Formation of High Affinity C5 Convertase of the Classical Pathway of Complement* 

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C3/C5 convertase is a serine protease that cleaves C3 and C5. In the present study we examined the C5 cleaving properties of classical pathway C3/C5 convertase either bound to the surface of sheep erythrocytes or in its free soluble form. Kinetic parameters revealed that the soluble form of the enzyme (C4b,C2a) cleaved C5 at a catalytic rate similar to that of the surface-bound form (EAC1,C4b,C2a). However, both forms of the enzyme exhibited a poor affinity for the substrate, C5, as indicated by a high $K_m$ (6–9 μM). Increasing the density of C4b on the cell surface from 8,000 to 172,000 C4b/cell did not influence the $K_m$. Very high affinity C5 convertases were generated only when the low affinity C3/C5 convertases (EAC1,C4b,C2a) were allowed to deposit C3b by cleaving native C3. These C3b-containing C3/C5 convertases exhibited $K_m$ (0.0051 μM) well below the normal concentration of C5 in blood (0.37 μM). The data suggest that C3/C5 convertase assembled with either monomeric C4b or C4b-C4b complexes are inefficient in capturing C5 but cleave C3 opsonizing the cell surface with C3b for phagocytosis. Deposition of C3b converts the enzymes to high affinity C5 convertases, which cleave C5 in blood at catalytic rates approaching $V_{max}$, thereby switching from C3 to C5 cleavage. Comparison of the kinetic parameters with those of the alternative pathway convertase indicates that the 6–9-fold greater catalytic rate of the classical pathway C5 convertase may compensate for the fewer numbers of C5 convertase sites generated upon activation of this pathway.

Activation of complement is essential to the innate and adaptive immune response of the body to infections. Complement helps fight infections and clear immune complexes in blood by generating products that have numerous biological activities (1–3). Cleavage products, C3b and C4b, generated upon the activation of the third (C3) and fourth (C4) components of complement serve the important function of opsonizing foreign particles. Opsonized particles are recognized by C3/C4 receptors on macrophages, and upon attachment are cleared by phagocytosis. In addition, C3b and C4b form the noncatalytic subunits of the C3/C5 convertase. Cleavage of C5 by C5 convertase is the last enzymatic step in the complement activation cascade. The cleavage products of C5, C5a and C5b, initiate important defensive reactions. C5b, the larger fragment, initiates the formation of membrane attack complex (cytolytic C5b-9 complex) that helps destroy microorganisms. C5a, the smaller fragment, is a spasmogen and chemotactic anaphylatoxin that mediates inflammatory responses at the site of injury (4, 5) and may have systemic as well as neurological effects (6–10).

Analysis of C5 convertases has been difficult because the enzyme in its most active form is a multimeric complex that is formed on the surfaces of biological organisms. Earlier studies on C5 convertases examined the affinity ($K_a$) of C5 for the non-enzymatic subunits, C3b or C4b, in the fluid phase and on surfaces (11–15), whereas more recent studies (11, 12, 14, 15) have analyzed kinetic properties ($K_m$ and $k_{cat}$) of C3b- and cobra venom factor (CVF)-dependent C5 convertases of the alternative pathway (16–20). The bimolecular serine protease, C3b,Bb assembled with monomeric C3b, cleaves C5 without the help of a second C3b molecule at a catalytic rate similar to that of the surface-bound C5 convertase on zymosan particles (ZymC3b,Bb). The findings were unexpected because it was thought that activation of C5 by a C3 convertase occurred only in the presence of an additional C3b molecule.

The natural surface-bound C5 convertases are formed from the simple bimolecular C3 convertases by attachment of additional C3b molecules (11–14, 16, 18–25). In these surface-bound complexes the added C3b molecules form high affinity sites for the substrate (11). C3b-C3b, C4b-C4b, and mixed dimers have been proposed (14, 26) to be the structures responsible for forming high affinity C5 sites. According to current theory the corresponding convertases of classical and alternative pathways would have the structures C4b$_{2a}$, C3b,C4b,C2a, and C3b,Bb (13, 14, 25, 27), but these high affinity enzymes have not been isolated. Characterization of the formation and enzymatic properties of alternative pathway C5 convertase (16–20) demonstrated that high densities of C3b and C3b complexes increased the affinity for C5 up to a 1000-fold.

In the present study we have determined the kinetic properties ($K_m$ and $k_{cat}$) of the classical pathway C5 convertase by analyzing C5b,6 production under physiological conditions of temperature, pH, and ionic strength. The data show that both soluble and surface-bound forms of the classical pathway C3/C5 convertase (C4b,C2a), assembled with C4b as the noncatalytic subunit, cleave C5 without the aid of an additional C3b molecule. In contrast to the formation of high affinity C5 convertases of the alternative pathway, high densities of the noncatalytic subunit (C4b), resulting in the generation of C4b-C4b complexes, did not form high affinity C5 sites via the classical pathway. The C4b-dependent convertases of the clas-

