Formation of High-Affinity C5 Convertases of the Alternative Pathway of Complement Formation

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Formation of High-Affinity C5 Convertases of the Alternative Pathway of Complement

Nenoo Rawal and Michael K. Pangburn

Cleavage of C5 by C5 convertase is the last enzymatic step in the complement activation cascade leading to the formation of the cytolytic proteolytically activated form of C5 (C5b)-9 complex. In the present study, we examined the effect of the density of C3b (the proteolytically activated form of C3) on the function of the noncatalytic subunit of natural surface-bound forms of the enzyme. A comparison of the kinetic parameters of C5 convertases assembled on three surfaces (zymosan, rabbit erythrocytes, and sheep erythrocytes) were similar and revealed that the average $K_m$ decreased $\sim$28-fold ($5.2-0.18 \mu M$) when the density of C3b was increased from $\sim$18,000 to 400,000 C3b/cell. Very-high-affinity C5 convertases were generated when preformed C3 convertases were allowed to self-amplify by giving them excess C3. These convertases exhibited $K_m$ from 0.016 to 0.074 $\mu M$, well below the normal plasma concentration of C5 in blood ($0.37 \mu M$). The results suggest that in serum convertases formed with monomeric C3b will be relatively inefficient in capturing C5 but will continue to cleave C3 opsonizing the cell surface for phagocytosis, whereas convertases formed with C3b-C3b complexes in areas of high C3b density will primarily cleave C5. The catalytic rate of these convertases approaches maximum velocity, thereby switching the enzyme from cleavage of C3 to cleavage of C5, and production of the cytolytic C5b-9 complex. The Journal of Immunology, 2001, 166: 2635–2642.

Activation of complement via the innate and the adaptive immune systems is vital to the body’s defenses in fighting infections. Complement activation triggers a series of reactions that involves $>30$ different proteins (1–3). These proteins are activated in a sequential manner resulting in the generation of products that have important biological activity. Cleavage of C5, the fifth component of complement, by the serine protease C5 convertase is an important enzymatic reaction that occurs during the activation cascade. C5 convertase cleaves C5 into two products, C5a and C5b (1). Both fragments play a vital role in killing microorganisms and in eliminating targets of complement action. C5b, the larger fragment, initiates the assembly of the cytolytic (C5b-9) complex, which results in the lysis of bacteria and other microorganisms. C5a, the smaller fragment, is a potent chemotactic and spasmogenic anaphylatoxin that mediates inflammatory responses by stimulating platelets, endothelial cells, eosinophils, neutrophils, and phagocytes to the site of complement activation (4, 5).

C5 convertases of the alternative and classical pathways of complement are complex serine proteases that are made up of two or more subunits (1, 6–11). The study of C5 convertases has been difficult because the enzyme complex that cleaves C5 is the same serine protease (C3 convertase) that cleaves C3 (1). Cleavage of C3 produces the proteolytically activated form of C3 (C3b), which forms the nonenzymatic subunit of the enzyme complex responsible for C3 and C5 cleavage (1, 3). C3 convertases of the alternative pathway (monomeric C3/C5 convertase assembled with monomeric C3b (C3b,Bb)) and the classical pathway (C4b,C2a) are bimolecular complexes (1, 3). The alternative pathway C5 convertase (C3b,Bb) has C3b as the noncatalytic subunit attached in a Mg$^{2+}$-dependent fashion to the catalytic subunit (Bb) of the enzyme complex. In the classical pathway C3 convertase C4b,C2a has a structure similar to the alternative pathway C3 convertase in having C2a as the catalytic subunit noncovalently bound to a C4b molecule. The soluble serine protease C3 convertase (C3b,Bb) assembled with monomeric C3b has been shown to be the simplest form of C5 convertase (12). The natural surface-bound C5 convertases are the more complex forms of the enzyme and are made up of a C3 convertase and an additional C3b molecule (6–11, 13, 14). Attachment of the additional C3b molecules near the C3 convertase was originally thought to be required for generating C5 convertase activity (6, 8, 15). Vogt et al. (13) revealed that the role of the additional C3b was to bind the substrate, C5. Later a more specific structure was described. High C5 convertase activity was associated with the formation of C3b-C3b or C3b-C4b dimers in which the additional C3b molecule was shown to be covalently attached to a specific site on the first C3b or C4b molecule (10, 16). The dimers were suggested to form complexes C3b-C4b,C2a and C3b$_2$-Bb, which are currently considered to be functional C5 convertases of the classical and the alternative pathways, respectively (9, 10, 17, 18).

