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A molecular concept of the properdin pathway

(complement activation/initiating factor/properdin activation/properdin receptor/multisubunit enzymes)

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ABSTRACT **The sequential events of the properdin system were analyzed. Properdin-depleted serum allows the formation of a Factor B- and D-dependent C3 convertase. This enzyme, called the properdin-receptor-forming enzyme, was shown to utilize a novel serum component, the initiating factor. The protein is a β-globulin in precursor form and is distinct from immunoglobulins. The function of the enzyme is to deposit C3b on the surface of activator particles. Apparently doublets of C3b are required for the formation of the properdin-activating principle. It consists of a complex containing surface-bound C3b and activated Factor B. Properdin precursor is activated by binding to this complex without detectable change in molecular weight. The transition of properdin precursor to activated properdin is probably caused by a conformational change. The complex, consisting of bound C3b, properdin, and activated Factor B, represents the enzyme that acts on C5, thereby initiating self-assembly of the membrane attack system. Native C3 is not needed for the function of the enzyme. It is disassembled by soluble C3 or C3b and its formation is under the control of the properdin-receptor-destroying enzyme, which may be identical with the C3b inactivator.**

More than 20 years ago Pillemer and his associates approached the problem of natural resistance to infections on the molecular level. A novel serum protein, termed properdin, was envisaged to cooperate with hydrazine-sensitive "Factor A" and heat-labile "Factor B" in the activation of complement resulting in natural resistance against certain viruses and bacteria (1, 2).

Today we recognize the properdin system or the alternative pathway of complement activation to consist of five components: properdin (P) (3), the third component of complement, C3 (Factor A) (4, 5), proactivator (Factor B) (6), proactivator convertase (Factor D) (5, 7, 8) and the initiating factor (Factor I or IF) (9), which will be introduced in this communication as a heretofore unknown component.

Four key questions have confronted us during the current investigation. First, how is the pathway initiated by such activators as bacterial or fungal cell walls? Second, how is properdin recruited and what is its function? Third, what is the nature of the enzyme that activates the membrane attack system of complement? And fourth, what are the regulatory principles of the pathway?

On the basis of a large body of experimental evidence, which will be fully documented elsewhere (9–11), we wish to propose a comprehensive molecular concept of the properdin pathway. In brief, the dynamics of the pathway involve assembly of the properdin-receptor-forming enzyme (PRFE) as a consequence of activation of the initiating factor, assembly of the properdin-activating principle, and formation of the properdin-dependent convertase which catalyzes opsonization and activation of the membrane attack system.

Abbreviations: P, properdin; C, complement, a lower-case letter indicates a fragment of a component, an overbar, —, indicates an activated component; IF, initiating factor; PRFE, properdin-receptor-forming enzyme; NF, nephritic factor; S, surface site; E, erythrocyte; PRDE, properdin-receptor-destroying enzyme

Initiation of the properdin pathway without properdin (P)

In exploring the possible role of P in the initiation of the pathway, serum was immunochemically depleted of P. In addition, to block the classical pathway, C4 was removed from the same serum. Treatment of the depleted serum with yeast cell walls (zymosan) resulted in uptake of C3 by the particles. This uptake was dependent upon the presence of Factors B and D and magnesium ions and, as will be described below, on Factor I (Table 1). Treatment for 60 min at 37° caused 15–22% consumption of serum C3. In absence of zymosan, B, or D, C3 consumption did not exceed 7%. This finding pointed out the existence of a C3 convertase that utilized B and D but not P, and that deposited C3 on particles, probably in the form of C3b (9). Our observations preclude a role of properdin in the initiation of the pathway and lend no support to the concept proposed by Fearon and Austen according to which properdin is required for formation of the initial C3 convertase (12).

Recognition of an initiating factor distinct from properdin

Another clue for the existence of a P-independent C3 convertase was derived from work on nephritic factor (NF), which is an unusual serum protein occurring in certain nephritides (13). NF associates with B, D, C3, and Mg to form a soluble C3 convertase which is functionally indistinguishable from its P-dependent counterpart (14).

It was hypothesized that an NF-analogue is present in normal serum in precursor form and constitutes an essential component of the initial C3 convertase. An antiserum to NF was utilized to explore normal serum for the presence of an NF-analogue. Anti-NF-treated serum failed to sustain C3 consumption upon prolonged incubation with zymosan. The incubated particles, examined by the immunofluorescence technique, were devoid of C3 and properdin. This totally negative result was observed in spite of the availability of physiological amounts in the serum of P, B, D, C3, and Mg (Table 1).