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‡ The abbreviations used are: C3b, C4b, and C5b, the proteolytically activated form of C3, C4, and C5, respectively; E$_a$, antibody-coated sheep erythrocytes; TBS, Tris-buffered saline; EA, antibody-coated sheep erythrocytes; CVF, cobra venom factor.
tical pathway because of their weak affinity for C5 primarily by using chicken erythrocytes (E,C), which are sensitive to reactive lysis by human C5b,6, as described (16). An aliquot of a diluted sample from C5 convertase assays, E,C (1.2×10⁷), and 5 µl of pooled normal human serum as a source of complement proteins C7–C9 were added in a final volume of 250 µl of GVB. After 10 min of incubation at 37°C, unlysed cells were removed by centrifugation, and the amount of hemoglobin released was quantitated spectrophotometrically at 414 nm. To determine 100% lysis, E,C were lysed in 2% Nonidet P-40. Controls, containing C5 and C6 but no C2, were subtracted as background. Controls, containing C5 convertase but no purified C5 or C6, were kept to ensure that C5b,6 was not formed from the C5 and C6 in the pooled normal human serum used as a source of C7–9 during the lysis of E,C. C5b,6 concentration was quantitated from a standard curve using purified C5b,6 as described previously (16).

**Preparation of the Number of C4b Molecules Bound Per Cell**—C4b was deposited on cells by incubating EA1 with radiolabeled C4 at 4.0 mg/ml (specific activity = 0.08 µCi/µg). The procedure was repeated to obtain cells bearing different densities of radiolabeled C4b ranging from 8,000 to 172,000 C4b/cell. The cells were washed and incubated for 10 min at 37°C in 25 µl of GVB+ after which they were diluted with 75 µl of GVB+. Bound and free radiolabel were separated by layering 75 µl of the mixture on 250 µl of 20% sucrose in GVB+ and centrifuging for 1 h at 100,000 × g at 22°C. The amount of radiolabeled C4b bound to cells was measured by cutting the tube and counting the amount of radioactivity in the pellet (35). Nonspecific background binding of radiolabeled C4 to EA cells was subtracted.

**Determination of the C5 Convertase Concentration**—The number of C5 convertase sites formed on EA1,C4b or EA1,C3b,C4b was determined by measuring only those C4b molecules that were capable of forming an enzyme with radiolabeled C2 under saturating conditions. Various amounts of ¹²⁵I-C2 were incubated with EA1,C4b or EA1,C3b,C4b for 10 min at 37°C. Bound and free radiolabel were separated as described above. Distribution of radioactivity in the 70-kDa C2a and the 30-kDa C2b fragments was determined by SDS-PAGE as described previously (16). The cpm radioactivity in the label of the specific C2a uptake, the C2a-specific activity, and the C2a molecular weight, the concentration of C5 convertase in the experiment was calculated. Specific C2a uptake was determined after subtracting non-specific binding and contribution from ¹²⁵I-labeled C4b and C3b if present in the experiment.

**Measurements of Surface-bound C5 Convertase**—Enzyme velocities were determined under saturating concentrations of C2 and C6 in 0.5-ml siliconized microcentrifuge tubes. Assay mixtures contained C2 (0.36 µg), C6 (2.5 µg), 0.5 mM MgCl₂, and varying concentrations of C5. The reaction was started by the addition of EA1,C3b,C4b or EA1,C4b cells in a final volume of 25 µl of GVB so that 0.5–2 × 10⁷ cells were present during the assay. Enzyme velocity was determined by measuring the number of C2a bound as described in the preceding paragraph. After 10 min of incubation at 37°C, further cleavage of C5 was prevented by transferring the assay tubes to an ice bath and adding 225 µl of ice-cold GVB. C5b,6 was titrated by hemo-

**Experimental Procedures**

Reagents—Chicken erythrocytes (E,C) were isolated from chicken blood purchased from Colorado Serum Co. (Denver, CO). Nonidet P-40, a non-ionic detergent, Thimerosal, Tween 20, and EDTA were purchased from Sigma. Immobilon-P transfer membrane was purchased from Millipore. Veronal-buffered saline contained 5 mM barbital, 145 mM NaCl, pH 7.4. Gelatin veronal-buffered saline (GVB) was veronal-buffered saline containing 0.1% gelatin, and GVB+ was GVB containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂. GVB was GVB containing 10 mM EDTA. Tris-buffered saline (TBS) was 20 mM Tris base, 500 mM NaCl, pH 7.5. Tween/Tris-buffered saline (T-TBS) was TBS containing 0.05% nonidet P-40, 0.5% nonfat dry milk, and 1% Thimerosal.