To enhance our understanding of the structure/function of natural surface-bound C5 convertases we examined the effect of C3b density on the functional role of the noncatalytic subunit of surface-bound C5 convertases. In this study we show that, whereas the density of C3b/cell influences the $K_m$ and not the catalytic rate...
constant ($k_{cat}$) of C5 convertases, the type of surfaces do not influence either the $K_m$ or the $k_{cat}$ of the enzyme. The data suggest that the initial monomeric C3/C5 convertases formed on the surface because of their weak affinity for C5 will function as C3 convertases amplifying C3b around them. As C3b deposition occurs, C3b-C3b complexes are formed. These complexes form convertases that have higher affinities for C5 and will primarily cleave C5 leading to a higher production of the cytotytic C5b-9 complex. Based on the present kinetic analysis of the natural surface-bound C5 convertases together with results published by other groups on the structure of C5 convertase, we propose a model for the formation of high-affinity C5 convertases during complement activation.

Materials and Methods

Reagents

Chicken erythrocytes (EC) were isolated from chicken blood purchased from Colorado Serum (Denver, CO). Nonidet P-40, a nonionic detergent, and EDTA were purchased from Sigma (St. Louis, MO). Veronal-buffered saline (VBS) contained 5 mM barbital and 145 mM NaCl (pH 7.4). Gelatin VBS (GVB) was VBS containing 0.1% gelatin, while GVB containing 10 mM EDTA (GVB-E) was also used.

Purified proteins

Complement proteins, C3 (19, 20), factor B (21), factor D (22), and C6 (23) were all purified from normal human plasma as described in the references cited. B and D and C6 in 0.5 ml siliconized microfuge tubes as described (24) except that ceramic hydroxyapatite (Bio-Rad, Richmond, CA) was used instead of hydroxyapatite. C3b was prepared from C3 by cleavage with factors B and D in the presence of Ni$^{2+}$ at 37°C as described previously (12). Purified C5b,6 was obtained from Advanced Research Technologies (San Diego, CA). All proteins were homogenous by polyacrylamide gel electrophoresis. Protein concentrations of C3b, C5, C6, factor B, factor D, and C5b,6 were spectrophotometrically determined using 11.0, 11.1, 10.3, and 11.0 as the values for $E_{280}$, respectively. All purified proteins were stored at $-76^\circ$C. $M_i$ values employed in the calculations were 176,000 for C3b, 190,000 for C5, 179,000 for C5b, 120,000 for C6, 299,000 for C5b,6, 93,000 for factor B, 63,000 for Bb, and 24,000 for factor D.

Preparation of ZymC3b, E$\alpha$C3b, and E$\beta$C3b

ZymC3b was prepared as described previously (12). C3b was deposited on zymosan by resuspending 1 $\times$ 10$^9$ zymosan particles in 0.2 ml of 10 mg/ml C3 and adding 5 g/ml of trypsin, followed by a 10-min incubation at 22°C. The deposition of C3b by trypsin was repeated and the cells were washed six times with 5 ml GVB. The zymosan particles were resuspended in 22°C. The amount of C3b deposited on zymosan was amplified by cutting the tube and counting the amount of radioactivity in the pellet (26).

Formation and assay of surface-bound C5 convertases

Because formation of C5 convertase took less than 1 min, enzyme was formed in the same reaction mixture in which the assays were performed. Enzyme velocities were determined under saturating concentrations of factors B and D and C6 in 0.5 ml siliconized microfuge tubes as described previously (12). Assay mixtures contained varying concentrations of C5 (preincubated for 20 min at 37°C to eliminate freeze/thaw-generated background C5b-6-like activity), factor B (1.2 g, 516 nM), factor D (0.1 g, 167 nM), C6 (2.5 g, 833 nM), and 0.5 mM NiCl$_2$. The reaction was started by the addition of ZymC3b, E$\alpha$C3b, or E$\beta$C3b. Depending on the density of C3b per cell, the concentration of C5 was adjusted so as to have $9-35$ ng of bound C3 in a final volume of 25 g VB resulting in 2-8 mM enzyme concentration. After 15 min of incubation at 37°C, further cleavage of C5 was prevented by transferring the assay tubes to an ice bath and adding ice-cold GVB-E. Appropriately diluted assay mixtures were immediately titrated for C5b,6 formation by hemolytic assays using EC. C5b,6 was quantitated using standard curves generated with purified C5b,6. Controls established that the cold temperature and the dilution were sufficient to reduce the cleavage of C5 during subsequent steps to undetectable levels. Lysis of rabbit erythrocytes (ER) or sheep erythrocytes (ES) was shown to contribute $<2$% to C5b,6 titer's use of EC as the endpoint.

