Enzymes of the complement system exhibit, unlike most other proteases, a high substrate specificity, hydrolyzing only a single peptide bond in their protein substrates. This property allowed the performance of a detailed analysis of the enzymatic activity of a protease acting on its natural high molecular weight substrate. The enzyme investigated was the cobra venom factor-dependent C3 convertase (EC 3.4.21.47) of human complement. The enzyme constitutes a bimolecular complex of cobra venom factor and the catalytic site bearing fragment Bb of human Factor B. It hydrolyzes peptide bond 77 (Arg-Ser) of the α-chain of the human complement protein C3, thereby producing the fragments C3a and C3b. The enzyme was generated from isolated proteins. It exhibited spontaneous decay-dissociation into its subunits with a half-life of 7 h at 37 °C. The following kinetic parameters for C3 hydrolysis were determined: the Michaelis constant, $K_m$, the catalytic constant, $k_{cat}$, the turnover number, the catalytic cycle time, the specific activity, the apparent second-order rate constant, $k_{cat}/K_m$, and the apparent first-order rate constant for the low substrate concentration range. The encounter of enzyme and substrate proceeded under rapid equilibrium conditions. For the formation of the enzyme-substrate complex, the equilibrium constant, $K$, the standard enthalpy, $\Delta H^\circ$, standard entropy, $\Delta S^\circ$, and standard Gibb's energy, $\Delta G^\circ$, were determined. For the rate-limiting step of the overall reaction, the activation energy, $E_a$, activation enthalpy, $\Delta H^*$, activation entropy, $\Delta S^*$, and Gibb's energy of activation, $\Delta G^*$, were derived. The results demonstrate that action of a protease of high molecular weight ($M_0 = 210,000$) on its substrate of high molecular weight ($M_0 = 185,000$) can be described in terms of Michaelis-Menten kinetics. The data are consistent with a double intermediate catalytic mechanism and a kinetic mechanism of a Tetra Uni Ping Pong Bi Bi reaction reduced to a Uni Bi reaction and therefore support the serine protease concept of Factor B-derived enzymes.

The enzymatic nature of many reactions occurring upon activation of complement is well established (1–4). Unlike most other proteases, complement enzymes exhibit a high substrate specificity, i.e., in most instances, they hydrolyze only a single peptide bond in their natural substrates. All of the well investigated proteases like trypsin or chymotrypsin lack this high substrate specificity and, therefore, the kinetic and thermodynamic parameters characterizing these enzymes have been derived from their reactions with synthetic low molecular weight substrates such as esters, anilides, or oligopeptide (8). The present study was performed to determine the kinetic and thermodynamic parameters for the reaction of a complement enzyme with its natural high molecular weight protein substrate. The enzyme investigated is the cobra venom factor-dependent C3 convertase (EC 3.4.21.47) of human complement.

The enzyme constitutes a bimolecular complex of cobra venom factor ($M_0 = 147,000$) and of the catalytic site-bearing fragment of human Factor B (10, 11), designated Bb ($M_0 = 63,000$). The enzyme is formed when the proenzyme Factor B ($M_0 = 93,000$) binds to CVF in the presence of Mg$^{2+}$ and is subsequently cleaved by its activating enzyme Factor D (EC 3.4.21.48) (12–15). The CVF:Bb enzyme hydrolyzes bond 77 (Arg-Ser) in the α-chain of C5 ($M_0 = 185,000$) which results in the liberation of the activation peptide C3a ($M_0 = 9,000$) and the formation of metastable C3b ($M_0 = 176,000$). The formation and action of the CVF-dependent C3 convertase are analogous to formation and action of the C3b-dependent C3 convertase (C3b:Bb). The latter enzyme is formed during activation of the alternative complement pathway (2). C3a constitutes one of the three complement-derived anaphylatoxins and C3b fulfills various functions in humoral and cellular mechanisms of host defense against infections. Metastable C3b contains a highly reactive carbonyl group through which it may form a covalent bond with surface constituents of biological particles. Through this mechanism, C3b becomes firmly attached to targets of complement attack and may serve as ligand for specific complement receptors on the surface of phagocytic cells and as structural subunit of the C3 and C5 activating enzymes of complement (18–20). The CVF-
dependent C3 convertase was selected for these investigations because it is characterized by a markedly greater stability than the C4b-dependent and analytical isoelectric focusing.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human blood and plasma were obtained from the San Diego Blood Bank. Guinea pig serum was purchased from Pel-Freeze; sheep erythrocytes were from Colorado Serum Co., lyophilized Staphylococcus aureus (Naja naja kauaii) was from Biologicals Unlimited, and Calbiochem-Behring; strain Cowan I, was from Calbiochem-Behring. The following chemicals were obtained commercially: gelatin (Difco), agarose (Marine Colloids Div., FMC Corp.), complete Freund’s adjuvant (Grand Island Biological Co.), EDTA, hydrazine, and 5,5-diethylbarbituric acid (Sigma), potassium thiocyanate (Malinckrodt).