**Purified Proteins**—Complement proteins, C2 (28), C3 (29, 30), C4 (31), and C6 (32), were all purified from normal human plasma as described in the references cited. C5 was purified from normal human plasma as described (33) except that ceramic hydroxylapatite (Bio-Rad) was used as an ion-exchange component. C5 was separated from C4 during the ion-exchange chromatography step of C4 purification on a Mono-Q column (Amersham Biosciences). The C4b, 95% monomeric in composition was separated from trace amounts of C4b-C4b dimers by gel filtration on G4000SW column (Superior). The purified C4b, 95% pure) as well as the 70-kDa C2a and the 30-kDa C2b fragments was determined by SDS-PAGE, was observed to be greater than 95%. Protein concentrations of C2, C3, C4, C5, C6, and C5b,6 were determined spectrophotometrically using 1.0, 11.0, 10.3, 11.0, 10.8, and 10.3, as the value for E₂₈₀nm respectively. All purified proteins were stored at −76°C. Mₚ values employed in the calculations were 204,000 for C4, 195,000 for C4b, 176,000 for C3b, 190,000 for C5, 179,000 for C5b, 120,000 for C6, 298,000 for C2, 102,000 for C2a, and 70,000 for C2b. The amount of C2a bound was determined by subtracting non-specific background binding from the C5b,6 concentration as described previously (16).

**Specific Binding and Contribution from ¹²⁵I-labeled C4b and C3b**—The cells were incubated with radiolabeled C4 at 4.0 mg/ml (specific activity = 0.08 µCi/µg). The procedure was repeated to obtain cells bearing different densities of radiolabeled C4b ranging from 8,000 to 172,000 C4b/cell. The cells were washed and incubated for 10 min at 37°C in 25 µl of GVB+ after which they were diluted with 75 µl of GVB+. Bound and free radiolabel were separated by layering 75 µl of the mixture on 250 µl of 20% sucrose in GVB+ and centrifuging for 1 h at 100,000 × g at 22°C. The amount of radiolabeled C4b bound to cells was measured by cutting the tube and counting the amount of radioactivity in the pellet (35). Nonspecific background binding of radiolabeled C4 to EA cells was subtracted.

**Determination of the C5 Convertase Concentration**—The number of C5 convertase sites formed on EA1,C4b or EA1,C3b,C4b was determined by measuring only those C4b molecules that were capable of forming an enzyme with radiolabeled C2 under saturating conditions. Various amounts of ¹²⁵I-C2 were incubated with EA1,C4b or EA1,C3b,C4b for 10 min at 37°C. Bound and free radiolabel were separated as described above. Distribution of radioactivity in the 70-kDa C2a and the 30-kDa C2b fragments was determined by SDS-PAGE as described previously (16). The cpm radioactivity in the label of the specific C2a uptake, the C2a-specific activity, and the C2a molecular weight, the concentration of C5 convertase in the experiment was calculated. Specific C2a uptake was determined after subtracting non-specific binding and contribution from ¹²⁵I-labeled C4b and C3b if present in the experiment.

**Measurements of Surface-bound C5 Convertase**—Enzyme velocities were determined under saturating concentrations of C2 and C6 in 0.5-ml siliconized microcentrifuge tubes. Assay mixtures contained C2 (0.36 µg), C6 (2.5 µg), 0.5 mM MgCl₂, and varying concentrations of C5. The reaction was started by the addition of EA1,C3b,C4b or EA1,C4b cells in a final volume of 25 µl of GVB so that 0.5–2 × 10⁷ cells were present during the assay. Enzyme velocity was determined by measuring the number of C2a bound as described in the preceding paragraph. After 10 min of incubation at 37°C, further cleavage of C5 was prevented by transferring the assay tubes to an ice bath and adding 225 µl of ice-cold GVB. C5b,6 was titrated by hemo-

**Structure/Function of C5 Convertases of the Classical Pathway**
lytic assays using \( E_c \) and quantitated using standard curves generated with purified C5b,6.

**Kinetic Measurements of Fluid Phase C5 Convertase**—The assays employed saturating amounts of C2 (0.36 \( \mu \)g), 20 ng of monomeric soluble C4b, and 0.5 mM MgCl\(_2\) varying concentrations of C5, excess C6 (2.5 \( \mu \)g), and GVB\(^{-1}\) in a final volume of 25 \( \mu \)l. The concentration of C5 convertase was assumed to be equivalent to the C4b concentration because preliminary experiments were used to determine the amounts of C2 and activated C1s needed to saturate C4b. Reactions were started with the addition of 4 ng of activated C1s. After 10 min of incubation at 37 \( ^\circ \)C, reactions were stopped by dilution with 200 \( \mu \)l of ice-cold GVB, and the amount of C5b,6 formed was quantitated using \( E_c \).

**Data Analysis**—The reaction velocity data were analyzed according to the Michaelis-Menten equation, \( v = \frac{V_{\text{max}}}{K_m + [S]} \). The results were fit to this equation using nonlinear regression analysis, and the kinetic parameters \( K_m \), \( V_{\text{max}} \), and \( k_{\text{cat}} \) were determined using Grafit version 4.12 software (Erithacus Software, Staines, Middlesex, London, UK).

**Preparation of Radiolabeled Proteins**—C2, C3, and C4 (100 \( \mu \)g) were radiolabeled with \(^{125}\)I for 30 min at 0 \( ^\circ \)C in a glass tube coated with IODO-GEN (Pierce). Free \(^{125}\)I was removed by centrifugal desalting kinetic parameters were fit to this equation using nonlinear regression analysis, and the specific activities of radiolabeled C2 ranged from 0.12 to 0.95 \( \mu \)Ci/\( \mu \)g. Radiolabeled C3 and C4 were diluted with cold C3 or C4 to give specific activities of 0.01–0.06 \( \mu \)Ci/\( \mu \)g.

