

ALTERNATIVE PATHWAY OF COMPLEMENT: RECRUITMENT
OF PRECURSOR PROPERDIN BY THE LABILE
C3/C5 CONVERTASE AND THE POTENTIATION OF THE
PATHWAY*,‡

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This is the first description of the physiological mode of recruitment of precursor properdin (pre-P)¹ and of the physiological role of the recruited protein. It has now been established that properdin does not participate in the initiation of the properdin pathway (1). It has also been demonstrated that the properdin pathway can operate in absence of properdin, although with a reduced efficacy (1). These observations do not support the interpretation of earlier studies (2, 3), which were performed with activated properdin (\bar{P}), an altered form of the protein, which does not occur in fresh normal serum. Using \bar{P} , it was possible to generate a fluid phase C3 convertase in whole human serum (2, 4) and to stabilize the cell-bound C3/C5 convertases, which are exceedingly labile enzymes (5, 6). It was therefore assumed that the activation of properdin precedes the formation of the enzymes. In fact, properdin has been invoked as an essential component for the initiation of the pathway (7).

In this paper we wish to report that unaltered properdin, as it occurs in fresh serum and as it may be isolated under specified conditions,² enters the pathway as the final component. It is activated and bound by the solid phase complex of C3b and proactivator (Factor B). The net effect of properdin recruitment is entirely confined to the preservation of the existing enzymes by conferring

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¹ Abbreviations used in this paper: C3bINA, C3b inactivator; GPS, guinea pig serum; GVB⁺⁺, VB⁺⁺ containing 0.1% gelatin; GVBE, GVB without Ca⁺⁺ and Mg⁺⁺ containing 0.01 M EDTA; \bar{P} , activated properdin; pre-P, precursor properdin; VB⁺⁺, sodium Veronal-buffered saline, pH 7.3, containing 0.15×10^{-3} M CaCl₂ and 1×10^{-3} M MgCl₂; Z, 63% lysis.

² Götze, O., P. G. Medicus, and H. J. Müller-Eberhard. Alternative pathway of complement: nonenzymatic, reversible transition of precursor to active properdin. *J. Immunol.* In press.

physical stability upon them and by protecting them against the destructive action of the C3b inactivator.

Materials and Methods

Purified Components. Highly purified human C3 (8), proactivator (Factor B) (9), proactivator convertase (Factor D) (10), \bar{P} (2), C5 (8), C6 (11), and C7 (11) were isolated as described elsewhere. The components were trace labeled with ^{125}I by the chloramine-T method (12). C3b inactivator (C3bINA) was purchased from the Cordis Corp., Miami, Fla.

Diluents and Reagents. The following isotonic Veronal-NaCl buffers were used (13): VB^{++} , sodium Veronal-buffered saline, pH 7.3, containing 0.15×10^{-3} M CaCl_2 and 1×10^{-3} M MgCl_2 ; GVB^{++} , VB^{++} containing 0.1% gelatin; GVBE , GVB without Ca^{++} and Mg^{++} containing 0.01 M EDTA. VB^{++} of half physiological ionic strength (0.075) consisted of 1 vol VB^{++} and 1 vol 10% sucrose in water containing 1×10^{-3} M MgCl_2 .

C5-9 reagent consisted of guinea pig serum treated with 1 M KCNS at 4°C overnight and 0.015 M hydrazine at 37°C for 45 min. The dialyzed reagent is reconstituted with isolated human C5.

Partial Purification of pre-P. pre-P was partially purified from normal human serum by sequential TEAE and CM Sephadex chromatography. Normal human serum dialyzed against 0.02 M sodium phosphate buffer, pH 7.3, was applied to a TEAE column equilibrated with the same buffer. The first protein fraction which emerged from the column contained pre-P and was dialyzed against 0.02 M sodium phosphate buffer, pH 6.0, and applied to a CM Sephadex column equilibrated with the same buffer. The column was washed with starting buffer adjusted to 16 mmho/cm with NaCl. The pre-P was then eluted with starting buffer adjusted to 32 mmho/cm with NaCl. Although this preparation of pre-P still contained other proteins, the protein was completely in the precursor form and was found to behave functionally as highly purified pre-P.²

Preparation of EAC4b,3b. This intermediate complex was prepared by incubating EAC1,4b with nonoxidized C2 for 3 min at 30°C (14). The resulting EAC1,4b,2a cells were then converted to EAC4b,3b by treatment of 10^9 cells with 95 μg C3 for 15 min at 37°C and subsequent incubation for 120 min at 37°C in 0.01 M EDTA to remove C1 and C2a. These conditions produced cells containing from 15,000 to 30,000 C3b molecules per cell. In certain experiments the amount of C3 was varied to produce cells with less than 1,000 to over 60,000 C3b molecules per cell. The C3b molecules on these cells are focally distributed as was shown by electron microscopy (15).

Preparation of EC3b(try). Nonsensitized sheep erythrocytes were suspended at a concentration of 1×10^9 cells/ml. 1-ml aliquots of this cell suspension were centrifuged and the supernate decanted. From 0.1 to 7 mg of purified C3 was added to the cell pellets in a final vol of 1 ml VB^{++} . To each tube, trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added to achieve an enzyme to C3 ratio of 1:100 (weight per weight) and incubated at room temperature for 3 min. Thereafter, a 10-fold excess of soybean trypsin inhibitor over trypsin was added, and the cells were washed with buffer three times. The resulting complex, EC3b(try), contained from 500 to 33,000 C3b molecules per cell which were presumed to be randomly distributed over the cell surface.

Assay of pre-P. The reaction mixture contained 0.5 μg Factor B, 5 ng Factor D, 1×10^7 EAC4b,3b, and an appropriate amount of pre-P in a total vol of 50 μl GVB^{++} . This mixture was incubated at 37°C for 5 min. The reaction was stopped by the addition of EDTA to a final concentration of 0.01 M, and the labile convertase was decayed by further incubation for 10 min at 37°C. The pre-P-stabilized convertase was measured by the extent of hemolysis observed after the addition of 1 ml of 1:50 diluted guinea pig serum (GPS) in 0.04 M EDTA and incubation for 30 min at 37°C.

Assay of \bar{P} . Samples containing \bar{P} were incubated with 1×10^7 EAC4b,3b for 10 min at 37°C. The cells were then washed with GVB^{++} two times and the pellets resuspended in 50 μl GVB^{++} containing the same amounts of Factors B and D as in the pre-P assay, and the stabilized convertase was measured as above. The ratio of \bar{P} to total properdin activity represents the fractional content of \bar{P} in a given properdin preparation. This ratio was generally at least 0.8 for preparations of \bar{P} and no higher than 0.05 for preparations of pre-P.

Intermediate Complexes and Activity Assays Used for the Demonstration of the C3bINA Effect. The intermediate complex EC3b,P,B was prepared by incubating 1×10^7 EC3b containing 22,000 C3b molecules per cell with 1 μg of Factor B, 20 ng of Factor D, and 200 ng of pre-P in a total

vol of 50 μ l for 10 min at 37°C. EC3b, \bar{P} was prepared by incubating another aliquot of EC3b with 200 ng of \bar{P} for 10 min at 37°C in a total vol of 100 μ l GVB⁺⁺. EC3b, \bar{B} was prepared with the identical procedure, except that buffer instead of properdin was added. All intermediates were incubated in a final vol of 200 μ l with 50 U of C3bINA for 5 min at 20°C.