Quantitation of reaction products

C5b,6 was measured hemolytically using the sensitivity of EC to hemolytic lysis by human C5b-9 as described (12). To an aliquot (25 l) of the diluted sample from C5 convertase assays was added a mixture of 1 $\times$ 10$^3$ Ec and 5 l of pooled normal human serum (NHS) as a source of complement proteins C7-C9 in a final volume of 225 g GVB. The reaction mixtures were incubated for 10 min at 37°C after which the unlysed cells were removed by centrifugation for 1 min at 10,000 $\times$ g. The amount of hemoglobin released was quantitated spectrophotometrically at 414 nm. One-hundred percent lysis was measured as EC lysed in 2% Nonidet P-40. Controls containing C5 and C6 but no C5 convertase were subtracted as the background. Controls containing C5 convertase but no purified C5 or C6 demonstrated that no significant amount of C5b,6 was formed from NHS used as a source of C7-9 during the lysis of EC.

Data analysis

The reaction velocity data was analyzed according to the Michaelis-Menten equation: $v = (V_{max} [S]) / (K_m + [S])$. The results were fit to this equation using nonlinear regression analysis and the kinetic parameters, $K_m$, $V_{max}$, and $k_{cat}$ were determined using Grafit version 4.12 software (Erithacus Software, London, U.K.).

Preparation of 125I-labeled factor B and C3

Factor B (100 g) was radiolabeled with 125I for 30 min at 0°C in a glass tube coated with Iodoagen (Pierce, Rockford, IL). Free 125I was removed by centrifugal desalting (27). Specific activities of radiolabeled factor B ranged from 0.12 to 0.95 Ci/g/ug. C3 was labeled as described for factor B. The radiolabeled C3 was diluted with cold C3 to give a specific activity of 0.08 Ci/ug.

Determination of the number of C3b molecules bound per cell

Cells were amplified with C3b using radiolabeled C3 at 9.8 mg/ml (specific activity = 0.08 Ci/g/ug) instead of unlabeled C3 as described above. The amplification procedure was repeated to obtain cells bearing different densities of radiolabeled C3b ranging from $18,000$ to 700,000 C3b/cell. Ca$^2+$ binding radiolabeled C3b were incubated for 5 min at 37°C in 25 g GVB after which the reaction mixture was diluted with 75 g GVB containing 0.5 mM NiCl$_2$. Bound and free radiolabeled C3b was separated by layering 75 g of the mixture on 250 g of 20% sucrose in GVB containing 0.5 mM NiCl$_2$ followed by centrifugation for 1 min at 10,000 $\times$ g at 22°C. The amount of radiolabeled C3b bound to cells was determined by cutting the tube and counting the amount of radioactivity in the pellet (26). The number of C3b bound to E$\alpha$C3b calculated from the radioactivity (cpm) bound to E$\alpha$C3b to verify after subtracting nonspecific binding to control EC.

Determination of the number of Bb binding sites (C5 convertase sites)

The number of C5 convertase sites formed on ZymC3b, E$\alpha$C3b, or E$\beta$C3b was determined by measuring only those C3b molecules that were capable of forming an enzyme with radiolabel factor B under saturating assay conditions (12). ZymC3b, E$\alpha$C3b, or E$\beta$C3b employed in the binding assays were washed three times with GVB to remove any free C3b. Various amounts of 125I-labeled factor B were added to assay mixtures containing factor D (0.1 g, 167 nM), C5 (5 g, 1053 nM), C6 (2.5 g, 833 nM), and 0.5 mM NiCl$_2$. Formation of enzyme was started with the addition of ZymC3b, E$\alpha$C3b, or E$\beta$C3b to give a final volume of 25 g GVB. After 5 min of incubation at 37°C, assays were diluted with 75 g GVB containing 0.3 mM NiCl$_2$. Bound and free radiolabeled factor B were separated by layering 75 g of the mixture on 250 g of 20% sucrose in GVB containing 0.5 mM NiCl$_2$ followed by centrifugation for 1 min at 10,000 $\times$ g at 22°C. The amount of factor B bound to ZymC3b as Bb was determined by cutting the tube and counting the amount of radioactivity in the pellet (26).
Results