**Measurement of the Formation and Decay of C5 Convertase**—The rate of formation of surface-bound C5 convertases was determined by assembling the enzyme under saturating concentrations of C2 (0.36 \( \mu \)g) in GVB\(^{-1}\). The reaction was started with the addition of EAC1,C4b or EAC1,C3bC4b. Reaction mixtures were incubated at 37 \( ^\circ \)C for different time intervals (0.5, 1, 2, 3, 5, and 7 min), and enzyme formation was stopped by addition of EDTA. A mixture of C5 (5 \( \mu \)g) and C6 (2.5 \( \mu \)g) was added and incubated at 37 \( ^\circ \)C and the amount of C5b,6 formed was quantitated hemolytically using \( E_c \). The rate of formation of the soluble C4b,C2a was determined similarly except that assay mixtures contained limited amounts of activated C1s (4 ng) and C4b (20 ng), and the reaction was started with the addition of C2.

The half-life of C5 convertases was measured by pre-making the enzyme for 2 min with C2 (1.2 \( \mu \)g), 0.5 mM MgCl\(_2\), and 50 \( \mu \)l of EAC1,C4b (0.38 \( \times \) 10\(^7\)pm) bearing 62,000 C4b/C2 cell in a final volume of 100 \( \mu \)l of GVB\(^{-1}\). At zero time additional formation of enzyme was stopped with EDTA. At various time intervals, aliquots (19.5 \( \mu \)l) of the enzyme preparation were withdrawn and assayed for remaining C5 convertase activity by adding 3.5 \( \mu \)l containing C5 (10.6 \( \mu \)g) and C6 (1.94 \( \mu \)g). The amount of C5b,6 formed after 10 min at 37 \( ^\circ \)C was determined hemolytically using \( E_c \). The half-life of surface-bound C5 convertase assembled with C3b-containing complexes (EAC1,C3bC4b,C2a) was determined under assay conditions similar to those described above for EAC1,C4b,C2a, except that EAC1,C3bC4b bearing 55,000 C4b and 292,000 C3b per cell were employed for assembling the enzyme, and a lower amount of C5 (0.05 \( \mu \)g) was used. The rate of decay of soluble C4b,C2a was determined by pre-making the enzyme for 2 min at 37 \( ^\circ \)C with saturating concentrations of C2 (1.94 \( \mu \)g), 0.5 mM MgCl\(_2\), limited amount of activated C1s (0.16 \( \mu \)g), and C4 (0.4 \( \mu \)g) in a final volume of 135 \( \mu \)l of GVB. Additional formation of enzyme was stopped with the addition of EDTA, and the remaining C5 convertase activity was measured using C5 (3.4 \( \mu \)g) and C6 (2.5 \( \mu \)g). The amount of C5b,6 formed after 10 min at 37 \( ^\circ \)C was determined hemolytically using \( E_c \).

**Western Blot Analysis of C4b Complexes Formed on EAC1,C4b Cells**—EAC1,C4b cells were prepared as described above. EA cells bearing C4b or no C4b (25 \( \mu \)l of 0.26 \( \times \) 10\(^7\)EA/ml) were lysed with water and centrifuged for 10,621 \( \times \) g. The ghost cells obtained were washed again with water, centrifuged, and suspended in 20 \( \mu \)l of SDS-PAGE sample buffer with and without 1 \( \mu \)l of 1 M dithiothreitol. Samples were boiled and applied on a SDS-PAGE gradient NOVEX gel (4–12%, Invitrogen). The proteins were transferred to an Immobilon-P membrane for Western blotting. After blocking with blotto, the membrane was incubated with goat anti-human C4 1:5000 in T-TBS as the primary antibody for 30 min at 22 \( ^\circ \)C. The membrane was washed with T-TBS and incubated with alkaline phosphatase-antibiotic goat anti-diluted 1:1000 in T-TBS for 30 min at 22 \( ^\circ \)C. The membrane was washed with T-TBS and then TBS and then incubated with substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Fast Tablets, B 5655), to visualize bands. Soluble C4b and EA cells bearing no C4b were employed as controls.

**RESULTS**

**Formation and Decay of C5 Convertase**—Rapid formation of enzyme during assays was necessary for accurate measurement of activity. Time courses of enzyme formation done under saturating concentrations of C2 (143 nm) at 0.5 mM MgCl\(_2\) demonstrated that maximum levels of cell-bound (EAC1,C4b,C2a or EAC1,C3bC4b,C2a) and fluid phase (C4b,C2a) enzymes were achieved in less than a minute under these conditions. The rate of decay of the three C5 convertases was also measured (Fig. 1A). The three C5 convertases decayed at similar rates as indicated by \( t_{1/2} \) between 2 and 3 min at 37 \( ^\circ \)C (EAC1,C4b,C2a = 2 min, EAC1,C3bC4b,C2a = 2.5 min, and C4b,C2a = 3.0 min).