The residual activity of the EC3b, \bar{P} and EC3b was determined by washing the C3bINA-treated cells with GVB⁺⁺ and assembling the stabilized C5 convertase as above with Factors B, D and B, D, and \bar{P} , respectively. The activity remaining was measured by the addition of GPS-EDTA 1:50 for 20 min at 37°C (after 10-min decay of the labile enzyme) and compared with controls that were not treated with C3bINA.

The residual activity of EC3b, \bar{P} , \bar{B} was determined after washing the C3bINA-treated cells by the addition of GPS-EDTA 1:50 and incubation for 20 min at 37°C. The same procedure was followed for EC3b, \bar{P} , \bar{B} , except that C5-9 reagent was used for lysis instead of diluted GPS-EDTA.

The residual activity of the labile convertases was measured in the same way except that the washing and decay steps were omitted. Either C5-9 reagent or GPS-EDTA 1:50 were added directly after incubation with C3bINA for 5 min at 20°C. Lysis was allowed to develop for 20 min at 37°C.

Results

Activation of Properdin Precursor. The most important question asked in this investigation was: how is properdin activated? Although properdin occurs in serum as a precursor, it is usually isolated in an altered form. Because the isolated protein is capable of forming a soluble C3 convertase with Factors B, D, and C3, it has been termed activated properdin or \bar{P} (2, 4). Under specified conditions,² properdin may be isolated in precursor form. This form behaves like properdin in untreated, normal serum. Added to properdin-depleted serum, it causes no C3 consumption. But it participates in the alternative pathway after its initiation by particulate activators.

To investigate the mode of properdin recruitment, pre-P was labeled with ¹²⁵I and offered at physiological ionic strength to C3b-bearing erythrocytes. Unless stated otherwise, these cells are EAC4b,3b cells which will be referred to in the following simply as EC3b. As seen in Fig. 1, no uptake of ¹²⁵I pre-P occurred, although ¹²⁵I \bar{P} was bound in a dose-related fashion. In contrast, uptake of pre-P was observed reproducibly under conditions that allowed formation of the labile C3/C5 convertase (Fig. 2). Uptake was dependent on the simultaneous presence of Factors B, D, and Mg, or on the prior formation of the enzyme to which pre-P binding occurred even in the presence of EDTA. Uptake required a concentration of pre-P approaching that in normal serum to saturate all pre-P binding-activation sites. In spite of the high pre-P concentration, uptake did not exceed 0.5% of input with the number of cells employed. Repeated exposure of the supernate to aliquots of fresh EC3b, \bar{B} resulted in identical pre-P uptake, showing that all of the pre-P was potentially capable of undergoing activation. There was no detectable activation or inactivation of pre-P in the fluid phase. The loss of pre-P activity in the fluid phase was fully accounted for by the cellular uptake. Thus, evidence for turnover of pre-P by EC3b, \bar{B} was not obtained. Although the activating principle constitutes an enzyme, activation of pre-P does not appear to be due to an enzymatic reaction. Evidence for the physical nature of the transition of pre-P to \bar{P} will be presented.²

The Functional Manifestations of Binding-Activation of pre-P. The effect of pre-P binding on the cell-bound C3b, \bar{B} enzyme is illustrated in Fig. 3. After addition of pre-P, the cells were held at 37°C in 0.01 M EDTA for 10 min to effect decay of the labile C3b, \bar{B} complex. Subsequent exposure to C3-9 led to lysis of

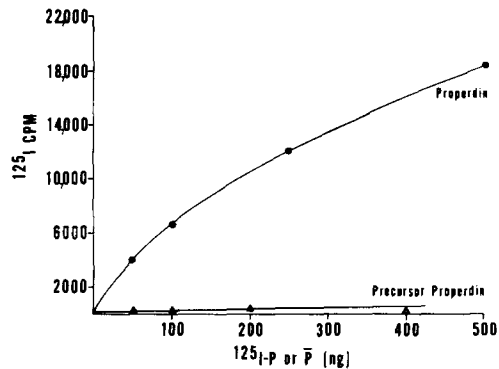


FIG. 1. Binding of \bar{P} but not of pre-P to EC3b at physiological ionic strength. To 1×10^7 EC3b containing 27,000 C3b molecules per cell, either $^{125}\text{I}-\bar{P}$ or ^{125}I -pre-P were added in a total vol of 100 μl . After 10 min at 37°C, the cells were washed and the uptake of radioactivity was determined. The specific radioactivity was 6.4×10^5 cpm/ μg for \bar{P} and 1.1×10^5 cpm/ μg for pre-P.

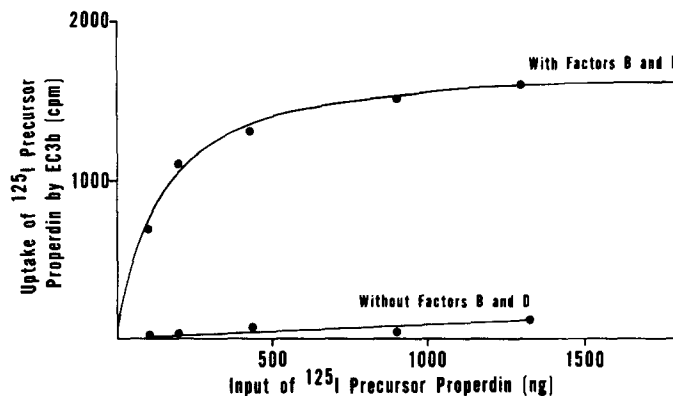


FIG. 2. The mode of recruitment of pre-P at physiological ionic strength. To 1×10^7 EC3b containing 15,700 C3b molecules per cell was added 2 μg of Factor B and 30 ng of Factor D and precursor properdin in a final vol of 130 μl . After 10 min at 37°C, the cells were washed and the uptake of radioactivity determined.

the cells, indicating that a proportion of the enzyme complexes had been protected by pre-P against rapid decay. Therefore the effect of pre-P on the enzyme is the same as that of \bar{P} (5, 6).

The results show that binding-activation of pre-P is accomplished by the cell-bound complex of C3b and activated Factor B. Stoichiometric measurements using radiolabeled C3 and pre-P indicated that at saturation of all pre-P binding sites, one molecule of pre-P was bound per 30–50 molecules of cell-bound C3b. The results of five different experiments are listed in Table I. They may be interpreted to indicate that a multiplicity of bound C3b molecules is required for the pre-P-activating complex.