In order to verify that immune absorption had specifically removed an essential component of the properdin system, the material dissociated from the immune absorbent was added back to the absorbed serum. The mixture exhibited zymosan-dependent C3 consumption comparable to untreated serum. It was concluded that the factor had been recovered in precursor form, that it was capable of restoring the impaired serum, and that it therefore must represent an essential constituent of the properdin system.

Component IF is a pseudoglobulin which behaves as a β-

Table 1. Properdin-independent initiation: Requirements for formation of the properdin-receptor-forming enzyme

C4-deficient serum depleted of	Material added	% C3 consumption		Deposition on zymosan*	
		+ Zymosan	+ NaCl	C3	P
P		22.4	6.9	2+	0
P, B		2.8	1.8	0	0
P, D		0.6	4.1	0	0
C3				0	0
Me^{++} (EDTA)		0	0	0	0
IF		1.8	4.5	0	0
IF	IF	54.6	7.8	4+	4+
P, IF		10.1	10.0	0	0
		50.0	10.0	4+	4+
	C4	78.6	14.7	4+	4+

* Twenty microliters of serum, 2 μl of zymosan (100 μg), or NaCl and 1 mM Mg^{++} were incubated 60 min at 37°. C3 consumption was determined by effective molecule titration, and deposition of C3 and properdin on zymosan was detected with fluorescent antibody.

globulin when examined in precursor form by agarose block electrophoresis at pH 8.6. It is stable to heating at 56° for 30 min and it is distinct from immunoglobulins and properdin by immunochemical and physical criteria (9).

The question of the exact role of immunoglobulins in the activation of the pathway is unanswered. Available evidence indicates that activation by inulin or zymosan may occur in absence of immunoglobulins (9). On the other hand, antibody of the IgG class was shown to participate in the initiation of the pathway by measles-virus-infected cells (15).

IF-C3 convertase, the properdin-receptor-forming enzyme

That bound C3b functions as a receptor for properdin has been documented (14, 16). That it is critically involved in the activation of properdin will be described below. The enzyme responsible for the formation of the receptor, that is, for the initial deposition of C3b on activating surfaces, is the IF-C3 convertase (9). The enzyme is generated from activated IF, B, D, native C3, and Mg. In analogy to the previously described NF-C3 convertase (14), the enzyme may be denoted as $\overline{IF,B,C3}$; the notation reflects the experimental result that interactions within the complex occur primarily between IF and B and between B and C3. Properdin deposition on zymosan particles was observed only under conditions which allowed formation of the enzyme and subsequent binding of C3b. C3 and P binding to zymosan were monitored by the immunofluorescence technique. The generation of $\bar{P}$-C3 convertase was also detected by activity measurements. Two experiments are particularly illuminating. When zymosan was treated with P- (and C4)-depleted serum C3b was bound as a function of the IF-C3 convertase. The particles were then removed from the reagent and the enzyme was allowed to decay for 2 hr at 22° and 16 hr at 4° in 0.01 M EDTA. When the washed particles were subsequently transferred to IF- (and C4)-depleted serum, P deposition on the particles and marked C3 consumption were observed. In contrast, no properdin uptake was seen when zymosan was treated with serum from an individual with homozygous C3 deficiency (17). The latter experiment stresses the uniqueness of C3b as the properdin receptor.

The properdin-activating principle

In the past P was isolated from serum, usually in the form denoted $\bar{P}$. This protein is able to bind directly to surface-bound C3b. It is also able to form soluble $\bar{P}$-C3 convertase together with B, D, C3, and Mg. In serum, P occurs in precursor form which does not bind directly to C3b and does not form the fluid phase enzyme. Precursor P was obtained by a method of purification employing QAE-Sephadex and Sepharose 6B chromatography (11). Using P in radiolabeled form, it was found that it was bound and activated by the complex consisting of the properdin receptor and activated Factor B (S—$\overline{C3b,B}$, where S denotes a surface site) (10). The reaction proceeds in EDTA and therefore does not require conditions allowing continued formation of S—$\overline{C3b,B}$ (Table 2). This complex is identical with the previously described C3b-dependent C3 convertase which is also known as the feedback enzyme (5, 18). Although the complex has an enzymatic function, present evidence suggests that P binding and activation is a non-enzymatic process. For instance, no turnover of P was detected. No activated or irreversibly inactivated P accumulated in the fluid phase. The loss of precursor from the fluid phase was totally accounted for by the amount of specifically bound P. Utilizing differentially radiolabeled B and P and C3b-bearing erythrocytes (EC3b), maximally one molecule of P was bound per molecule of $\bar{B}$. A further characteristic of the properdin-activating principle is that only some of the bound C3b molecules can serve to form the activating principle. Approximately