Determination of the number of cell-bound C3b and the number of Bb binding sites

To count the number of C3b/cell, E<sub>R</sub> cells bearing different amounts of C3b ranging from ~18,000 to 700,000 C3b/cell were prepared using radiolabeled C3. The number of C5 convertase sites that could be generated on these E<sub>R</sub> cells bearing different densities of radiolabeled C3b was determined as the number of Bb binding sites. Only those C3b molecules capable of binding [sup]125[I]-labeled factor B as Bb under saturating concentrations of factor D, Ni<sup>2+</sup>, and radiolabeled factor B were quantitated. The specific activity of [sup]125[I]-labeled C3 (0.079 µCi/µg) was adjusted such that it was ~9-fold less than that of [sup]125[I]-labeled factor B (0.70 µCi/µg). This was necessary so as to have a 3-fold increase in the radioactivity over the background from C3b if all the bound C3b molecules could be saturated with Bb (12). The assays employed Ni<sup>2+</sup> to minimize the decay of the Bb subunit of the enzyme during the 1-min spin through cold 20% sucrose. Quantitation of the number of C3b/cell and the number of Bb binding sites was done at the same time to minimize the possible effect of C3b decay from the cell surface. As seen in Fig. 1, the ratio of the number of Bb binding sites to the number of C3b/cell did not vary significantly with a 35-fold variation in density of C3b. The average ratio of Bb per C3b was determined to be 0.83 ± 0.13, (i.e., one Bb per 1.2 molecules of C3b) suggesting that most of the C3b can bind Bb even when the C3b density is greater than 400,000 C3b/cell. Given the errors associated with determining radioactive and specific activities of the two proteins, these results suggest that equivalent numbers of C3b and Bb bind at all levels of C3b/cell. Therefore, throughout this study we have determined the number of C5 convertase sites formed by measuring Bb binding under saturating levels of factor B and used it also as a measure of the number of C3b bound per cell.

Measurement of kinetic parameters of C5 convertases formed on activators of the alternative pathway of complement

Zymosan particles bearing C3b ranging from 18,600 to 405,000 C3b/Zym were employed for assembling C5 convertase. Enzyme activity was measured as the amount of C5 cleaved by quantitating the amount of C5b,6 formed during a 15-min assay under saturating concentrations of factors B and D and C6. Initial velocities were determined at various concentrations of C5. Data for ZymC3b,Bb made with 31,000 and 300,000 C3b/Zym are shown in Fig. 2, whereas data obtained with ZymC3b,Bb formed with other densities of C3b/cell are summarized in Fig. 3A and in Table 1. The velocity data obtained were found to fit well to the theoretical curve based on the Michaelis-Menten equation: $V = \frac{V_{max}}{K_m + [S]}$ (Fig. 2A). The data were also observed to fit well to the linearized form of the Michaelis-Menten equation, shown as the Eadie-Hofstee plot (Fig. 2B). C5 convertase formed with 300,000 C3b/cell had a 10-fold lower $K_m$ (0.3 µM) than the enzyme formed with 31,000 C3b/cell (2.8 µM). These results suggest that the density of C3b/cell influences the $K_m$ of the enzyme. The effect of the density of C3b/cell on the $K_m$ of C5 convertase is apparent in the results shown in Fig. 3A, which illustrates a spectrum of different $K_m$ values depending on the number of C3b/cell employed to assemble the enzyme. The average $K_m$ of the enzyme decreased from 5.15 µM at 18,600 C3b/cell to 0.177 µM at
The measured as the amount of C5b,6 formed at 37°C as described in Fig. 2. C5 cleavage by C5 convertases assembled with varying C3b/cell were the number of Bb binding sites as described in Fig. 1. Initial velocities of amounts of C3b/cell as indicated. The number of C3b/cell was measured as the number of Bb binding sites as described in Fig. 1. Initial velocities of C5 cleavage by C5 convertases assembled with varying C3b/cell were measured as the amount of C5b6 formed at 37°C as described in Fig. 2. The $K_m$ of each C5 convertase was determined from individual velocity vs substrate concentration plots. B. Comparison of the $k_{cat}$ of C5 convertases ZymC3b,Bb (○), EqC3b,Bb (□) and EoC3b,Bb (△) formed with cells bearing varying amounts of C3b/cell as indicated. The number of C3b/cell was measured as the number of Bb binding sites as described in Fig. 1. Initial velocities of C5 cleavage by C5 convertases assembled with varying C3b/cell were measured as the amount of C5b6 formed at 37°C as described in Fig. 2. The $K_m$ of each C5 convertase was determined from individual velocity vs substrate concentration plots. B. Comparison of the $k_{cat}$ of C5 convertases ZymC3b,Bb (○), EqC3b,Bb (□) and EoC3b,Bb (△) with the number of C3b/cell as indicated. The $k_{cat}$ for each C5 convertase was calculated from the $V_{max}$ obtained from individual velocity vs substrate concentration plots and the enzyme concentration determined from the number of 125I-labeled Bb bound/cell under saturating concentrations of factor B. The data obtained with all the three surfaces at the various indicated densities of C3b per cell were considered as one set of data points and were fitted by linear regression using Grafit version 4.12 Erithacus software. C. Comparison of $k_{cat}/K_m$ ratio of C5 convertases ZymC3b,Bb (○) for EqC3b,Bb (□) and for EoC3b,Bb (△) with the number of C3b per cell as indicated. The $k_{cat}/K_m$ ratio was calculated from the data in Table I.