**Demonstration of Constant C5 Convertase Levels during Assays**—Because C5 convertases have a short half-life of 2–3 min (Fig. 1A), the enzyme decays during an assay. Preliminary experiments were done to determine the concentrations of C2 required to maintain constant amounts of enzyme during a 10-min assay. Fig. 1B shows that with the C2 concentrations used a constant amount of enzyme was maintained throughout the 10-min assay even though the convertase was decaying and reforming during this assay period. For the soluble form of the enzyme (C4b,C2a), it was also necessary to determine the concentration of activated C1s that would maintain a constant amount of enzyme because C1s can consume C2 unproductively during the assay. As seen in Fig. 1B constant levels of the soluble C5 convertase were maintained throughout the 10-min assay period using 4 ng of activated C1s and 360 ng of C2 per 25-\( \mu \)l reaction mixture.

**Fig. 1. Characterization of the C5 convertase assay.** A, rate of decay of classical pathway C5 convertases. C5 convertases were pre-made for 2 min at 37 \( ^\circ \)C. After adding GVB to prevent additional formation of enzyme, the enzyme was allowed to decay at 37 \( ^\circ \)C. Aliquots were removed at the indicated times and assayed for remaining C5 convertase activity by incubating with C5 and C6. C5b,6 formed was quantitated using lysis of \( E_c \) and EDTA containing pooled normal human serum as a source of C7–C9. C4b,C2a; EAC1,C4b,C2a; and C4b,C2a. Reaction mixtures of the convertases were incubated for the indicated length of time. C5 and C6 were then added, and C5b,6 formation was allowed to continue for the next 5 min.

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Determination of the Number of Cell-bound C4b and the Concentration of C5 Convertase—The number of C4b/cell was determined with radiolabeled C4. EAC1 cells were prepared bearing amounts of C4b ranging from about 8,000 to 172,000 C4b/cell. The number of C5 convertase sites that could be generated on these EAC1,C4b cells was determined as the number of C2a-binding sites. As seen in Fig. 2A, the binding of $^{125}$I-C2a to C4b-coated cells was saturable. The maximum amount of C2a bound was used to determine the number of C5 convertase sites. The ratio of C2a per C4b (Fig. 2B) did not vary significantly over a 20-fold range of C4b/cell. The average ratio of C2a per C4b was found to be $0.28 \pm 0.10$. These results suggest that one in every four C4b molecules is capable of forming a C5 convertase. Because the number of C2a molecules bound to EAC1,C4b or EAC1,C3b-C4b indicates only those C4b molecules that are capable of forming a C5 convertase, throughout this study we have determined the number of C5 convertase sites formed by measuring C2a binding rather than relying on the number of C4b/cell.

Measurement of Kinetic Parameters of Surface-bound C3/C5 Convertase (EAC1,C4b,C2a)—Initial velocities of C5 activation were determined at various concentrations of C5, and a fixed concentration of enzyme was calculated from the binding of radiolabeled C2a to the EAC1,C4b cells. The velocity data obtained for the surface-bound C3/C5 convertase, shown in Fig. 3A, were found to fit well to the theoretical curve based on the Michaelis-Menten equation, $v = V_{\text{max}} [S]/(K_m + [S])$. The data were also observed to fit well to the linear form of the Michaelis-Menten equation, shown as the Eadie-Hofstee plot (inset in Fig. 3A). The $K_m$ of the enzyme for C5 was $5.7 \pm 2.6 \mu M$ suggesting that surface-bound C3/C5 convertase assembled with C4b as the noncatalytic subunit has a weak affinity for the substrate, C5, compared with the normal plasma concentration.
the density of C4b on the cell does not influence the complexes (C3b-C4b complexes) as the noncatalytic subunits of C3/C5 convertases assembled with C3b-containing substrate C5. The Km, or the affinity C5 convertases of the classical pathway. In contrast to the 30-fold effect of C3b density on the alternative pathway C5 convertase varied greatly with C3b density on cell surfaces (18), the effect of C4b density on the kinetic parameters of the classical pathway enzyme was examined. EAC1 cells bearing different densities of C4b, 8,000–172,000 C4b/cell, were used for assembling surface-bound C3/C5 convertase. In contrast to the 30-fold effect of C3b density on the alternative pathway enzyme, C4b density had no significant effect on the Km value of classical pathway C5 convertase (range of 4.6–6.6 μM, Fig. 3B and Table I). The Km values observed suggest a weak binding interaction between the enzyme complex assembled with C4b as the noncatalytic subunit and the substrate C5 and therefore will be referred to as the low affinity C3/C5 convertases in the present study. Like the Km, the rate of C5 cleavage (kcat) by C3/C5 convertases assembled with different densities of C4b/cell did not vary significantly (Fig. 3C and Table I). Together, the data suggest that the density of C4b on the cell does not influence the Km or the kcat values of C3/C5 convertases of the classical pathway for the substrate C5.