The C3 and C5 Convertase Function of the Labile and the pre-P-Stabilized Enzyme. In the following it will be shown that the cell-bound complex of C3b and activated Factor B can enzymatically act on C3 and on C5. Fig. 4 shows C3 uptake and overall consumption by the cell-bound, pre-P-stabilized enzyme. C3

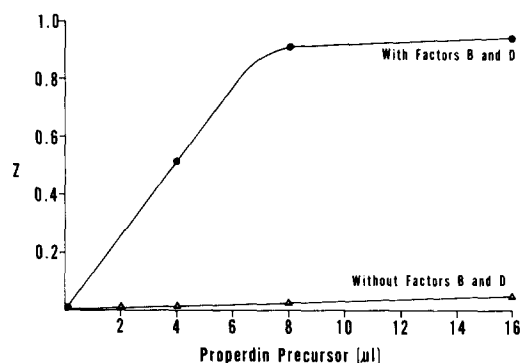


FIG. 3. Stabilization of the labile $EC3b, P, B$ complex by binding-activation of pre-P. To 1.4×10^7 $EC3b$ containing 7,000 C3b molecules per cell was added pre-P, 0.5 μg of Factor B, and 40 ng of Factor D in a final vol of 44 μl . After 10 min at 37°C, 100 μl of GVBE was added and the cells were washed and decayed for 10 min at 37°C in GVBE. Control: $EC3b$ plus pre-P, 10 min at 37°C, washed in GVB^{++} , Factors B and D added in amounts indicated above, decayed 10 min at 37°C in GVBE. Lysis was initiated in both sets of cells by addition of GPS-EDTA 1:50 and developed for 20 min at 37°C.

TABLE I
Properdin/C3b Ratio at Saturation of all Binding-Activation Sites for Precursor Properdin on $EC3b$

| C3b molecules bound per cell | pre-P molecules bound per cell | $\frac{\text{Molecules C3b}}{\text{Molecules pre-P}}$ |
|------------------------------|--------------------------------|---|
| 10,700 | 200 | 54 |
| 15,700 | 476 | 33 |
| 26,800 | 783 | 34 |
| 27,000 | 900 | 30 |
| 27,200 | 610 | 45 |

To 5×10^7 $EC3b$, containing the indicated number of C3b molecules per cell, was added 5 μg of Factor B, 200 ng of Factor D, and 1 μg of ^{125}I -labeled pre-P in a total vol of 250 μl GVB^{++} . After 10 min at 37°C, the cells were washed and the uptake of radioactivity determined. The sp act of the ^{125}I pre-P was 1.1×10^5 cpm/ μg . The number of C3b molecules per cell was determined using C3 with low specific radioactivity.

uptake approached 23% of total consumption. The apparent turnover of C3 was 35 molecules per single $C3b, P, B$ site per 15 min at 37°C.

Fig. 5 shows the ability of $C3b, P, B$ to act directly on C5 without requiring a supply of native C3. In this experiment, which is representative of more than 10, highly purified C5, C6, and C7 were reacted with cells bearing the stabilized enzyme and the formation of C5b,6,7 sites was measured subsequently. The turnover of C5, although hemolytically effective, was exceedingly slow. Uptake of C5 did not exceed one to two molecules per single pre-P-stabilized enzyme site per 15 min at 37°C. Consumption of C5 in the fluid phase was not measurable.

It should be stressed that the unstabilized enzyme also is capable of acting on C5 and of initiating cell lysis in the absence of C3. The C5 convertase function of the labile enzyme can be demonstrated only under conditions allowing regeneration of the enzyme, that is, in presence of excess Factors B and D.

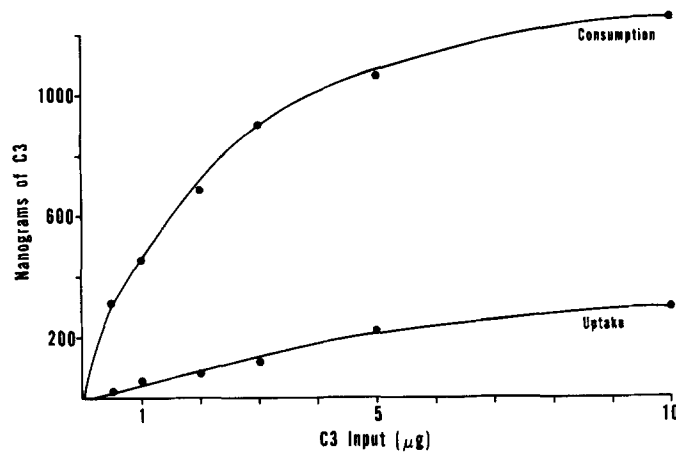


FIG. 4. The C3 convertase function of the pre-P-stabilized enzyme. To 1.4×10^8 EC3b containing 10,700 C3b molecules per cell was added 2.5 μg of Factor B, 150 ng of Factor D, and 4 μg of pre-P in a total vol of 100 μl . After 10 min at 37°C, the cells were washed in GVBE, the labile enzyme was decayed for 10 min at 37°C, and ^{125}I -C3 was added in amounts from 0 to 10 μg in a final vol of 100 μl . After 15 min at 37°C, the cells were centrifuged, and C3 was measured by hemolytic assay and on the cells by radioactivity measurement.

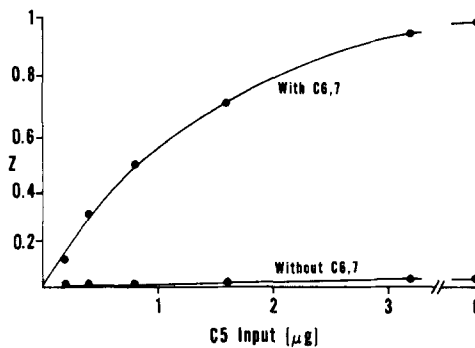


FIG. 5. The C5 convertase function of the pre-P-stabilized enzyme and the lack of native C3 requirement. To 1×10^7 EC3b containing 10,500 C3b molecules per cell was added 0.5 μg of Factor B, 20 ng of Factor D, and 150 ng of P. After 10 min at 37°C, the cells were washed and suspended in 60 μl GVB⁺⁺ containing C5 and 0.1 μg each of C6 and C7. After 10 min at 37°C, 1 ml of GPS-EDTA 1:500 was added as a source of C8,9, and lysis was allowed to develop for 30 min at 37°C.

Assembly of the Stabilized C3/C5 Convertase as a Function of Number and Distribution of Cell-Bound C3b Molecules. Does the C5 convertase differ in composition from the C3 convertase, specifically in C3b content? Since the alternative C5 convertase so far could only be generated on a surface and not in free solution, cells were prepared bearing C3b in two distinct distributions. EAC4b,3b cells exhibit on their surface circumscribed clusters of bound C3b molecules (14). In contrast, EC3b(try) cells, prepared by treating E with C3 and trypsin, are likely to contain a random distribution of C3b molecules on their surface, because attachment to the cells occurred from the fluid phase and not through the membrane bound C3 convertase. ^{125}I -labeled C3 was used for the preparation of these complexes in order to be able to enumerate the bound C3b

