000 daltons each (8, 9).  $\bar{P}$  eluted from a zymosan-serum complex or purified from the euglobulin obtained from serum at pH 5.4 interacts with normal serum in the absence of zymosan, resulting in cleavage of C3 and B (9, 10). Müller-Eberhard and Götze (6) have showed that the hydrazine-sensitive factor (factor A) of the properdin system is C3 and that its major cleavage product, C3b, interacts with B and  $\bar{D}$  to form a C3b-dependent C3 convertase. Formation of this convertase on the surface of an erythrocyte, EAC43, permitted stoichiometric hemolytic titrations of B and  $\bar{D}$  to be performed (5, 11) and revealed that  $\bar{P}$  profoundly augmented the number of effective convertase sites formed (12). This action, previously attributed to a capacity of  $\bar{P}$  to convert D to  $\bar{D}$ , is shown now to be the result of binding of  $\bar{P}$  to C3b with concomitant retardation in decay of the C3b-dependent convertase.

## Materials and Methods

Functionally pure guinea pig C1 and C2 were prepared as described (13). Human C3 (14, 15) and B (4) were purified to homogeneity as assessed by alkaline disc gel electrophoresis and quantitated by radial immunodiffusion against monospecific antisera.  $\bar{D}$  was purified from citrated plasma which was adjusted to pH 7.8, diluted with 0.0035 M  $\text{PO}_4$ , 0.002 M EDTA, pH 7.8, to a conductivity of 5 mS at 4°C and applied directly to quaternary aminoethyl (QAE) Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) that had been equilibrated with the  $\text{PO}_4$  buffer brought to 5 mS by addition of NaCl.  $\bar{D}$  eluted at 7 mS and was further purified by gel filtration twice on Sephadex G-75 Superfine. The final preparation of  $\bar{D}$  had no detectable trypsin-activable D

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<sup>1</sup> In the nomenclature for the alternative pathway the components are designated by capital letters, with a bar over the letter indicating the active factor. B (1) is the properdin factor which also has been termed C3 proactivator (2) and glycine-rich beta glycoprotein (3); D (4,5) is also termed precursor of C3 proactivator convertase (6); factor A (1) is now known to be C3 (6); and P is the symbol for properdin (7).

*Other abbreviations used in this paper:* C-EDTA, rat serum diluted in GVB-EDTA; DFP, diisopropylphosphorfluoridate; DGVB<sup>++</sup>, GVB<sup>++</sup> plus dextrose; GVB<sup>++</sup>, gelatin veronal-buffered saline with Mg<sup>++</sup> and Ca<sup>++</sup>; QAE, quaternary aminoethyl; Z, average number of hemolytic sites per cell.

activity, and exhibited a single band on alkaline disc gel electrophoresis.  $\bar{D}$  protein concentration was estimated by Folin analysis (16).  $\bar{P}$  was isolated from the euglobulin fraction of citrated plasma at pH 6.0 by chromatography on QAE Sephadex, sulphopropyl Sephadex, and Sephadex G-200 (12). The final product exhibited a single band on acidic disc gel electrophoresis and induced cleavage of C3 and B when added to serum in the absence of zymosan.  $\bar{P}$  was quantitated by radial immunodiffusion (12).

Isotonic veronal-buffered saline containing 0.1% gelatin,  $5 \times 10^{-4}$  M  $Mg^{++}$ , and  $1.5 \times 10^{-4}$  M  $Ca^{++}$  (GVB $^{++}$ ); half-isotonic gelatin veronal buffer with dextrose (DGVB $^{++}$ ); and GVB in which cations were omitted and containing 0.04 M EDTA (GVB-EDTA) were used as diluents for hemolytic assays (13). Tube and microtiter plate hemolytic assays for B,  $\bar{D}$ , and trypsin-activable D were performed (5, 11, 12, 17) with rat serum diluted 1:15 in GVB-EDTA (C-EDTA) as the source of C3-C9. Activity determinations obtained by tube titration are expressed as the average number of hemolytic sites per cell (Z) and microtiter plate titrations are expressed as CH50 units per milliliter. Diisopropylphosphorfluoridate (DFP) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