Analysis of C4b Complexes Formed on EAC1,C4b Cells—C4b deposited on EAC1,C4b cells was analyzed by Western blot. Three major bands of approximate molecular masses of 200, 400, and 600 kDa were observed under non-reduced conditions. This pattern of bands was similar to that observed by Takata et al. (13) and by Kozono et al. (25) for C3b containing C4b complexes. Together, these results demonstrate formation of C4b dimers and higher complexes on EA cells employed in the present study. Under reduced conditions, several bands with approximate molecular masses of 95, 140, 230, 280, and 350 kDa were observed in addition to the β- (75 kDa) and γ-bands (33 kDa) of C4b. This pattern of bands was found to be consistent with those shown for C4b-C4b and C3b-C4b dimers by various other groups (13, 24, 25, 37, 38). These results indicate that although C4b dimers form on the surface of EA1 cells employed in the current study, they do not generate high affinity C5 convertases of the classical pathway.

Effect of C3b Density on Kinetic Parameters of Surface-bound C3/C5 Convertase—Because the Km value of C4b-dependent C3/C5 convertases (EAC1,C4b,C2a) indicated a weak interaction with the substrate, C5, the effect of C3b molecules on the affinity for C5 was examined. Kinetic parameters of C3/C5 convertases assembled with C3b-containing complexes (C3b-C4b complexes) as the noncatalytic subunits were measured. EAC1,C3b,C4b,C2a was prepared by allowing C3/C5 convertase, EAC1,C4b,C2a, to cleave native C3 and deposit C3b on and around themselves. After C3b deposition, the cells were washed to remove excess C3. Kinetic data (Fig. 4) were generated for C5 cleavage by enzymes assembled with saturating levels of C2 and EAC1,C3b,C4b cells that had 17,000 C4b and 70,000 C3b per cell. The enzymes formed on these cells exhibited Km in the nanomolar range (1.1–8.3 μM). This was observed to be the case with all convertases assembled at all levels of C4b/cell (Fig. 3B and Table I). Because of the low Km, 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 less than 5%; nevertheless, at substrate concentrations below 5 μM cleavage of C5 approached 25% at the end of the 10 min assay. The very low Km value of these C3b-containing convertases indicates an interaction with C5 that is stronger by about 3 orders of magnitude compared with convertases assembled with C4b alone. Although the Km of the two enzymes differed by 1000-fold, the rate of C5 cleavage (kcat) was nearly identical (Fig. 3C and Table I).

Effect of C3b Density on Kinetic Parameters of Surface-bound C3/C5 Convertase—Because generation of high affinity C5 convertases required deposition of C3b molecules on the cell surface, the results suggested that C5 convertases with varying affinities for C5 exist and that the observed Km would depend on the ratio of the number of C3b molecules deposited to the number of C4b molecules present on the cell surface. If the observed Km value was an average of a high affinity C5 convertase (average Km = 5.1 μM) and a low affinity C3/C5 convertase (average Km = 5.6 μM), then the two convertases differ a thousand-fold in their affinity for C5, the Edie-Hofstee plot would have a curvature to it. To investigate this possibility, kinetic properties of convertases assembled with varying ratios of C3b:C4b were examined. EAC1,C3b,C4b cells with a C3b:C4b ratio of 2:1, 1:1, 0.5:1, and 0.2:1 were prepared by allowing EAC1,C4b,C2a to cleave native C3 in limited amounts. Initial velocities for C5 cleavage were measured, and data for C3/C5 convertase assembled with EAC1,C3b,C4b cells that had a C3b:C4b ratio of 0.2:1 (i.e. 20,000 C3b/cell and 100,000 C4b/cell) are shown in Fig. 5. C5 cleavage was measured in the presence of saturating concentrations of C2, excess C6, and varying concentrations of C5 ranging from 0.0016 to 26 μM. The kinetic data obtained did not fit well to the theoretical curve based on the Michaelis-Menten equation, which assumes a homogeneous enzyme (Fig. 5A). The presence of C5 convertases having different affinities for C5 was very apparent in the Edie-Hofstee plot, which had a strong curvature to it (Fig. 5B). The initial and final slopes