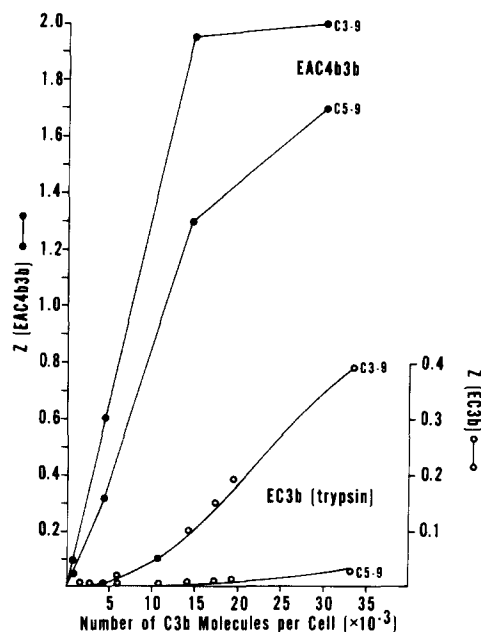


FIG. 6. Dependence of pre-P-stabilized C5 convertase on number and distribution of cell-bound C3b molecules. To 1×10^7 EAC4b,3b or EC3b(trypsin) was added $4.2 \mu\text{g}$ of Factor B, 20 ng of Factor D, and 100 ng of pre-P in a total vol of $30 \mu\text{l}$. After 10 min at 37°C , $20 \mu\text{l}$ of GVBE was added, and the labile enzyme was decayed for 10 min at 37°C . Either $200 \mu\text{l}$ of C3-9 or $200 \mu\text{l}$ of C5-9 were added, and lysis was determined after 30 min at 37°C .

molecules. The two distinct cell preparations were then subjected to the assembly of pre-P-stabilized C3/C5 convertase with the precursor proteins of the enzyme present in excess. As is demonstrated in Fig. 6, EAC4b,3b allowed the formation of the enzyme at low numbers of C3b molecules per cell, EC3b(trypsin) only at high numbers of C3b molecules per cell. Treating the cells with C3-9, which allows to assay for C3 convertase activity, both types of cells underwent lysis, although at different C3b multiplicities per cell. Treating the cells with C5-9, which allows to assay for C5 convertase activity, EAC4b,3b was strongly positive, whereas EC3b(trypsin) was not. This shows that pre-P-stabilized C5 convertase can be formed on C3b-bearing cells without further C3 provided C3b is focally distributed on the cell surface. These results lend support to the concept that generation of C5 convertase, unlike that of C3 convertase, necessitates multiple C3b molecules in close proximity. They further show that these molecules may be firmly bound to the cell surface before assembly of the C5 convertase.

Functional Comparison of pre-P and \bar{P} . The functional difference between pre-P and \bar{P} has been pointed out in Fig. 1 which shows that at physiological ionic strength \bar{P} can bind to EC3b and pre-P cannot. That pre-P does recognize C3b may be demonstrated at half physiological ionic strength. As shown in Table II, binding under these conditions is specific and reversible by elevating the ionic strength to 0.15. The loose complex between EC3b and pre-P can be converted to the cell-bound stabilized C5 convertase upon addition of Factors B and D. That after binding-activation pre-P continues to differ in function from \bar{P}

TABLE II
Reversible Interaction of Precursor Properdin with Cell Bound C3b

| Treatment of ^{125}I -pre-P | Initial ionic strength | |
|--------------------------------------|------------------------|--------|
| | 0.15 | 0.075 |
| EC3b | | |
| Uptake (cpm) | 378 | 10,700 |
| C5 Convertase (z)* | 0.03 | 0.91 |
| Elution of pre-P at $\mu = 0.15$ | | |
| Supernatant (cpm) | — | 8,800 |
| Pellet (cpm) | — | 1,040 |
| EA | | |
| Uptake (cpm) | 389 | 1,966 |

* Measured after addition of Factor B, D, and Mg to cell pellet.
 Buffers contained 0.5 mM MgCl_2 . EC3b and EA: 5×10^7 cells. Total reaction vol: 200 μl . Reaction time: 10 min, 37°C.

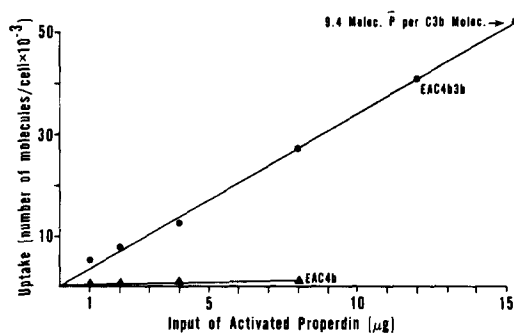


FIG. 7. Uptake of $\bar{\text{P}}$ by EC3b without apparent saturation. To 1.5×10^7 EAC4b,3b in GVB^{++} containing 5,500 C3b molecules per cell was added radiolabeled $\bar{\text{P}}$ in a total vol of 200 μl . After incubation for 10 min at 37°C, the cells were washed, and the radioactive uptake was determined. Uptake did not exceed 1% of input. At the highest input the concentration of properdin is $3\frac{1}{2}$ times that in serum. Control experiments were performed using EAC4b.

is apparent from uptake measurements. Fig. 7 illustrates an apparently unlimited capacity of $\bar{\text{P}}$ to bind to EC3b. As many as 10 molecules of $\bar{\text{P}}$ may bind to 1 molecule of C3b on the surface of EC3b. This is in striking contrast to the behavior of activated pre-P. Uptake of pre-P which only occurs in presence of Factors B and D yields initially a linear dose response curve and then becomes independent of input, indicating saturation of all binding sites (Fig. 2). At saturation the molar ratio of bound pre-P to bound C3b is 1 to 30-50. Fig. 8 indicates that the half-life of pre-P-stabilized C3/C5 convertase is 5 min at 37°C compared to 1.5 min of the unstabilized enzyme. This stabilization is independent of the number of pre-P molecules bound to the cells. When $\bar{\text{P}}$ is offered to EC3b under identical conditions, namely simultaneous with Factors B and D, the degree of stabilization observed is identical to that effected by pre-P. In contrast, when $\bar{\text{P}}$ is offered before the addition of Factors B and D, variable degrees of stabilization are found. The variability is dose dependent and is undoubtedly related to the uptake of $\bar{\text{P}}$ which, as shown in Fig. 7, is proportional to input.

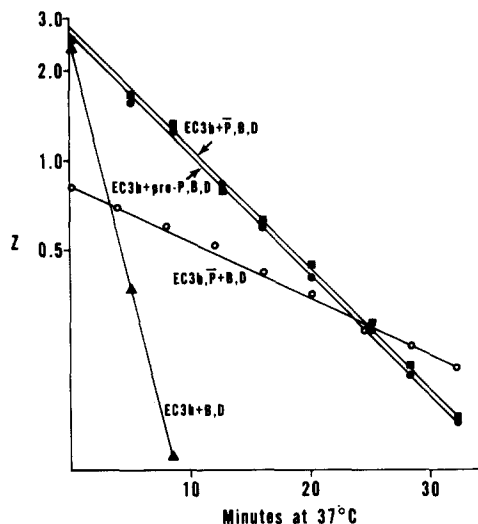


FIG. 8. Comparable enzyme stabilization by pre-P and \bar{P} and increased stabilization by \bar{P} upon reverse assembly of the enzyme. To 1×10^8 EC3b containing 30,000 C3b molecules per cell was added 3 μ g of Factor B, 200 ng of Factor D, and 400 ng of \bar{P} (EC3b + \bar{P} , B, D) or 400 ng of pre-P (EC3b + pre-P, B, D) in a final vol of 275 μ l. After 10 min at 37°C, 200 μ l GVBE was added, and the unstabilized enzyme was decayed for 10 min at 37°C. 40- μ l samples were then withdrawn at 4-min intervals and added to 1 ml GPS-EDTA 1:100. Lysis was quantitated after 20 min at 37°C. Reverse assembly (EC3b, \bar{P} + B, D) was performed under identical conditions, except that \bar{P} was offered during a 10-min preincubation period. For comparison, the decay of the unstabilized enzyme is shown (EC3b + B, D).