**Isolation of Factor B, Factor D, C3, and Cobra Venom Factor**—Factor B (12), Factor D (15), and C3 (22) were isolated from human serum or plasma as described. CVF was isolated from lyophilized Staphylococcus aureus according to Ref. 23. All proteins were pure as judged by sodium dodecyl sulfate-polycrylamide gel electrophoresis, immunoelectrophoresis, and analytical isoelectric focusing.

**C3 Titration**—Titration of C3 hemolytic activity was performed as described (24). The C5-C9 reagent was prepared from guinea pig serum by incubation with KSCN and hydrazine (25) and subsequent addition of functionally purified human C3 (22). Sheep erythrocytes bearing the complement proteins C1, C4, and C2 were prepared acting to published procedures (26, 27).

**Buffers**—VBS is isotonic veronal-buffered saline, pH 7.4, containing 3.5 mM 5,5-diethylbarbituric acid, 143 mM NaCl; VBE is VBS containing 10 mM EDTA; GVBE is VBS containing 0.1% (w/v) gelatin.

**Formation of the CVF-dependent C3 Convertase**—CVF (12 μg) was incubated at 37°C with a 3-fold molar excess of Factor B and 0.2 μg of Factor D in a total volume of 100 μL of VBS containing 1 mM MgCl₂. After 60 min, the reaction was stopped by addition of 100 μL of VBS containing 20 mM EDTA and the mixture was further diluted with VBE to the desired concentration.

**Kinetic Analyses**—Experiments were performed with five different C3 concentrations in GVBE (8.01 × 10⁻⁶ M, 3.56 × 10⁻⁶ M, 2.13 × 10⁻⁶ M, 1.52 × 10⁻⁶ M, and 1.18 × 10⁻⁶ M). At 0°C, the C3 solutions were mixed with the CVF-dependent C3 convertase and the reaction was started by transferring the mixtures to a thermostated water bath of the desired temperature. Kinetic experiments were performed at 10, 20, 30, and 37°C. At 1.5 or 2-min intervals, 30-μl aliquots were withdrawn and diluted with ice-cold GVBE to stop the reaction. The C3 concentration in these samples was adjusted to comparable values by addition of GBGE. The total dilution was between 150 and 500X. Dilution and temperature drop were found highly efficient in stopping further substrate hydrolysis, whereas addition of anti-CVF was without effect and anti-B was only partially inhibitory. Twenty μL of these dilutions, in duplicate, were assayed for remaining C3 by hemolytic titration. Progress curves were obtained by plotting remaining C3 versus time. The enzyme concentrations used at a given temperature were chosen to result in linear progress curves over a period of 8-10 min (10°C, 2.86 × 10⁻⁷ M; 20°C, 7.14 × 10⁻⁸ M; 30°C, 3.57 × 10⁻⁸ M; 37°C, 2.86 × 10⁻⁸ M). Initial velocities were calculated from the slopes of the progress curves by regression analysis using a Hewlett-Packard computer (Model 9815A) with plotter.

Since C3 titration requires incubation at 37°C, a reactivation of the CVF-dependent C3 convertase present in the samples occurs. The presence of the enzyme has two effects on the C3 titration for which corrections were made by including the appropriate controls. First, reactivation of the enzyme caused C3 turnover which lowered the apparent amount of C3. Second, the CVF-dependent C3 convertase also hydrolyzes C5 and thereby causes C3-independent hemolysis. This effect resulted in an increase of the apparent amount of C3. Both effects were minor or negligible at low concentrations of the CVF-dependent C3 convertase, but required correction in experiments where high concentrations of the enzyme were used.