### Table I

| Enzyme | C4b/cell | C3b/cell | Km | kcat | kcat/Km | Turnover no. |
|--------|----------|----------|----|------|---------|-------------|
|        |          |          | μM | s⁻¹  | s⁻¹ μM⁻¹ | min⁻¹       |
| Surface-bound C3/C5 convertase |       |          |    |      |         |             |
| EAC1,C4b,C2a | 8,000 | 0 | 4.6 ± 2.5 | 0.023 ± 0.001 | 5.0 × 10⁻³ | 1.36 ± 0.08 |
| 27,000 |          |          | 5.4 ± 2.3 | 0.047 ± 0.013 | 8.7 × 10⁻³ | 2.82 ± 0.75 |
| 62,000 |          |          | 6.6 ± 3.5 | 0.023 ± 0.010 | 3.5 × 10⁻³ | 1.40 ± 0.57 |
| 172,000 |          |          | 5.7 ± 2.6 | 0.025 ± 0.010 | 4.4 × 10⁻³ | 1.48 ± 0.58 |
| High affinity C5 convertase |       |          |    |      |         |             |
| EAC1,C3b,C4b,C2a | 17,000 | 73,000 | 0.0025 ± 0.0004 | 0.015 ± 0.002 | 6000 × 10⁻³ | 0.89 ± 0.12 |
| 37,000 |          |          | 0.0083 ± 0.0007 | 0.015 ± 0.003 | 1800 × 10⁻³ | 0.81 ± 0.27 |
| 55,000 |          |          | 0.0085 ± 0.0013 | 0.026 ± 0.005 | 3100 × 10⁻³ | 1.54 ± 0.30 |
| 147,000 |          |          | 0.0114 ± 0.0001 | 0.016 ± 0.005 | 1500 × 10⁻³ | 1.20 ± 0.12 |
| Soluble monomeric C3/C5 convertase |       |          |    |      |         |             |
| C4b,C2a | 1 | 0 | 8.9 ± 3.9 | 0.022 ± 0.005 | 2.4 × 10⁻³ | 1.21 ± 0.26 |

*Kinetic parameters of surface-bound C5 convertase were determined by nonlinear regression as described under “Experimental Procedures” and shown in Figs. 2–4 and 6. Values are mean ± S.D. (n = 3 or 4).*
The Eadie-Hofstee plot suggested two C5 convertase species with a $K_m$ of 18 nM for the high affinity and 2.2 μM for the low affinity enzyme, in agreement with the other kinetic data presented here. The biphasic nature of the Eadie-Hofstee was observed in all experiments that employed cells with a C3b:C4b ratio of 0.5:1 or less but not with cells that had a C3b:C4b ratio of one or more. Considered together, these results suggest that deposition of C3b molecules equal to or more than the number of C4b molecules or four times the number of C4b,C2a enzymes resulted in the conversion of all low affinity C3/C5 convertases to high affinity C5 convertases.

Measurement of the Kinetic Parameters of Soluble C3/C5 Convertase (C4b,C2a)—To compare the kinetic properties of surface-bound C4b-dependent C3/C5 convertases with those of the soluble form of the enzyme, the rate of cleavage of C5 by C4b,C2a was analyzed. The monomeric C4b employed in the assays to form a C2b, C4b-dependent C3/C5 convertase, either soluble or surface-bound, exhibits a poor affinity for the substrate, C5, but a 6-9-fold higher catalytic rate than the alternative pathway enzyme (Table I). Similar results were obtained when purified C4b-C4b dimers or C4 was used instead of monomeric C4b to assemble the soluble form of the enzyme. Considered together, the results imply that C4b-dependent C3/C5 convertase, either soluble or surface-bound, exhibits a poor affinity for the substrate, C5, but a 6-9-fold higher catalytic rate than the alternative pathway enzyme (Table I).
binding C5 and therefore inefficient in cleaving C5. But cleavage of C3 by these C3/C5 convertases lowers the $K_m$ value for C5 to well below the normal plasma concentration of C5 in blood (0.37 $\mu M$). This enzyme (EAC1,C3bC4b,C2a) will be occupied by C5 most of the time and will cleave C5 at a catalytic rate close to $V_{\text{max}}$.

**DISCUSSION**

The present study examined the structure/function of C5 convertases of the classical pathway of complement. The classical pathway bimolecular serine protease, C4b,C2a, which cleaves C3, was found to cleave C5 at a catalytic rate similar to the surface-bound form of the enzyme (EAC1,C4b,C2a) (Table I). Determination of kinetic parameters revealed that C4b,C2a, either soluble (assembled with purified C4b monomers or C4b-C4b dimers) or surface-bound, exhibited a weak affinity for C5 as indicated by a high $K_m$ value for C5 (8.9 and 4.6–6.6 $\mu M$, respectively) (Table I). Increasing the density of C4b on the cell surface did not generate high affinity C5 convertases. This was evident when a 20-fold increase in C4b density on the cell surface (8,000–172,000 C4b/cell) generated C5 convertases that exhibited similar $K_m$ values (Fig. 3B). Western blot analysis of the C4b deposited on EAC1 cells showed about 50% to be present as high molecular weight complexes of C4b, indicating that although the surface-bound C5 convertases assembled in this study were comprised of C4b-C4b complexes, they did not form high affinity C5 convertases.

Conversion of low affinity C3/C5 convertases to high affinity C5 convertases was achieved by allowing preformed C3/C5 convertases (C4b,C2a), assembled on EA cells, to cleave native C3. Cleavage of C3 results in the deposition of C3b on and around the low affinity C3/C5 convertases (C4b,C2a), consequently generating C3b-containing complexes (C3b-C4b). Examination of the C5 cleaving properties of these complexes revealed an ~1000-fold decrease in the $K_m$ (Fig. 3B and Table I). The average $K_m = 5.1$ $\mu M$ was well below the normal plasma concentration of C5 in blood (0.37 $\mu M$), suggesting that under normal physiological conditions these convertases will be 90% or more saturated with C5. These enzymes will cleave C5 at velocities approaching maximum velocity ($V_{\text{max}}$) (Fig. 6). In this way low affinity C3/C5 convertases are converted to high affinity C5 convertases, and the enzymes switch from primarily cleaving C3 to almost exclusively activating C5 and producing cytolytic C5b-9.