Three Modes of Regulation of the C3/C5 Convertase and of Its Subunits. The first mode of control is represented by the intrinsic decay of the C3/C5 convertase which is modified by pre-P as well as by \bar{P} , as described above.

The second mode of control is effected by the serum enzyme C3bINA. It acts on at least five different entities, which contain C3b and which are inactivated by the action of the enzyme on C3b. The relative susceptibility of the five complexes containing C3b and of C3b alone is indicated in Table III. The most susceptible entity is C3b per se, and the least susceptible is the complex C3b, \bar{P} , B. Properdin and Factor B exercise a protective function against C3bINA which properdin alone continues to provide to some extent after dissociation of Factor B. When the C3bINA succeeds in acting on the residual C3b, \bar{P} complex, properdin is released into the fluid phase as was demonstrated using ^{125}I - \bar{P} (Fig. 9).

The third mode of control involves the disassembly of the properdin-stabilized convertase by the accelerator of C3bINA (30). In experiments not shown here, it was found that the accelerator dissociates only Factor B from the complex while \bar{P} remains bound to C3b. After washing of the accelerator-treated cells, the stabilized enzyme activity could be fully recovered upon addition of Factors B and D.

Reverse Assembly of the Stabilized C3/C5 Convertase. Reverse assembly of the properdin-stabilized enzyme begins with the complex EC3b, \bar{P} which results from decay or active disassembly of the pre-P-stabilized form of the enzyme. Alternatively, it may begin with the binding of soluble \bar{P} to EC3b. Addition of Factor B to EC3b, \bar{P} leads to the Mg-dependent formation of EC3b, \bar{P} , B in absence

TABLE III
Susceptibility of Bound C3b and Various C3b Containing
Complexes to Inactivation by C3b Inactivator

| Intermediate complex | Activity remaining after treatment with C3bINA (5 min, 20°C) |
|------------------------|--|
| | % |
| EC3b,P,B | 100 |
| EC3b,P̄ | 90 |
| EC3b,B | 60 |
| EC3b _n ,B | 40 |
| EC3b _n ,P,B | 30 |
| EC3b | 5 |

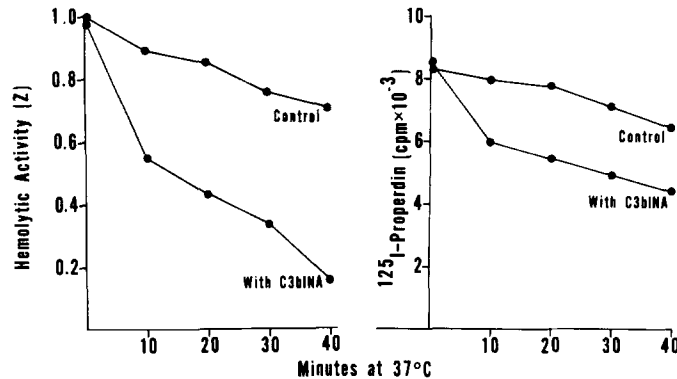


FIG. 9. Release of radioactive properdin upon destruction of EC3b,P̄ by C3bINA. The left panel shows the loss of hemolytic reactivity of EC3b,P̄ and the right panel the release of ¹²⁵I-properdin from the same cells during treatment with C3bINA. To 5×10^7 EC3b containing 34,700 C3b molecules per cell was added 5 μ g of ¹²⁵I P̄ in a final vol of 100 μ l. After 10 min at 37°C, the EC3b,P̄ was washed and exposed to C3bINA or buffer (control) at 37°C in a total vol of 110 μ l. At intervals, 10- μ l aliquots were removed and the cells washed and suspended in 100 μ l GVB⁺⁺ containing 1 μ g of Factor B and 20 ng of Factor D. After 10 min at 37°C, 100 μ l of GVBE was added and the labile enzyme decayed for 10 min at 37°C. 1 ml of GPS-EDTA 1:50 was added and lysis determined after 20 min at 37°C. Residual P̄ was detected by measuring the cell-associated radioactivity.

of Factor D. This complex exhibits no measurable enzymatic activity. On addition of Factor D, enzyme activity appears, and the uptake of Factor B is reduced by 30% (Fig. 10). The reduction in cell-bound radioactivity corresponds numerically to the relative size of the activation fragment (Ba) of Factor B, which is released from the complex (10). A low degree of Factor B binding was also observed with EC3b in absence of P̄. The Mg-dependent complexes of Factor B, EC3b,B and EC3b,P̄,B were dissociated by EDTA. The appearance of C3/C5 convertase activity upon reverse assembly of the enzyme is shown in Fig. 11 as a function of P̄ input.

Discussion

In this study we addressed ourselves to these questions: (a) What is the physiological mechanism of properdin activation? (b) What is the function of

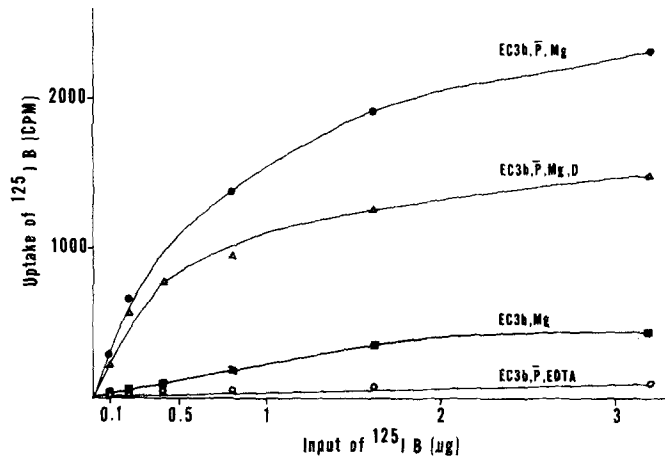


FIG. 10. Magnesium-dependent binding of nonactivated Factor B to cell-bound C3b or C3b, \bar{P} sites. To 1.4×10^7 EC3b containing 27,000 C3b molecules per cell was added 750 ng of \bar{P} . After 10 min at 37°C, the cells were washed in GVB⁺⁺ and suspended in 64 μl GVB⁺⁺ containing Factor B and, when indicated, 30 ng of Factor D. After 10 min at 37°C, the cells were washed either in GVB⁺⁺ or GVBE, and the radioactivity was measured. The uptake of Factor B on EC3b cells was performed the same way. The sp act of the ^{125}I -labeled Factor B was 150,000 cpm/ μg .