Table 2. Activation of properdin precursor by cell-bound $\overline{C3b,B}$ as revealed by formation of the stable $\overline{\bar{P}\text{-}C5}$ convertase*

Initial treatment of EC3b†	Second treatment	$\overline{\bar{P}\text{-}C5}$ convertase (z‡)
B, D, Mg	EDTA, P	0.95
P, B, D, Mg	B, D, Mg	1.35
P, B, D, EDTA	B, D, Mg	0.02
$\underline{P}$, Mg	B, D, Mg	0.07
$\underline{\bar{P}}$, Mg	B, D, Mg	1.40
$\underline{\bar{P}}$, EDTA	B, D, Mg	1.40

* Initial incubation, 10 min, 37°, followed by wash in EDTA; second treatment, 10 min, 37°, followed by 10 min, 37° in EDTA and 20 min, 37° with 1:50 guinea pig serum, 0.04 M EDTA.

† Quantities: 1×10^7 EC3b, 1 μg of B, 30 ng of D, 100 ng of P, 50 ng of $\bar{P}$, 0.5 mM Mg, 10 mM EDTA, 50 μl total volume.

‡ Effective molecules. One z equals 63% hemolysis.

one molecule of P was bound per 50 molecules of C3b. The possibility is raised, therefore, that at least two critically spaced C3b molecules are an essential requirement for the generation of one properdin activation-binding site.

Transition of P to $\bar{P}$

Upon dissociation of B from S—C3b,P,B by spontaneous decay or active disassembly, $\bar{P}$ remains firmly attached to C3b. The site S—C3b,$\bar{P}$ can reform the complex S—C3b,P,B upon addition of B, D, and Mg. These and the previously mentioned observations demonstrate a functional difference between P and $\bar{P}$. In exploring the chemical basis for the P–$\bar{P}$ transition, the subunit molecular weights of both forms were determined. Since the molecular weights were indistinguishable, we propose that the P–$\bar{P}$ transition is a nonenzymatic process that, in all probability, involves a critical conformational change of the properdin molecule (11).

Two forms of $\bar{P}$ may be distinguished. The physiological form of $\bar{P}$ is properdin bound to the properdin receptor through the above-described reaction. Upon dissociation of bound $\bar{P}$ it appears to revert largely to the precursor state. The second form of $\bar{P}$ has been obtained in the laboratory from the serum euglobulin fraction at low pH. The activity of this form may be due to the purification procedure because, under physiological conditions, transition of P to $\bar{P}$ in solution has so far not been observed. It is conceivable, however, that soluble $\bar{P}$ arises by spontaneous dissociation from S—C3b,$\bar{P}$ or by enzymatic destruction of the properdin receptor.

Assembly and function of C3 and C5 convertase

In the classical pathway, C3 convertase constitutes a bimolecular complex of fragments of C2 and C4 (C4b,2a) (19). Incorporation of C3b into this complex gives rise to C5 convertase activity (20). The catalytic site of both enzymes resides in the C2a subunit (21). By analogy, we anticipated a close relationship between the alternative pathway C3 and C5 convertases. The C3 convertase was shown several years ago to have the composition C3b,B (5, 22, 23). Does the C5 convertase require an additional C3b molecule in order to recognize its substrate? Unlike the classical counterpart, bound C3b,B functions as C3 and C5 cleaving enzyme. Specifically, *native* C3 is not a requirement for its action on C5. It is important to stress that C5 converting activity is measurable only under solid phase conditions. It is probable, therefore, that two C3b molecules are necessary to achieve the steric conditions for generation of C5 convertase activity. If this is true, the first C3b molecule performs a function analogous to C4b, while the second molecule fulfills a function analogous to C3b in the C4b,2a,3b enzyme. The apparent inability of soluble C3b,B to turn over C5 could then be explained by the lack of a second C3b molecule in the soluble complex. Formalistically, it is therefore possible that formation of C3 convertase precedes that of C5 convertase on a surface, but in that case, the latter retains C3 convertase activity.

That P is part of an efficient form of the enzyme was pointed out in 1974, when we proposed that P might have a stabilizing effect on the molecular interactions of the multisubunit C5 convertase (24). In fact, Fearon and Austen have since demonstrated retardation of decay of C3b,B by P (16).