## Results

*Capacity of Properdin to Increase the Hemolytic Titer of  $\bar{D}$  and B.* To examine whether  $\bar{P}$  and trypsin were acting upon the same factor, plasma obtained from blood collected into hexadimethrine and EDTA (5) was chromatographed on QAE Sephadex as for isolation of  $\bar{D}$ , and fractions were assayed for  $\bar{D}$ , trypsin-activable D, and for the activity enhanced by  $\bar{P}$ . Trypsin-activable D activity eluted at 5.8 mS while  $\bar{P}$ -inducible activity was found only in fractions containing  $\bar{D}$  which eluted with peak activity at 6.8 mS. To assess further whether the expression of  $\bar{P}$  was dependent on  $\bar{D}$  activity,  $5 \mu\text{g } \bar{D}$ , purified to give a single band on disc gel electrophoresis and free of trypsin-activable D, was incubated with  $5 \times 10^{-3}$  M DFP or DGVB $^{++}$  for 30 min at 37°C, extensively dialyzed, and assayed for residual  $\bar{D}$  activity, with and without  $1 \mu\text{g } \bar{P}/10^8$  EAC43.  $\bar{D}$  incubated in DGVB $^{++}$  contained 0.08 U/ng alone and 1 U/ng in the presence of  $\bar{P}$ , while  $\bar{D}$  treated with DFP exhibited 0.007 U/ng alone and 0.086 U/ng with  $\bar{P}$ . Thus, the  $\bar{P}$ -enhanceable activity, which is DFP-sensitive and separable from trypsin-activable D, is  $\bar{D}$ .

To assess the capacity of  $\bar{P}$  to increase the titer of a set, limited amount of B in the presence of excess  $\bar{D}$  and to establish a standard assay for  $\bar{P}$ ,  $1 \times 10^7$  EAC43 with 10 ng  $\bar{D}$ , 0.16 ng B, and variable amounts of  $\bar{P}$  in 0.2 ml DGVB $^{++}$  were incubated for 30 min at 30°C. After addition of 0.3 ml C-EDTA and further incubation for 60 min at 37°C, 1.5 ml saline was added to each reaction mixture, the extent of hemolysis was determined, and the Z was calculated. In the absence of  $\bar{P}$ , 0.16 Z were generated and the presence of increasing amounts of properdin was associated with a linear increase in the number of hemolytic sites per cell (Fig. 1). In the absence of B, no hemolytic sites were formed, so that this activity of  $\bar{P}$  cannot be attributed to contamination with B. In subsequent hemolytic titrations,  $\bar{P}$  activity is expressed as the increase in Z achieved over that obtained with EAC43, limited B and excess  $\bar{D}$  alone; reaction mixtures containing B and  $\bar{D}$  alone always generated less than 0.2 Z.

*Binding of  $\bar{P}$  to EAC43.*  $1 \times 10^7$  EAC43, 0.14 ng B, and 10 ng  $\bar{D}$  were incubated with or without 10 ng  $\bar{P}$  in 0.2 ml DGVB $^{++}$  for 30 min at 30°C, followed by addition of C-EDTA to bring the hemolytic reaction to completion; the activity of 10 ng  $\bar{P}$  was 1.50 Z. An additional  $5 \times 10^7$  EAC43 were incubated with 50 ng  $\bar{P}$  in 0.5 ml DGVB $^{++}$  for 10 min at 15°C, after which the reaction mixture

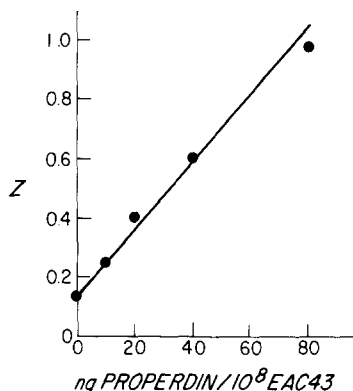


FIG. 1. Hemolytic activity (Z) generated during incubation of EAC43,  $\bar{D}$ , constant limited B, and variable amounts of  $\bar{P}$ .

was centrifuged, the supernate was removed, and the erythrocytes were washed twice in ice-cold DGVB<sup>++</sup>. Development of the hemolytic sites on  $1 \times 10^7$  washed erythrocytes with 0.14 ng B and 10 ng  $\bar{D}$  resulted in generation of 1.14 Z above that obtained with cells not exposed to  $\bar{P}$ , while hemolytic assay of 0.1 ml of the supernate yielded 0.13 Z of residual  $\bar{P}$  activity. Thus, of the total  $\bar{P}$  activity initially available, 76% bound to EAC43, creating EAC43P, with 20% recovered in the supernate.