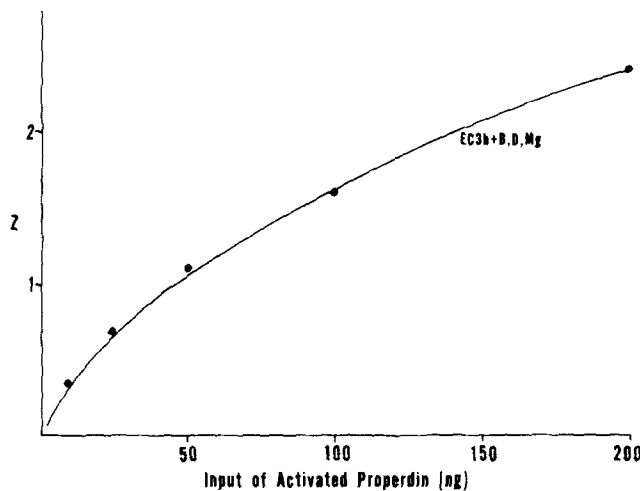


FIG. 11. Reverse assembly of the \bar{P} -stabilized C5 convertase on EC3b. To 8×10^6 EC3b(try) containing 13,800 C3b molecules per cell was offered \bar{P} , 470 ng of Factor B, and 60 ng of Factor D in a total vol of 27 μl . After 16 min at 37°C, 1 ml of GPS-EDTA 1:10 was added and lysis was allowed to develop for 20 min at 37°C.

activated pre-P under physiological conditions? (c) What is the composition of the multisubunit C3 and C5 convertases of which properdin appears to become a part?; and (d) How are these enzymes controlled?

At the beginning of this study we considered the possibility that particle-bound C3b might serve as the properdin-activating principle by imposing a conformational change on pre-P. Bound C3b had previously been recognized as

the receptor for \bar{P} (4). In contradistinction to \bar{P} , pre-P was found to be entirely incapable of interacting with bound C3b at physiological ionic strength, except in presence of Factors B, D, and Mg (Fig. 1, 2). Those, of course, are the conditions of formation of the labile C3/C5 convertase. Since pre-P was found to be bound by the assembled, cell-bound enzyme even in presence of EDTA, the complex of C3b and activated Factor B could be identified as the binding and activating principle of pre-P.

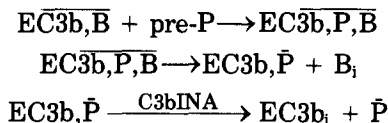
Immediately the question arose as to whether the binding-activation of pre-P is an enzymatic reaction. This possibility seemed remote, because the enzyme specificity of the complex is known to be directed toward C3 and C5. Nevertheless, the claim of an enzymatic activation of pre-P had been reported (16). Such a mechanism has virtually been excluded by the following findings: (a) there is no detectable turnover of pre-P; (b) one enzyme site causes binding of maximally one molecule of pre-P, as exploratory experiments with radiolabeled Factor B and pre-P indicate;³ and (c) no difference in molecular size can be detected between pre-P and \bar{P} subunits.² We therefore propose that the recruitment of pre-P by the labile C3/C5 convertase involves a nonenzymatic, conformational change of the molecule.

The fact that pre-P does not bind to EC3b at physiological ionic strength does not rule out its entering into reversible interaction with C3b. That the molecule does possess affinity for C3b could be shown by its adsorption to EC3b at unphysiologically low ionic strength (Table II). When after washing of EC3b,pre-P, Factors B and D were added, stabilized C5 convertase activity appeared. However, when EC3b,pre-P was exposed to physiological ionic strength, all of the pre-P was released from the cells into the fluid phase. This reversible interaction between pre-P and C3b is analogous to the behavior of other complement proteins which are designed to function in concert. Thus, reversible complexes are formed by the following proteins: C1q, C1r, and C1s (17, 18), C2 and C4 (19), C5, C6, C7, C8, and C9 (20), and Factor B and C3b (21, Fig. 11). That at low ionic strength pre-P is present in whole human serum as a complex, possibly with native C3, has been reported by Chapitis and Lepow (22).

The functional differences between pre-P and \bar{P} are considerable. \bar{P} appears to aggregate on the surface of EC3b, as evidenced by the fact that uptake of \bar{P} molecules may greatly exceed the number of C3b molecules on EC3b (Fig. 7). Such unlimited binding does not occur with pre-P. In the presence of Factors B and D, pre-P binding approaches saturation when one molecule is bound per 30 molecules of C3b (Table I, Fig. 2). With regard to stabilization of the enzymes, the effect of pre-P and \bar{P} is quantitatively identical and independent of dose, provided the two properdin forms are reacted with EC3b in the presence of Factors B and D (Fig. 8). If \bar{P} is first allowed to attach to EC3b and Factors B and D are added subsequently, enzyme stabilization is variable and dose dependent as also reported by Fearon and Austen (5). The dose-related increase of enzyme stabilization may be due to the above mentioned aggregation of \bar{P} on EC3b, which occurs in absence of Factors B and D but not in their presence. The more \bar{P} molecules are clustered around on enzyme site, the greater the stabilization. With Factors B and D in the reaction mixture, \bar{P} produced a given number of

³ Medicus, R. G., O. Götze, and H. J. Müller-Eberhard. Unpublished observations.

stabilized C5 convertase sites which decayed with a half-life of 7 min. When the same amount of \bar{P} (same preparation) was added to EC3b before Factors B and D, fewer stabilized C5 convertase sites were produced, but these sites had a half-life of 18 min (Fig. 8). Whether \bar{P} is a physiological form of properdin or a laboratory artifact remains to be established. It is conceivable that soluble \bar{P} arises through the action of the C3bINA according to the following expression:



The composition of the C3 and the C5 convertases differs in the number of C3b molecules. The C3 convertase in free solution (23) or bound to the surface of a cell (24) probably is composed of only one molecule of C3b and one molecule of activated Factor B. In contrast, the C5 convertase requires more than one molecule of C3b. The fact that C5 convertase activity could not be detected in free solution (2, 4, 25), but appears under appropriate conditions on particles, indicated that assembly of the enzyme requires a surface. As we have shown in this study, a surface allows assembly of C5 convertase because it imposes the constraint upon two or more adjacent, surface bound C3b molecules to remain in a critical topographical relationship. When C3b is randomly distributed on the surface of a cell, only C3 convertase activity appears after addition of Factors B and D. When C3b is distributed in clusters so as to provide a critical density of these molecules, C3 and C5 convertase activity is generated upon addition of Factors B and D (Fig. 6). Alternatively, a C3 convertase site can convert itself into a C5 convertase site by catalyzing uptake of additional C3b molecules. These experiments show that the additional C3b molecule is as firmly attached to the cell membrane as the initial molecule and not as a terminal subunit to the $\overline{C3b, B}$ complex. A difference in subunit composition between the C3 and the C5 convertase was previously inferred from kinetic studies by Brade et al. (26).

Examining more closely the data presented in Fig. 6, it becomes necessary to distinguish between the C3b multiplicity required for binding-activation of pre-P and that required for expression of stabilized C5 convertase activity. For instance, to achieve the equivalent of 0.42 Z ($Z = 63\%$ lysis) of stabilized C3 convertase activity, 3,000 focally distributed or 33,000 randomly distributed C3b molecules were needed. At the level of 33,000 randomly distributed C3b molecules, the activity of the stabilized C5 convertase (0.02 Z) measured only one-twentieth of that of the stabilized C3 convertase. Accordingly, we must assume that if binding-activation of pre-P requires two C3b molecules, expression of C5 convertase activity requires at least three C3b molecules. This conclusion is further supported by the marked susceptibility to C3bINA action of the stabilized C5 convertase ($\overline{C3b_n, P, B}$) and the relative resistance of the stabilized C3 convertase ($\overline{C3b, P, B}$) (Table III). For simplicity's sake, we propose to omit reference to the C3b multiplicity required for pre-P binding-activation in the systematic denotations of the enzymes and to use the letter n to refer to the greater C3b multiplicity required for C5 convertase activity ($\overline{C3b_n, B}$ or $\overline{C3b_n, P, B}$) compared to the requirements for C3 convertase activity ($\overline{C3b, B}$ or $\overline{C3b, P, B}$).