The initial velocity, v, was plotted versus the C3 concentration in the double reciprocal manner according to Lineweaver and Burk (28). Michaelis constants, Kₘ, and maximum velocities, Vₘₐₓ, were calculated from the Lineweaver-Burk plots by regression analysis. Turnover numbers and catalytic constants, kₐₜₖ, were calculated according to kₐₜₖ = Vₘₐₓ/[Eₜₖ], where Eₜₖ is the total enzyme concentration. The specific activities are reported in nkat·mg⁻¹ following the recommendations of the International Union of Biochemistry (29). The apparent first order rate constants for the low substrate concentration range, k₀, were determined (a) using the experimental values of substrate concentrations and initial velocities in the concentration range of apparent first order kinetics, k₀ = v/[S]; and (b) according to k₀ = kₐₜₖ/[Eₜₖ], a formula that is derived from the Michaelis-Menten equation assuming low substrate concentrations.

The C3 concentration in the stock solution was determined by amino acid analysis. Molar concentrations were calculated using a protein M = 182,000.

**Thermodynamic Calculations**—Activation energy, Eₜₖ, was calculated from the slope of a plot of ln kₐₜₖ versus 1/T using the Arrhenius equation. The integrated form of this equation was used to calculate the temperature coefficient Qₜₖ. Standard enthalpy, ΔH*, was calculated from the slope of a plot of ln kₐₜₖ versus 1/T applying the van’t Hoff law. Activation enthalpy, ΔH*, was calculated from the slope of a plot of (ln kₐₜₖ)/T versus 1/T according to the transition state theory of absolute reaction rates using the equation

\[
\ln \frac{k_{a tweak}}{T} = -\frac{\Delta H^*}{R} + \ln \frac{k_0}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{R}
\]

where R is the molar gas constant, k₀ is the Boltzmann entropy constant, h is the Planck constant, and x is the transmission coefficient (assumed to be unity (8, 31)). The standard Gibbs energy, ΔG*, was calculated from ΔG* = -R·T·ln K and the Gibbs energy of activation, ΔG*, from ΔG* = -R·T·ln kₐₜₖ = h·x = K₀·T (8, 32). Entropies, ΔS₀ and ΔSₜₖ, were calculated using the Gibbs-Helmholtz equation. All slopes of linear graphs were determined by regression analysis.

**Other Methods**—Monospecific antisera to Factor B and CVF were obtained by immunization of rabbits and goats using complete Freund’s adjuvant. Immunoelectrophoresis was performed in 1% (w/v) agarose in 43.5 mM 5,5-diethylbarbituric acid, pH 8.6, for approximately 2 h at 5 V·cm⁻¹ at 4°C. Protein determination was performed by the Lowry method (33) and by amino acid analysis. Amino acid analysis was performed after hydrolysis in 6 N HCl with a Beckman amino acid analyzer (Model 121M) (34), modified to the two-column system (35).

1 The reported equilibrium constants and thermodynamic parameters are apparent ones for a given pH and are based on concentration calculations rather than activities. A prime and the subscript c as recommended by the Interunion Commission on Biothermodynamics to indicate these facts (30) have been omitted to simplify symbols.
Kinetic and Thermodynamic Analysis of Complement C3 Convertase

Fig. 2. Formation and decay-dissociation of the CVF-dependent C3 convertase. Shown are agarose gels after immunoelectrophoresis and corresponding line drawings. The anode is at the right. A, applied were isolated Factor B (top), isolated CVF (bottom), and the reaction mixture after formation of the enzyme in 2-fold molar excess of CVF (center). The partial incorporation of CVF into the enzyme, CVF,Bb, is shown with anti-CVF. Anti-B precipitated the free split products Ba (anodal) and Bb (cathodal) as well as CVF,Bb (intermediate position). No uncleaved Factor B can be detected. Since Bb is a subunit of the enzyme, the precipitation lines of free Bb and the enzyme fuse, while the precipitation lines of Ba and the enzyme spur over each other. In addition, A demonstrates the similar electrophoretic mobilities of the isolated proteins CVF and Factor B. B, applied were isolated CVF (bottom) and the reaction mixture after formation of the enzyme in 3-fold molar excess of Factor B (top). This demonstrates the complete incorporation of CVF into the enzyme. C, applied were isolated CVF (bottom) and the reaction mixture as in B after incubation for 35 h at 37 °C in the presence of 10 mM EDTA prior to immunoelectrophoresis (top). As can be seen, no enzyme is detectable and CVF returned to its original electrophoretic position indicating complete decay-dissociation of the enzyme.