The specificity of  $\bar{P}$  binding for an intermediate bearing C3b was examined with EAC4 and EAC43. 20 ng  $\bar{P}$  were incubated alone or with  $2 \times 10^7$  EAC4 or EAC43, ranging from  $1.25 \times 10^6$  to  $2 \times 10^7$  in 0.2 ml DGVB<sup>++</sup> for 10 min at 15°C, after which the reaction mixtures were centrifuged, and the supernates removed and assayed for  $\bar{P}$  as in the previous experiment.  $\bar{P}$  input was 0.64 Z and an identical amount was recovered in the supernate of the reaction mixture containing  $2 \times 10^7$  EAC4 (Fig. 2). In contrast, incubation of  $\bar{P}$  with increasing numbers of EAC43 resulted in a dose-related removal of  $\bar{P}$  activity plateauing at 85% depletion by  $1 \times 10^7$  EAC43.

The kinetics of binding of  $\bar{P}$  to EAC43 at 15°C was compared to that at 0°C because of the failure of  $\bar{P}$  in serum to bind to zymosan at 0°C (7). Samples of  $1 \times 10^7$  EAC43 in 0.1 ml DGVB<sup>++</sup> at either 0°C or 15°C were added to 10 ng  $\bar{P}$  in 0.1 ml DGVB<sup>++</sup> equilibrated at the appropriate temperature, and incubated at 0°C or 15°C for up to 20 min. The reactions were stopped by the addition of 5 ml ice-cold DGVB<sup>++</sup>, immediate centrifugation at 0°C, and washing twice with DGVB<sup>++</sup>. The erythrocyte intermediates, and EAC43 that had been incubated with DGVB<sup>++</sup> alone, were each resuspended in 0.2 ml DGVB<sup>++</sup> containing 0.14 ng B and 10 ng  $\bar{D}$  and then lysed with C-EDTA. Binding of  $\bar{P}$ , as assessed by incremental lysis, occurred rapidly and was complete in 5 min at 15°C and in 10 min at 0°C. No significant difference in the amount of  $\bar{P}$  activity bound was apparent at these two temperatures and there was no decay in 20 min (Fig. 3).

Binding of  $\bar{P}$  was compared in GVB<sup>++</sup> and GVB-EDTA to examine the cation requirement. Samples of  $1 \times 10^7$  EAC43 were incubated for 10 min at 15°C with 10 ng  $\bar{P}$  in 0.2 ml of GVB<sup>++</sup> or GVB-EDTA. The intermediates were washed once with their initial buffers and twice with ice-cold DGVB<sup>++</sup>, and assayed for

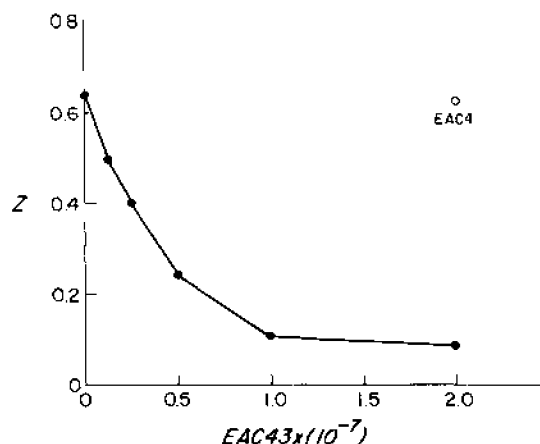


FIG. 2. Depletion of supernatant  $\bar{P}$  by incremental concentrations of EAC43 (●) as compared to  $2 \times 10^7$  EAC4 (○).