The composition of the alternative pathway C3 and C5 convertases is thus very similar to that of the classical convertases (27). $C3b, \bar{B}$ corresponds to $C4b, \bar{2}$, where C3b and C4b fulfill the function of the acceptor and modulator of Factor B and C2, respectively, and where the latter two entities carry the serine protease site (28). The additional C3b in $C3b_n, \bar{B}$ and C3b molecule in $C4b, 2, 3b$ serve to confer C5 convertase function on the respective enzymes.

How does properdin fulfill its stabilizing function? Properdin is an asymmetrical, string-like molecule composed of four presumably identical subunits (10, 29). Assuming a mol wt of 212,000 an s rate of 5.2S, and a \bar{v} of 0.70, the f/f_0 may be calculated to be approximately 2.0. This value corresponds to an axial ratio of 1:20. For reasons mentioned above, it is likely that one molecule of properdin enters in physical contact with at least two adjacent C3b molecules and one or two molecules of activated Factor B. It is therefore not improbable that the string-like conformation of pre-P becomes rearranged upon binding-activation, such as to impose constraints on the lateral movement of the C3b molecules and on the dissociation of Factor B from the complex.

Studying the effects of pre-P, it became apparent that it prolongs the half-life of the labile C3/C5 convertase only three-fourfold (Fig. 8). The more important biological function of properdin therefore may be its protection of C3b from the destructive action of the C3bINA (6). The C3b that is critical for C5 convertase activity is relatively unprotected (Table III). The C3b in the stabilized C3 convertase, $C3b, P, \bar{B}$, is highly protected. The C3b in the complexes $C3b, \bar{B}$ and $C3b, \bar{P}$ is partially protected. Accordingly, the physiological function of pre-P is a dual one: to protect C3b in most of its complexes against C3bINA and to diminish the entropy of the assembled C3/C5 convertase.

To date, three modes of regulation are known for the enzymes of the pathway: spontaneous decay, destruction by C3bINA, and active disassembly by β_{1H} . The protein β_{1H} was observed in this laboratory in 1965 as a protein without known function (9). Whaley and Ruddy (30) showed β_{1H} to be the accelerator of C3bINA and, more importantly, to have a controlling function of its own.

The main molecular events of the properdin pathway are summarized schematically in Fig. 12. Only those reactions are shown that proceed on or near the surface of an activating particle. The fluid phase side reactions and the regulatory events have been omitted for the sake of clarity. The pathway comprises four essential components: initiating factor, Factors B, D, and C3. Properdin itself functions as an accessory component. Factor B represents the principal zymogen, being the catalytic subunit in the initial C3 convertase ($S_1 \cdot \bar{F}, \bar{B}, \bar{C3}$), the solid phase C3 convertase ($S_{II} \cdot C3b, \bar{B}$), and in the C5 convertase ($S_{II} \cdot C3b_n, \bar{B}$). Factor D constitutes the second zymogen of the pathway. It participates in two reactions, the assembly of the initial C3 convertase and of the solid phase C3 convertase. It has a dual function. It activates Factor B by enzymatic cleavage when it is in complex with C3b. And it activates Factor B in a nonenzymatic, reversible reaction when it is in complex with nephritic factor and C3 (4), or by implication with initiating factor and C3. Reversible activation of Factor B was first observed by Day and coworkers (31, 32). The protein C3 fulfills multiple functions. It acts in native form as modulator of Factor B in the initial C3 convertase complex. As cell-bound C3b, it functions as anchor of the cell-bound

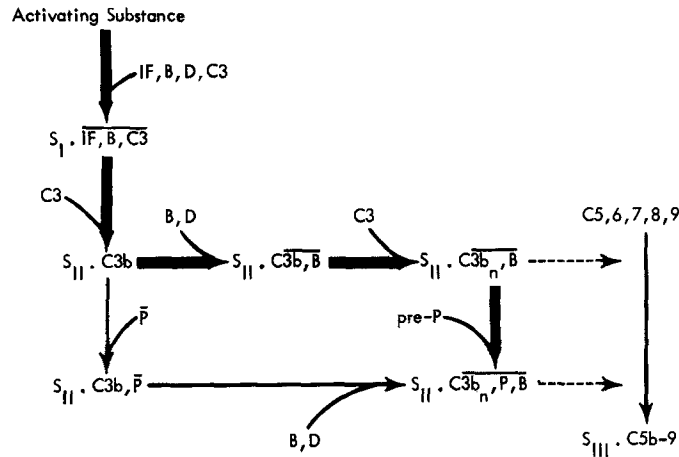


FIG. 12. Schematic representation of the major molecular events of the properdin pathway. For detailed explanation see text.

C3 and C5 convertases. It also constitutes the acceptor and modulator of activated Factor B within these enzymes.

After the deposition of C3b on the surface of activating particles by the initial C3 convertase, the C3 and C5 convertases are assembled. The C5 convertase requires for its formation multiple surface bound C3b molecules in close physical proximity. In acting on C5 the enzyme initiates the self-assembly of the membrane attack complex, C5b-9. That the pathway is effective without properdin has also been demonstrated by the lysis of rabbit erythrocytes in properdin and C4-depleted serum (1). The enzyme is labile and is the target of regulators such as the C3bINA. Properdin not only lends stability to the enzyme complex, but also provides some protection against the C3bINA. Precursor properdin recognizes C3b and enters into reversible interaction with it. Binding and activation of pre-P occurs only when it collides with the surface-bound complex of at least two molecules of C3b and one molecule of activated Factor B. Thus, the assembly of the enzyme is a prerequisite for pre-P recruitment. This behavior is in contradistinction to that of \bar{P} , which can bind directly to bound C3b and then engage in reverse assembly of the C3/C5 convertase. Although properdin fulfills a nonessential function *in vitro*, it appears probable to us that, because of its role as a potentiator of the alternative pathway, properdin deficiency *in vivo* might result in perturbation of natural resistance to infections.

Summary

In this study the physiological role of properdin and the differential subunit composition of the solid phase enzymes of the pathway have been explored.

Cell-bound C3 and C5 convertase differ in their C3b requirement. Apparently one molecule of C3b is sufficient to allow formation of C3 convertase ($C3b, \bar{B}$), whereas two or more are required for generation of C5 convertase ($C3b_n, \bar{B}$). This conclusion was drawn from results indicating the critical role of the spacial distribution of C3b molecules on the cell surface in enzyme formation.

While the C3/C5 convertase is fully capable of acting on C5 and thereby

initiating the assembly of the cytolytic membrane attack complex, it is exceedingly labile and vulnerable to destruction by the C3b inactivator. It is the apparent role of properdin to confer a degree of stability upon the labile enzyme and to protect its C3 convertase function against enzymatic destruction. To achieve these effects, precursor properdin (pre-P) is recruited in a binding-activation reaction by the labile C3/C5 convertase. Multiple C3b molecules appear to be needed for the formation of properdin-activating principle.