Seya and co-workers (24, 37) group have shown that C4b-dependent C3/C5 convertases generated in their studies were comprised of C4b-C4b dimers and suggested that these convertases may provide a mechanism for complement-mediated cytolysis in patients with complete C3 deficiency by serving as high affinity C5 convertases. In contrast, the data presented here show that C4b-C4b dimers either soluble or surface-bound (Figs. 3A, 3B, and 6 and Table I) do not generate high affinity C5 convertases of the classical pathway. Instead, the results show that only C3b-containing C4b complexes form high affinity C5 convertases of the classical pathway (Figs. 3B and 4). These findings agree with the binding studies of Takata et al. (13) that associated high C5 convertase activity to C3b-C4b formation. Although the C4b-dependent C5 convertases have a weak affinity for C5, cleavage of C5 by C4b,C2a ($k_{\text{cat}}$) assembled with monomeric C4b or multimeric C4b is about 10% that of C3b-C4b,C2a at the physiologic concentration of C5 in blood (0.37 $\mu M$), suggesting that C5 could be cleaved without C3 and without C4b-C4b complexes (Fig. 6).

Formation of high affinity C5 convertase was found to be dependent on the ratio of C3b:C4b molecules deposited on the cell surface. Deposition of C3b such that the ratio of C3b:C4b was less than 1:1 generated a heterogeneous population of C5 convertases (Fig. 5). This was evident from the biphasic nature of the Edie-Hofstee plot (Fig. 5B) suggesting the presence of both low and high affinity C5 convertases (C4b,C2a and C3b,C4b,C2a). Deposition of additional C3b/cell equal to or greater than the number of C4b/cell resulted in a homogeneous population of high affinity C5 convertases (Fig. 4). If every C3b deposited was covalently attached to a C4b, then C3b-C4b dimers would form, and the results would mean that a single C3b molecule is required to convert a low affinity C3b/C5 convertase to high affinity C5 convertase. However, because only one of four C4b molecules is capable of forming a convertase, the results suggest that for every C4b capable of forming an enzyme four C3b molecules must be deposited to convert it to a high affinity C5 convertase. It is not yet clear whether these C3b are on the C4b with the C2a, on nearby C4b, attached to other C3b, or attached directly to the cell. Additional studies examining the structural properties of the high affinity C3/C5 convertase are necessary to evaluate the precise nature of these complexes.

Studies by different groups (12–15, 17, 39) have indicated stronger binding interactions between C5 and C3b-C4b or C3b-C3b complexes deposited as clusters on sheep erythrocytes or zymosan than for soluble C3b. Reconstitution experiments by Hong et al. (26) have shown higher C5 activity with C3b oligomers and dimers than with C3b monomers, whereas C3b dimers in complex with IgG molecules have been reported to be better precursors of convertases than monomeric C3b (40). Studies of insertion/deletion patterns have suggested at least two binding sites on C5 for interaction with classical pathway C5 convertase in addition to the convertase cleavage site (41). Considered together with the kinetic data presented here, the findings suggest that the high affinity interaction between C5 and C3b-containing C4b complexes or C3b-C3b complexes is the result of bivalent or multivalent interactions.
As shown in our previous studies, the $K_m$ of alternative pathway enzymes for C5 was similar to the affinity ($K_d$) of C5 for the non-catalytic subunits of these enzymes (16–18). This was true for C3b-, C4b, and C3b multimer-dependent C5 convertases. Studies by others (12–15, 17, 39) have reported similar affinities between C5 and some of these non-catalytic subunits. In the present study, $K_m$ measured for high affinity C5 convertase of the classical pathway (average $K_m = 5.1 \text{nM}$) (Fig. 3B and Table II) was observed to be similar to the $K_d$ reported for the binding of C5 to C4b-C3b bearing sheep erythrocytes ($K_d = 5.0 \text{nM}$) (13). Together, these findings indicate that the $K_m$ of C5 convertases is primarily determined by the interaction of C5 with the noncatalytic subunits of the enzyme.

Although the $K_m$ values of classical pathway C5 convertases differed by 1000-fold, their catalytic rate constants ($k_{cat}$) were similar (Fig. 3C and Table I). The average $k_{cat}$ was $0.024 \pm 0.010 \text{s}^{-1}$ indicating that the classical pathway C5 convertase has a turnover number of 1.44 C5 molecules cleaved per min per enzyme. The low turnover number suggests that C5 convertases can be grouped with other enzymes such as ribozymes per enzyme. The low turnover number implies that equivalent numbers of C4b/C2a or C3b,Bb (Tables I and II). Both enzymes initially alter their specificity upon attachment of multiple C3b molecules. The acquisition of high affinity for C5 results in cessation of C3b deposition and initiation of cytolytic activity.

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