The investigation of the precise subunit composition of the $\bar{P}$-dependent convertases was greatly aided by the introduction by Fearon *et al.* of the erythrocyte-bound enzymes (22). Using this experimental technique we were able to define the essential structure of the alternative C5 convertase (10). Stoichiometric measurements using radiolabeled components revealed a probable composition of one B molecule per one P molecule per two C3b molecules. The enzyme acts on C3 and C5, exhibiting a higher turnover for C3 than for C5. Its direct action on C5, in absence of C3, was demonstrated by the formation of stable C5,6,7 sites on the surface of the enzyme-carrying erythrocytes upon incubation with highly purified C5, C6, and C7 (Table 3).

Table 3. $\bar{P}$ dependent convertase: Action on C3 or C5 as measured by cellular uptake*

^{125}I-C3		^{125}I-C5	
Input, μg/ml	Uptake, molecules/cell	Input, μg/ml	Uptake, molecules/cell
5	600	2.8	26
10	1200	8.4	56
20	1800	16.8	160
30	2760	28	440

* Treatment: 1.4×10^8 EC3b,P,B containing 200 $\bar{P}$ molecules/per cell were at 37° for 15 min with ^{125}I-C3 or 10 min with ^{125}I-C5 in presence of 100 ng of C6 and 100 ng of C7. Specific uptake was calculated by subtracting cellular uptake in absence of enzyme.

It is of physiological importance that the enzyme may undergo reverse assembly. Following dissociation of B, the residual complex S—C3b,$\bar{P}$ can reincorporate B and regain full enzymatic activity. EC3b may bind soluble $\bar{P}$, forming EC3b,$\bar{P}$ (Table 2). Such cells are able to bind B in the absence of D provided Mg is offered. The binding of B is totally reversed by EDTA. Upon addition of D to EC3b,$\bar{P}$,B, C5 convertase activity appears and a portion of the B-associated radioactivity is released from the cell. The released material represents a B product which arises from the activation of B to $\bar{B}$.

Regulation

Three distinct modes of control have become apparent in the course of this study. The enzymes decay spontaneously at 37°, the IF- and $\bar{P}$-dependent convertases with a half-life of approximately 15 min and 2 min for the non-stabilized C3b,B. Decay is accompanied by dissociation of inactive B.

The activity of the $\bar{P}$-dependent convertase was found to be inhibited by the product C3b, but not by C5b. The C3 fragment effects inhibition by disassembly of the active enzyme with release of inactive B. Native C3 has the same ap-

Table 4. Regulation of pathway by the properdin-receptor-destroying enzyme (PRDE)

Complex	Residual activity (z†) after 10 min, 37°	
	without PRDE	with PRDE
EC3b	2.0	0.05
EC3b, $\bar{P}$	0.95	0.46
EC3b, B*	0.6	0.6
EC3b, P, B	1.12	1.07

* 20° instead of 37°.

† Effective molecules. One z equals 63% hemolysis.