Three modes of regulation have been described, which involve spontaneous dissociation enzymatic degradation by C3b inactivator and disassembly by β_{1H} .

The functional differences of pre-P and activated properdin (\bar{P}) were delineated, pre-P displaying a weak affinity for C3b and \bar{P} the capacity of strong interaction, \bar{P} generating a soluble C3 convertase in serum and pre-P being unable to do so. Because of the profound differences between native pre-P and the laboratory product \bar{P} , the question was raised as to whether soluble \bar{P} represents an unphysiological form of the protein.

On the basis of this and other studies, the conclusion was reached that in vitro properdin recruitment constitutes the terminal event of the properdin pathway, and that properdin augments the function of C3/C5 convertase without changing its substrate specificity.

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References

1. Schreiber, R. D., O. Götze, and H. J. Müller-Eberhard. 1976. Alternative pathway of complement: demonstration and characterization of initiating factor and its properdin-independent function. *J. Exp. Med.* 144:1062.
2. Götze, O., and H. J. Müller-Eberhard. 1974. The role of properdin in the alternate pathway of complement activation. *J. Exp. Med.* 139:44.
3. Fearon, D. T., and K. F. Austen. 1975. Inhibition of complement-derived enzymes. *Ann. N. Y. Acad. Sci.* 256:441.
4. Schreiber, R. D., R. G. Medicus, O. Götze, and H. J. Müller-Eberhard. 1975. Properdin- and nephritic factor-dependent C3 convertases: requirement of native C3 for enzyme formation and the function of bound C3b as properdin receptor. *J. Exp. Med.* 142:760.
5. Fearon, D. T., and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J. Exp. Med.* 142:856.
6. Medicus, R. G., O. Götze, and H. J. Müller-Eberhard. 1976. Activation of properdin (P) and assembly and regulation of the alternative pathway C5 convertase. *J. Immunol.* 116:1741.
7. Fearon, D. T., and K. F. Austen. 1975. Properdin: initiation of alternative complement pathway. *Proc. Natl. Acad. Sci. U. S. A.* 72:3220.
8. Nilsson, U., and H. J. Müller-Eberhard. 1965. Isolation of β_{1F} -globulin from human serum and its characterization as the fifth component of complement. *J. Exp. Med.* 122:277.
9. Götze, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternate pathway of complement activation. *J. Exp. Med.* 134:90s.
10. Götze, O. 1975. Proteases of the properdin system. In *Proteases and Biological Control*. E. Reich, D. B. Rifkin, and E. Shaw, editors. Cold Spring Harbor Laboratory. 255.

11. Podack, E. R., W. P. Kolb, and H. J. Müller-Eberhard. 1976. Purification of the sixth and seventh component of human complement without loss of hemolytic activity. *J. Immunol.* 116:263.
12. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29:185.
13. Mayer, M. M. 1961. Complement and complement fixation. In *Experimental Immunochimistry*. E. A. Kabat, and M. M. Mayer, editors. Charles C Thomas, Springfield, Ill. 133.
14. Cooper, N. R., M. J. Polley, and H. J. Müller-Eberhard. 1970. The second component of human complement (C2): quantitative molecular analysis of its reactions in immune hemolysis. *Immunochemistry.* 7:341.
15. Müller-Eberhard, H. J., U. Nilsson, A. P. Dalmasso, M. J. Polley, and M. A. Calcott. 1966. A molecular concept of immune cytotoxicity. *Arch. Pathol.* 82:205.
16. Stitzel, A. E., and R. E. Spitzer. 1974. The utilization of properdin in the alternate pathway of complement activation: isolation of properdin convertase. *J. Immunol.* 112:56.
17. Lepow, I. H., G. B. Naff, E. W. Todd, J. Pensky, and C. F. Hinz, Jr. 1963. Chromatographic resolution of the first component of human complement into three activities. *J. Exp. Med.* 117:983.
18. Valet, G., and N. R. Cooper. 1974. Isolation and characterization of the proenzyme form of the C1r subunit of the first complement component. *J. Immunol.* 112:1667.
19. Müller-Eberhard, H. J., M. J. Polley, and M. A. Calcott. 1967. Formation and functional significance of a molecular complex derived from the second and the fourth component of human complement. *J. Exp. Med.* 125:359.
20. Kolb, W. P., J. A. Haxby, C. M. Arroyave, and H. J. Müller-Eberhard. 1972. Molecular analysis of the membrane attack mechanism of complement. *J. Exp. Med.* 135:549.
21. Vogt, W., W. Dames, G. Schmidt, and L. Dieminger. 1976. Complex formation of properdin Factor B with C3b, an essential step in the generation of the C3 cleaving enzyme of the properdin system. *J. Immunol.* 117: In press.
22. Chapitis, J., and I. H. Lepow. 1976. Multiple sedimenting species of properdin in human serum and interaction of purified properdin with the third component of complement. *J. Exp. Med.* 143:241.
23. Müller-Eberhard, H. J., and O. Götze. 1972. C3 proactivator convertase and its mode of action. *J. Exp. Med.* 135:1003.
24. Fearon, D. T., K. F. Austen, and S. Ruddy. 1973. Formation of a hemolytically active cellular intermediate by the interaction between properdin factors B and D and the activated third component of complement. *J. Exp. Med.* 138:1305.
25. Vallota, E. H., O. Götze, H. L. Spiegelberg, J. Forristal, C. D. West, and H. J. Müller-Eberhard. 1974. A serum factor in chronic hypocomplementemic nephritis distinct from immunoglobulins and activating the alternate pathway of complement. *J. Exp. Med.* 139:1249.
26. Brade, V., G. D. Lee, A. Nicholson, H. S. Shin, and M. M. Mayer. 1973. The reaction of zymosan with the properdin system in normal and C4-deficient guinea pig serum; demonstration of C3- and C5-cleaving multi-unit enzymes, both containing factor B, and acceleration of their formation by the classical complement pathway. *J. Immunol.* 111:1389.
27. Müller-Eberhard, H. J. 1975. Initiation of membrane attack by complement: assembly and control of C3- and C5-convertase. In *Proteases and Biological Control*. E. Reich, D. B. Rifkin, and E. Shaw, editors. Cold Spring Harbor Laboratory. 229.
28. Medicus, R. G., O. Götze, and H. J. Müller-Eberhard. 1976. The serine protease nature of the C3 and C5 convertases of the classical and alternative complement pathways. *Scand. J. Immunol.* In press.

29. Minta, J. O., and I. H. Lepow. 1974. Studies on the subunit structure of human properdin. *Immunochemistry*. 11:361.
30. Whaley, K., and S. Ruddy. 1975. C3b inactivator accelerator (A·C3bINA): a serum protein of the complement system required for full expression of C3b inactivator (C3bINA) activity. *Fed. Proc.* 35:654.
31. Day, N. K., and H. J. Müller-Eberhard. 1974. Cold-dependent activation of the alternate complement pathway. *Fed. Proc.* 33:775.
32. Day, N. K., R. D. Schreiber, O. Götze, and H. J. Müller-Eberhard. 1976. Reversible activation of proactivator (B) without cleavage of the molecule. *Scand. J. Immunol.* In press.