FIGURE 3. Comparison of the kinetic properties of C5 convertases assembled on three different surfaces (zymosan, Eq, and Eo) bearing different amounts of C3b. A. Comparison of the $K_m$ of C5 convertases ZymC3b,Bb (○), EqC3b,Bb (□), and EoC3b,Bb (△) formed with cells bearing varying amounts of C3b/cell as indicated. The number of C3b/cell was measured as the number of Bb binding sites as described in Fig. 1. Initial velocities of C5 convertases assembled with varying C3b/cell were measured as the amount of C5b6 formed at 37°C as described in Fig. 2. The $K_m$ of each C5 convertase was determined from individual velocity vs substrate concentration plots. B. Comparison of the $k_{cat}$ of C5 convertases ZymC3b,Bb (○), EqC3b,Bb (□) and EoC3b,Bb (△) with the number of C3b/cell as indicated. The $k_{cat}$ for each C5 convertase was calculated from the $V_{max}$ obtained from individual velocity vs substrate concentration plots and the enzyme concentration determined from the number of 125I-labeled Bb bound/cell under saturating concentrations of factor B. The data obtained with all the three surfaces at the various indicated densities of C3b per cell were considered as one set of data points and were fitted by linear regression using Grafit version 4.12 Erithacus software. C. Comparison of $k_{cat}/K_m$ ratio of C5 convertases ZymC3b,Bb (○) for EqC3b,Bb (□) and for EoC3b,Bb (△) with the number of C3b per cell as indicated. The $k_{cat}/K_m$ ratio was calculated from the data in Table I.

Measurement of kinetic parameters of C5 convertases formed on nonactivators (Eo) bearing 18,000 and 115,000 C3b/cell. A. Initial rates of C5 cleavage by EoC3b,Bb assembled with 18,000 C3b per cell were determined as described in Fig. 2 except that EoC3b bearing 46.7 ng C3b were added to the assay resulting in 10.6 nM of enzyme under saturating assay conditions. Inset. Analysis of the initial velocity of C5 convertase using the Eadie-Hofstee function. B. Velocity vs substrate concentration plot for EoC3b,Bb assembled with 115,000 C3b/cell is shown. Assay conditions were as described in Fig. 2 except that EoC3b bearing 14.5 ng C3b were added to the assay resulting in 3.3 nM of enzyme. Inset. Analysis of the initial velocity of C5 convertase using the Eadie-Hofstee function.

Measurement of the kinetic parameters of self-amplified C5 convertases

The conversion of low affinity to high-affinity C5 convertases was studied by examining the enzymatic properties of self-amplified C5 convertases. These were the highest affinity C5 convertases that were generated in this study. They were prepared by allowing pre-formed C3 convertases to amplify C3b around themselves in the presence of excess C3. After amplification, the cells were washed and the convertases that had not yet decayed were assayed for C5 cleavage. The velocity data obtained were found to fit well to the theoretical curve based on the Michaelis-Menten equation (Fig. 5). The data were also observed to fit well to the linearized form of the...
Table I. Kinetic parameters of C5 convertases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>C3b/cell</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
<th>Turnover Number (min$^{-1}$)</th>
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<tbody>
<tr>
<td>Surface-bound C5 convertases</td>
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<tr>
<td>ZymC3b,Bb</td>
<td>18,600*</td>
<td>5.2</td>
<td>0.003</td>
<td>0.6 × 10$^3$</td>
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<td>31,000</td>
<td>2.8 ± 0.8</td>
<td>0.0033 ± 0.0001</td>
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<td>Self-amplified C5 convertases</td>
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<td>ZymC3b,Bb</td>
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<td>0.016 ± 0.007</td>
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<td>Soluble monomeric C5 convertases</td>
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<td>24 ± 6.6</td>
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<tr>
<td>CVP$_6$Bb</td>
<td>None</td>
<td>0.036 ± 0.013</td>
<td>0.0071 ± 0.0026</td>
<td>197 ± 67 × 10$^3$</td>
<td>0.43 ± 0.16</td>
</tr>
</tbody>
</table>

* Kinetic parameters of surface-bound C5 convertases were determined by nonlinear regression as described in Materials and Methods and shown in Figs. 2–6. Kinetic parameters of C3b,Bb; CVP$_6$Bb; and CVP$_6$Bb have been reported from Refs. 12 and 28. Values are mean ± SD (n = 3 or 4) except for those marked with an asterisk, in which n < 3.

a C3b/cell was measured by radiolabeled Bb binding described in Materials and Methods.