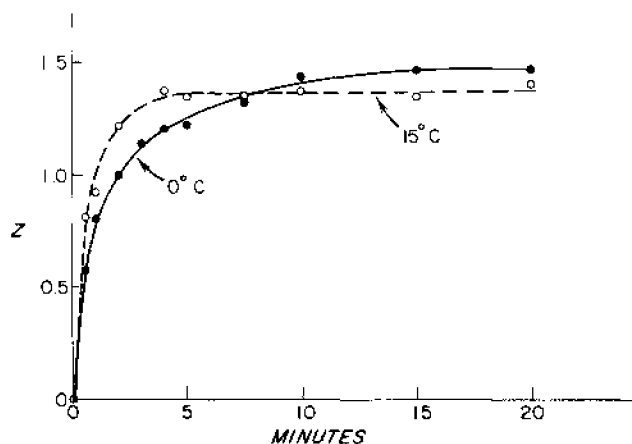


FIG. 3. Kinetics of formation of EAC43P (Z) at 0°C and 15°C.

cell-bound  $\bar{P}$  activity. 0.86 Z and 0.88 Z of  $\bar{P}$  activity bound to EAC43 in GVB<sup>++</sup> and GVB-EDTA, respectively.

*Stabilization of the Hemolytically Active Site on EAC43B by  $\bar{P}$ .* The capacity of  $\bar{P}$  to bind to EAC43 and to increase by more than 10-fold the hemolytic efficiency of a set and limited amount of B prompted an analysis of the effect of  $\bar{P}$  on the half-life ( $t_{1/2}$ ) of the C3b-dependent C3 convertase.  $4 \times 10^8$  EAC43, 80 ng B, and  $0.4 \mu\text{g } \bar{D}$  in 8 ml DGVB<sup>++</sup> were incubated for 30 min at 30°C to generate EAC43B. The reaction mixture was divided into four equal portions which were centrifuged at 4°C, the supernates were removed, and the cells washed once in ice-cold DGVB<sup>++</sup>. The washed EAC43B were resuspended at 30°C in 2 ml GVB-EDTA, or GVB-EDTA containing either 500, 100, or 20 ng  $\bar{P}$ . Incubation was continued at 30°C during which 0.2-ml samples were removed from each reaction mixture at timed intervals and added to 0.3 ml C-EDTA to assess residual convertase sites. The hemolytically active site on EAC43B decayed with a  $t_{1/2}$  of 4 min in the absence of  $\bar{P}$ , while the presence of  $\bar{P}$  prolonged the  $t_{1/2}$  up to 30 min

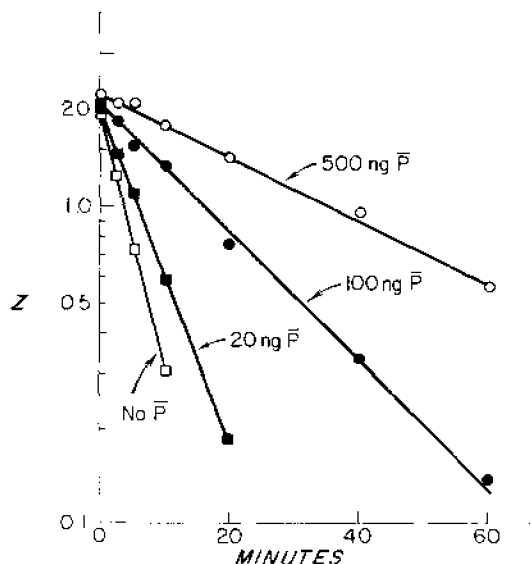


FIG. 4. Decay of EAC43B alone (□) and in the presence of 20 (■), 100 (●), and 500 ng  $\bar{P}$  (○), respectively.

in a dose-related manner (Fig. 4). There was no detectable lag in the stabilizing effect of  $\bar{P}$  so that decay was first order in each reaction mixture, and the presence of  $\bar{P}$  did not increase the number of active sites available at zero time. Washing the stabilized intermediate at 4°C in GVB-EDTA during the decay analysis did not alter the previously established decay rate upon subsequent resuspension and decay.