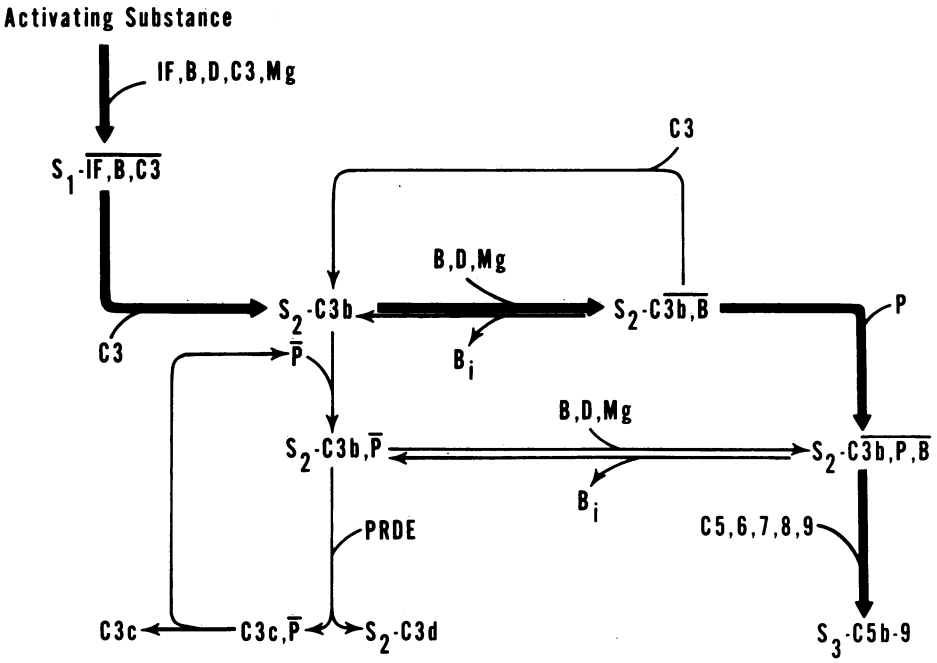

FIG. 1. Schematic representation of the molecular dynamics of the properdin pathway as proposed in the *text*. S_1 represents the site of initiation at which the properdin-receptor-forming enzyme is generated. S_2 denotes the site of attachment of C3b doublets, which serve as properdin receptor. S_2 also is the site of formation of the properdin-activating principle which acts as feedback enzyme and of the $\bar{P}$-C5 convertase. S_3 refers to the site of binding of the C5b–9 membrane attack complex. The properdin-receptor-destroying enzyme (PRDE) acts on S_2—C3b and S_2—C3b,$\bar{P}$ but not on S_2—$\overline{C3b,P,B}$. The $\overline{IF}$- and $\bar{P}$-convertases are disassembled by soluble C3b (not indicated).

parent effect. The $\overline{IF}$-dependent enzyme is, in all probability, similarly regulated by soluble C3b.

The third mode of control involves fragmentation of the properdin receptor C3b by a serum enzyme. The enzyme acts on bound C3b as well as on the bound C3b,$\bar{P}$ complex. In the former case it destroys the properdin receptor function and in the latter situation it releases properdin, probably in association with a C3 fragment. Action of the enzyme on the bound C3b,$\bar{P}$ complex could explain the appearance of soluble $\bar{P}$, provided this form of properdin does occur under physiological conditions. It is probable that the enzyme cleaves C3b into C3c and C3d (25) and that it represents the known C3b inactivator (26). Since its identity has not been established, it is here referred to as the properdin-receptor-destroying enzyme (PRDE). In contradistinction to its effect on S—C3b and S—C3b,$\bar{P}$, PRDE has little or no effect on S—$\overline{C3b,B}$ and S—$\overline{C3b,P,B}$, suggesting that the susceptible region in C3b is protected within these complexes (Table 4).

Synopsis

Fig. 1 depicts a schematic representation of the proposed concept. The initial events occur on the surface of the activating particle. It is envisioned that the precursor of IF establishes contact with the activating particle (S_1) and then interacts with B, D, and native C3 to generate the P receptor-forming enzyme (PRFE), which is the initial C3 convertase.* PRFE is a low magnitude enzyme which is limited in its action by spontaneous decay and disassembly through soluble C3b. PRFE deposits C3b at sites (S_2) in the vicinity of the enzyme. Properdin receptor function resides in at least two critically oriented and closely spaced C3b molecules. Binding of activated B to the receptor results in generation of the properdin-activating principle, $\overline{C3b,B}$. The complex, which is identical with the feedback enzyme, has C3 and C5 convertase activity and is exceedingly labile. Upon collision of native P with the complex, P undergoes a transition to its bound form, $\bar{P}$. $\bar{P}$ confers an increased degree of stability on $\overline{C3b,B}$ converting it to the $\overline{C3b,P,B}$ enzyme. The $\bar{P}$-enzyme effects activation of C5 and self-assembly of the membrane attack complex C5b–9 (28). C3 does not participate in this enzymatic reaction. When present, C3 is turned over and the enzyme is disassembled at a rate greater than that characteristic for its spontaneous decay. Reverse assembly of the enzyme may commence with uptake of $\bar{P}$ by S_2—C3b and is completed after incorporation of $\bar{B}$ into the complex. Action of the P-receptor-destroying enzyme (PRDE) on S_2—C3b abrogates its receptor function, and action of the enzyme on S_2—C3b,$\bar{P}$ releases properdin posssibly in association with C3c.

Although IF and P are subunits of closely related enzymes, they differ with respect to their essential functions. IF probably serves as the recognition factor of the properdin system, while P is limited to interaction with the $\overline{C3b,B}$ complex. Whereas IF fulfills the role of the nucleus of an assembling enzyme, P functions as modulator of an already assembled enzyme. IF exhibits affinity primarily for B and not for C3 or C3b. P exhibits affinity primarily for C3 or C3b and not for B. Three components, C3, B, and D, are operative at all stages of the pathway and B contains the essential catalytic site of all C3 and C5 convertases.

Properdin itself does not play the central role in the pathway it had once been assigned. It is recruited late in the sequence rather than early as was previously assumed. It greatly augments the cytolytic efficiency of the pathway, but its function is a nonessential one compared to that of the other components of the properdin system.

* Until larger quantities of highly purified initiating factor precursor become available, the participation of an accessory factor in initiation cannot be precluded (27).

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