Michaels-Menten equation, shown as the Eadie-Hofstee plot (Fig. 5, inset). The $K_m$ of self-amplified C5 convertases examined on all three surfaces exhibited values in the range of 0.074–0.016 μM (Table I). Due to the low $K_m$, care was taken to ensure that substrate consumption was kept low during the assays by using low concentrations of enzyme. In most assays, cleavage of C5 was <1%, whereas in those at substrate concentrations below 100 nM, the observed $K_m$ of self-amplified C5 convertases was observed to be the case in most of the experiments that employed different densities of C3b/cell to assemble the enzyme as observed in Figs. 2B, 4A, 4B, 5, and 6 indicating that the observed $K_m$ is not an average of just a high and a low-affinity C5 convertase but instead is an average of different affinities. To investigate this further, we determined the kinetic properties of C5 convertases formed on zymosan bearing 84,000 C3b/cell. Because these zymosan particles do not have a very high density of C3b/cell, we would expect a larger percent of C5 convertases formed at this density of C3b/cell to have a $K_m$ ($K_m$ = 5.0 or 24 μM, Table I) that is above the average $K_m$ of 1.39 μM obtained when all the C3b molecules are converted to enzymes under saturating levels of factor B (Table I). Therefore, initial velocities for C5 cleavage were determined up to 53 μM of C5 (10.0 mg/ml), a concentration that is 38-fold above the average $K_m$ (1.39 μM) of the enzyme (Fig. 6). Errors involved in measuring velocities at very high substrate concentrations are large. Nevertheless, within experimental error, the data obtained appeared to fit well to the theoretical curve for a homogeneous population of enzymes based on the Michaels-Menten equation and the Eadie-Hofstee plot had no curvature to it (Fig. 6, inset). Modeling the data indicated that subpopulations of C5 convertases that have $K_m$ values...
was observed to decrease with increasing C3b density (Fig. 3). The normal plasma concentration of C5 in blood, these convertases will vary in affinity monomeric C3/C5 convertases to high-affinity C5 convertases. These were the highest affinity C5 convertases that were observed on cell surfaces. Preformed C3 convertases were allowed to amplify C3b around themselves by providing excess C3. After amplification, the cells were washed and the remaining initial C3 convertases were assayed for C5 cleaving properties. The $K_m$ of these convertases for C5 ($0.016 – 0.074 \mu M$) was observed to be well below the normal plasma concentration of C5 in blood ($0.37 \mu M$) suggesting that they will cleave C5 at velocities approaching $V_{max}$ (Fig. 7) under physiological conditions. In contrast, monomeric C3/C5 convertase (C3b,Bb) because of its weak affinity for C5 ($K_m = 24 \mu M$) has been suggested to cleave 9,000 C3 for every C5 cleaved under physiological conditions (12, 28).

Considered together, these results suggest that because C5 convertases formed with few C3b per cell have a $K_m$ well below the normal plasma concentration of C5 in blood, these convertases will be occupied with C5 most of the time and the enzyme will cleave C5 at a catalytic rate close to $V_{max}$.

**Comparison of C5 convertase activities at the normal plasma concentration of C5**

The activities of surface-bound C5 convertases assembled with different densities of C3b/cell have been compared at the normal plasma concentration of C5. Because all the three surfaces exhibited similar enzymatic properties, only data for zymosan is shown in Fig. 7. Fig. 7 shows that because C5 convertases formed with few C3b/cell have a $K_m$ above the normal physiologic concentrations of C5 in plasma ($0.37 \mu M$; Fig. 7, vertical dashed line) these convertases will be relatively inefficient in binding C5 and, therefore, inefficient in cleaving C5. And because C5 convertases formed with a high density of C3b/cell have a $K_m$ well below the normal plasma concentration of C5 in blood, these convertases will be occupied with C5 most of the time and the enzyme will cleave C5 at a catalytic rate close to $V_{max}$.

**Discussion**

In an attempt to enhance our understanding of the mechanism of action of natural surface-bound C5 convertases, a systematic study of C3b-dependent and cobra venom factor-dependent C5 convertases (12, 28) led us to the discovery that the simplest form of the enzyme, the bimolecular serine protease C3 convertase (C3b,Bb), was as good a C5 convertase as the more complex forms. This was evident when soluble C3 convertase (C3b,Bb) made with monomeric C3b was shown to cleave C5 without the help of a second C3b molecule and at a catalytic rate similar to that of the surface-bound enzyme formed on zymosan particles, ZymC3b,Bb, containing multiple C3b. But surface-bound C5 convertases, which are the natural forms of the enzyme, exhibit higher affinities for C5 than soluble C3b,Bb. In the present study, examination of the enzymatic properties of surface-bound C5 convertases revealed a spectrum of $K_m$ values ranging from 5.2 to 0.016 $\mu M$ depending on the density of C3b/cell (Table I). The average $K_m$ of the enzyme was observed to decrease with increasing C3b density (Fig. 3A) to levels lower than the normal plasma concentration of C5 (0.37 $\mu M$; vertical dashed line in Fig. 7). These results imply that as amplification continues depositing C3b molecules on the cell surface, C5 convertases that have high affinities for C5 are generated.