The capacity of  $\bar{P}$  introduced into the fluid phase to stabilize  $C3\bar{B}$  raised the question of whether or not  $\bar{P}$  previously bound to C3b could transfer so as to achieve the same result. Donor cells were formed by interacting three batches of  $1 \times 10^7$  EAC43 with 400 ng  $\bar{P}$ , 200 ng  $\bar{P}$ , or buffer for 10 min at 15°C, after which the intermediates were washed twice with ice-cold DGVB<sup>++</sup> and resuspended in 1 ml GVB-EDTA at 30°C. Recipient EAC43B were generated by incubating  $3 \times 10^8$  EAC43 with 30 ng B and 0.3  $\mu$ g  $\bar{D}$  in 6 ml DGVB<sup>++</sup> for 30 min at 30°C. The recipient cells were washed once in DGVB<sup>++</sup>, resuspended in 3 ml GVB-EDTA at 30°C, and divided into three equal portions, each of which received 1 ml containing  $1 \times 10^7$  EAC43P or EAC43. Incubation was continued at 30°C and 0.2-ml samples containing  $1 \times 10^7$  recipient cells and  $1 \times 10^6$  donor cells were withdrawn at timed intervals, added to 0.3 ml C-EDTA, and incubated for 60 min at 37°C to develop the recipient cell convertase sites. The  $t_{1/2}$  of EAC43B interacted with EAC43 was 4.5 min, while the  $t_{1/2}$  of EAC43B interacted with EAC43P, which had been formed with 200 and 400 ng  $\bar{P}$ , was prolonged to 24 and 60 min, respectively (Fig. 5, left panel).

To confirm that stabilization of the recipient convertase was directly related to the quantity of  $\bar{P}$  made available by the donor cells, a fixed quantity of  $\bar{P}$  was interacted with two different concentrations of donor cells, which were then analyzed for their capacity to stabilize EAC43B.  $4 \times 10^7$  EAC43 and  $1 \times 10^7$

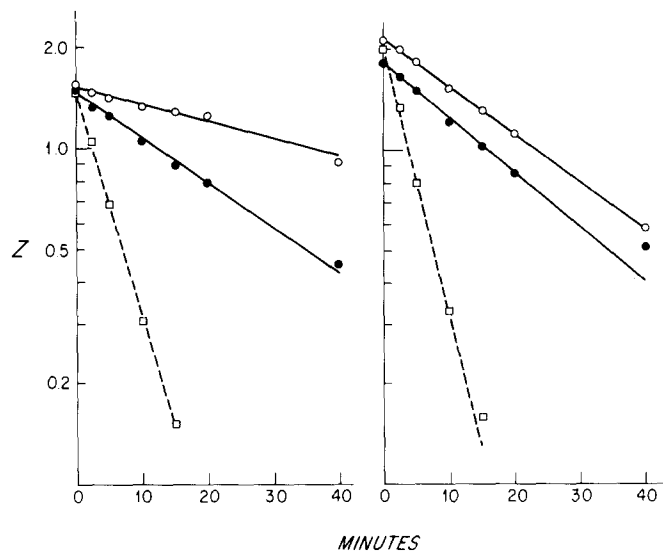


FIG. 5. (Left panel) Decay of EAC43B interacted with EAC43 ( $\square$ ) or EAC43P generated with 200 ( $\bullet$ ) and 400 ng  $\bar{P}$  ( $\circ$ ), respectively. (Right panel) Decay of EAC43B interacted with  $4 \times 10^7$  EAC43 ( $\square$ ),  $1 \times 10^7$  EAC43P ( $\bullet$ ) generated with 200 ng  $\bar{P}$ , or  $4 \times 10^7$  EAC43P ( $\circ$ ) generated with 200 ng  $\bar{P}$ .

EAC43 were incubated with 200 ng  $\bar{P}$  in 1 ml DGVB<sup>++</sup> for 10 min at 15°C, while  $4 \times 10^7$  EAC43 were incubated with DGVB<sup>++</sup> alone. After washing in ice-cold DGVB<sup>++</sup> and resuspension in 1 ml GVB-EDTA at 30°C, the donor cells were added to  $1 \times 10^8$  EAC43B in 1 ml GVB-EDTA, and transfer was assessed exactly as above. EAC43B incubated with EAC43 decayed with a  $t_{1/2}$  of 4 min, while addition of EAC43P, either  $1 \times 10^7$  or  $4 \times 10^7$  formed with the same amount of  $\bar{P}$ , resulted in a comparable degree of stabilization, extending the  $t_{1/2}$  to 19 and 20 min, respectively (Fig. 5, right panel). Since stabilization of the convertase site on EAC43B varied with the amount of bound  $\bar{P}$  made available and was independent of the number of EAC43P, transfer of  $\bar{P}$  to EAC43B must have occurred.