The study of self-amplified C5 convertases in particular has provided new insights into the mechanism of transformation of low-affinity monomeric C3/C5 convertases to high-affinity C5 convertases. These were the highest affinity C5 convertases that were observed on cell surfaces. Preformed C3 convertases were allowed to amplify C3b around themselves by providing excess C3. After amplification, the cells were washed and the remaining initial C3 convertases were assayed for C5 cleaving properties. The $K_m$ of these convertases for C5 ($0.016 – 0.074 \mu M$) (Table I) was observed to be well below the normal plasma concentration of C5 in blood ($0.37 \mu M$) suggesting that they will cleave C5 at velocities approaching $V_{max}$ (Fig. 7) under physiological conditions. In contrast, monomeric C3/C5 convertase (C3b,Bb) because of its weak affinity for C5 ($K_m = 24 \mu M$) has been suggested to cleave 9,000 C3 for every C5 cleaved under physiological conditions (12, 28).

Considered together, these results suggest that because C5 convertases formed with few C3b per cell have a $K_m$ values well above the normal concentration of C5 in blood, these convertases will be relatively inefficient in binding C5 like the monomeric C3/C5 convertase. Instead they will spend most of their time cleaving C3, thereby opsonizing the cell surface with C3b for phagocytosis. As amplification continues, C3b density increases and C3b complexes form convertases that have high affinities for C5. These convertases will predominately cleave C5 at catalytic rates close to $V_{max}$ (Fig. 7) thus switching the C3/C5 convertase to production of the cytolytic C5b-9 complex.

Several studies have examined the binding interaction between the substrate C5 and the noncatalytic subunit of C5 convertase and binding constants that vary by about three orders of magnitude have been reported (9, 10, 14, 28 –30). The binding of C5 to C3b molecules deposited in clusters on E S (0.016 $\mu M$) (9, 10), whereas that between C5 and soluble monomeric C3b has been shown to be the weakest ($K_d = 37 \mu M$) (28). Although the dissociation binding constant, $K_d$, and the $K_m$ of an enzyme are not always similar, the binding affinity measured between C5 and the noncatalytic subunit of the soluble forms of C3/C5 convertases (C3b,Bb; cobra venom factor from Naja naja kaouthia-dependent C5 convertase; and cobra venom factor from Naja haje haje-dependent C5 convertase) has been shown to be nearly identical with the $K_m$ of the respective enzyme (28). In the present study, the lowest $K_m$ measured was for the self-amplified C5 convertase assembled on E S ($0.016 \pm 0.007$...
crease in with increasing density of C3b/cell (Table I). Although this de-

soluble monomeric C3b,Bb but showed a 3- to 4-fold decrease

cleaved per 3 min at.

age is one of the slowest enzymatic reactions known with one C5

vertase, because of its weak affinity for C5, will function primarily

convertases of the classical and the alternative

pathway, respectively (9, 10, 17, 18). The covalent C3b-binding

site has been identified as a serine residue at position 1217 on the

α'-chain of C4b (9). Although no evidence is provided in the

present study for the structure of high-affinity C5 convertases, we

attribute the low K_m of the enzyme to the formation of C3b-C3b

complexes.

The functional role of C3b dimers has been suggested to be able to

hold the substrate C5 in a rigid conformation so as to enable

cleavage by Bb. Hong et al. (16) reconstituted C5 convertase ac-

tivity with purified C3b dimers or oligomers in the presence of

Ni²⁺ and factors B and D. Their studies showed higher C5 con-

vertase activity with C3b oligomers and dimers than with mono-

meric C3b. Studies by Jelezarova et al. (31) have suggested C3b-

C3b dimers in complex with IgG molecules to form better

precursors of convertases than C3b. Recent studies by Sandvol et al. (32)

involving indels have identified at least two binding sites

on C5 for the classical pathway C5 convertase in addition to the

convertase cleavage site. Based on all these studies it appears that

C5 binds with high affinity to multimeric C3b complexes through

interactions of at least two distinct sites on C5 and at least two sites

on C3b.

In contrast to the K_m of surface-bound C5 convertases, which

varied by approximately three orders of magnitude (Fig. 3A and

Table I), the k_cat of the enzyme varied very little (Fig. 3B). C5

convertases assembled on zymosan particles exhibited similar cat-

alytic rates at all densities of C3b/cell. Those formed on ES and E_S

at low density of C3b/cell exhibited k_cat that was similar to that of

the soluble monomeric C3b,Bb but showed a 3- to 4-fold decrease

with increasing density of C3b/cell (Table I). Although this de-

crease in k_cat was consistently observed with increasing density of

C3b/cell, its origin is not apparent. High densities of C3b,Bb, or

bound C5 could interfere with efficient C5 cleavage or restrict

access to neighboring active sites. The k_cat of the self-amplified C5

convertases might be revealing in this regard, but the k_cat of these

enzymes was impossible to measure accurately due to the low

numbers of enzymes per cell and to enzyme decay before and

during the assays (these enzymes were premade and no factor B

was available for enzyme reformation as in other kinetic assays).

Nevertheless, the average k_cat for cell-bound alternative pathway

C5 convertases was 0.0056 s⁻¹ indicating that the rate of C5 cleav-

age is one of the slowest enzymatic reactions known with one C5

cleaved per 3 min at V_max. Apparently this level of activity is

sufficient to generate biologically effective levels of cytolytic

C5b-9 and the chemotactic fragment C5a.

The proposed mechanism underlying the conversion of low af-

finity C5 convertase to high-affinity C5 convertase. At physiological

C5 concentrations these enzymes exceed 90% saturation with

C5, which prevents C3 cleavage and allows C5 cleavage at a

velocity approaching V_max. The C3b molecules at the edges of the

expanding amplification ring will be subjected to inactivation by factors H and I and

these convertases will stop production of both C3b and C5b-9. In this way the

sequence of waves of amplification, formation of high-affinity C5 conver-

tases and their inactivation continues outward from the initial C3b

resulting in both opsonization of the cell surface with C3b/C3b/C3d for

phagocytosis and to production of the cytolytic C5b-9 complex.

FIGURE 8. Model for formation and inactivation of high-affinity C3/C5

convertases during alternative pathway activation. Both side views (left)

top views (right) of the activating surface are shown. The first C3b

molecule deposited on the target surface forms a monomeric C3/C5 con-

vertase C3b,Bb. C3b,Bb cleaves C3, depositing C3b on and near itself.

Some of the newly deposited C3b molecules will form C3b-C3b complexes, which have higher affinities for C5, thereby switching the enzyme

from cleavage of C3 to cleavage of C5. These convertases will predomi-

nantly cleave C5 at a velocity close to V_max resulting in higher production

of the cytolytic C5b-9 complex. The C3b molecules on the outer edge of

the expanding ring of amplification have low affinities for C5. Because

these convertases will interact weakly with C5, they will predominantly

cleave C3, amplifying C3b on the surface. As amplification continues

expanding outward, additional C3b molecules are deposited and the process

of creating new high-affinity C5 convertases continues. This results in a

mixed population of C5 convertases with varying affinities for C5 and

accounts for the average K_m of the enzyme observed in the present study

(Table I) when all the C3b molecules are converted to convertases. Under

physiological conditions, the initial C5 convertases, located in the center of

the expanding ring, will be subjected to inactivation by factors H and I and

the original C3b in the center of the expanding ring will be mostly monomers and will

form monomeric C3/C5 convertase. The monomeric C3/C5 conver-

tases will spend most of their time cleaving C3 because of their

weak affinity for C5. As amplification continues outward, deposition

of C3b converts this region into high-affinity C5 convertases and

produces another ring of monomers. This process continues

until the surface is completely coated with C3b and most C3/C5

convertases have been converted to high-affinity C5 convertases.

At this point, net C3b deposition ceases and a plateau in C3b per

cell is reached (33).

Although, in the present study the effect of the complementary

protein factors H and I was not examined, it is informative to

envisage their functional roles in inactivating high-affinity C5 con-

vertases during complement activation. The higher affinity C5 con-

vertases formed in the center of the expanding ring of amplifica-

tion will be inactivated by the decay-accelerating activity of factor

H (decay of Bb) and the cofactor activities of factors H and I. The

effect of these regulatory proteins would be the conversion of C3b

to iC3b with the eventual formation of C3d. This process of C3

cleavage, C3b amplification, formation of high-affinity C5 conver-

tases, C5a release, C5b-9 formation, and C3b inactivation are all

critical for opsonization, phagocytosis, cytolysis, and presentation
of the cell surface of the invading microorganism to the adaptive immune system through receptors CD21/CD19.

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References