### Discussion

The capacity of  $\bar{P}$  to augment the hemolytic activity of  $\bar{D}$  was appreciated by three different approaches: chromatographic fractions of plasma that contained  $\bar{D}$  exhibited enhancement in their hemolytic titer when assayed in the presence of  $\bar{P}$ , while fractions containing trypsin-activable D were not augmented; the  $\bar{P}$  enhancement of  $\bar{D}$  activity was fully apparent with  $\bar{D}$  that presented a single band on alkaline disc gel electrophoresis coinciding with the position from which  $\bar{D}$  activity was eluted in replicate unstained gels; and treatment of purified  $\bar{D}$  with DFP reduced both the  $\bar{D}$  titer and the net increment observed with  $\bar{P}$  by 92%. The previous conclusion that  $\bar{P}$  permits the hemolytic assay of D by conversion to  $\bar{D}$  (12) is not supported and is now attributed to the presence of less than 10%  $\bar{D}$  in the preparations of D. The capacity of  $\bar{P}$  to increase also the hemolytic activity of limited B (Fig. 1) is consistent with the view that the action

of  $\bar{P}$  is not on an individual component but on the product of the interaction of several factors.

Although  $\bar{P}$ -dependent augmentation of the hemolytic titer of a set, limited amount of B in the presence of excess  $\bar{D}$  was appreciated initially by introduction of  $\bar{P}$  into the fluid phase (Fig. 1), the same result occurred when  $\bar{P}$  was bound to EAC43 before interaction with B and  $\bar{D}$ .  $\bar{P}$  did not bind to EAC4, and the extent of binding to EAC43 was related to the number of cells employed for adsorption (Fig. 2). Furthermore, the sum of the  $\bar{P}$  activity bound to EAC43 and that remaining in the supernate was equal to the initial input. Formation of EAC43P was slightly more rapid at 15°C than at 0°C, but was quantitatively equal at these temperatures (Fig. 3), and did not require divalent cations. In contrast to these findings are the temperature and  $Mg^{++}$  requirements (7) for binding of P in serum to zymosan, which probably relate to the conditions necessary for initial cleavage of C3 and binding of C3b to zymosan.

The mechanism by which  $\bar{P}$  augmented the hemolytic activity of either B or  $\bar{D}$  was elucidated when  $\bar{P}$  was shown to stabilize the otherwise labile convertase site on EAC43B, extending its  $t_{1/2}$  up to 10-fold in a dose-response fashion (Fig. 4). The fact that each intermediate exhibited the same number of initial sites, irrespective of its subsequent rate of decay, indicates that the effect of  $\bar{P}$  is to stabilize the cell-bound convertase rather than to uncover additional convertase sites. The additional observation that decay of convertase sites was first order at each dose of  $\bar{P}$  suggests that a limited amount of  $\bar{P}$  interacts equally among all sites. The reversible binding of  $\bar{P}$  to C3b on EAC43 was appreciated by transfer from EAC43P to EAC43B with the stability of the resulting intermediate, EAC43PB, being dependent on the amount of cell-bound  $\bar{P}$  available for transfer rather than the concentration of donor cells (Fig. 5). The capacity of  $\bar{P}$  to bind to C3b and stabilize  $C\bar{3}B$  contrasts with the inhibitory effect of the C3b inactivator on formation of this amplification convertase (15, 18).

### Summary

A function of  $\bar{P}$  in the alternative complement pathway is to prolong the first order decay of the hemolytic sites on EAC43B in a dose-dependent manner. As the number of initial convertase sites is not changed, even when activated properdin ( $\bar{P}$ ) increases the  $t_{1/2}$  10-fold or more,  $\bar{P}$  acts to stabilize rather than to uncover additional sites.  $\bar{P}$  binds to EAC43 to generate EAC43P in a reaction that proceeds slightly more rapidly at 15°C than at 0°C, but reaches the same plateau and does not require divalent cations. The presence of  $\bar{P}$  on EAC43P not only stabilizes the convertase subsequently formed on that cell, but, alternatively, permits transfer to convertase sites on other cells with the stability of the recipient intermediate being dependent on the  $\bar{P}$  available for transfer. The capacity of  $\bar{P}$  to bind to C3b and stabilize  $C\bar{3}B$  contrasts with the inhibitory effect of the C3b inactivator on formation of this amplification convertase.

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