Fig. 3. Decay-dissociation of the CVF-dependent C3 convertase. The enzyme was generated as described under "Experimental Procedures" and subsequently incubated at 37 °C in the presence of 10 mM EDTA. At time intervals indicated, aliquots were transferred into ice-cold GVBE and assayed for remaining convertase activity in a similar manner as described in Fig. 1.

RESULTS

Generation and Quantitation of the CVF-dependent C3 Convertase—The formation of the CVF-dependent C3 convertase from Factor B and CVF in the presence of Factor D and Mg"+ proceeds according to the following expression:

CVF + Factor B + Factor D + Mg"+ → CVF,Bb + Ba

Fig. 1 shows the generation of enzyme activity as a function of time. After approximately 10 min, the maximum activity is reached and remains constant because spontaneous decay-dissociation of the enzyme is compensated by reformation of the enzyme from liberated CVF and excess Factor B. The amount of enzyme formed was calculated from CVF input, since in excess of Factor B, CVF is quantitatively incorporated into the enzyme as shown by immunoelectrophoresis (Fig. 2). Measurements of the concentration of the CVF stock solution were performed by amino acid analysis. Molar concentrations of the enzyme are based on a \( M_r = 210,000 \) corresponding to the sum of the molecular weights of the CVF and the Bb fragment of Factor B.

Incubation of CVF in presence of Mg"+ but without Factor D generated a weak C3 convertase activity representing 1% of the activity generated in the presence of Factor D. A complex of CVF and native Factor B (CVF,B), in analogy to the complex C3b,B (36), believed to be responsible for this activity, could not be detected by immunoelectrophoresis.

Stability of the CVF-dependent C3 Convertase—The CVF-dependent C3 convertase shows a spontaneous decay-dissociation into its subunits CVF and Bb. The decay was quantitated in order to determine whether it could influence the outcome of kinetic experiments. At 37 °C and physiological pH and ionic strength, the decrease of enzyme activity was first order with a half-life of approximately 7 h (Fig. 3), which corresponds to a decay rate constant of \( 2.75 \times 10^{-5} \) s\(^{-1}\). Considering the duration of the kinetic experiments to be performed, the decay was negligible. The spontaneous dissociation of the enzyme into its subunits CVF and Bb could be shown by immunoelectrophoresis (Fig. 2). After incubation for 35 h at 37 °C, the liberated CVF assumed the electrophoretic position...
of the free protein. At 6°C, the half-life of the enzyme was 2 to 3 weeks, and at 0°C it exceeded that time. Freezing at -70°C and subsequent thawing resulted in a 30% loss of enzyme activity.

**Dependence of the Initial Velocity on the Concentration of the CVF-dependent C3 Convertase**—CVF-dependent C3 convertase at five different concentrations was incubated at 37°C, ranging from $6 \times 10^{-8}$ to $6 \times 10^{-8}$ M with C3 at a concentration of $1.1 \times 10^{-5}$ M. At 1.5-min intervals, aliquots were transferred to ice-cold GVBE and assayed for remaining C3. Initial velocities were determined and found to be a linear function of the enzyme concentration in the range tested. This result demonstrates that hydrolysis of C3 by the CVF-dependent C3 convertase exhibits the expected behavior of an enzymatic reaction.

**Determination of Kinetic Parameters of the CVF-dependent C3 Convertase**—Kinetic experiments were performed as described under “Experimental Procedures.” Progress curves were linear for 8-10 min, indicating that the reaction was pseudo-first order over that period. Fig. 4 shows the double reciprocal plots of the initial velocity, $v$, versus the C3 concentration for four different temperatures. The results indicate that the rate of hydrolysis of C3 by the CVF-dependent C3 convertase depends on the C3 concentration in accordance with the Michaelis-Menten equation. Table I lists the kinetic parameters determined for each of the four temperatures. Mean values and standard deviations have been calculated for the parameters $K_m$, turnover number, and $k_0 = v/[S]$ which were determined by serveral individual experiments. The other parameters were derived from these mean values. Since the CVF-dependent C3 convertase has only one catalytic subunit/molecule, the turnover number is identical with the catalytic center activity. Up to a C3 concentration of about $3 \times 10^{-6}$ M, the substrate turnover can be described as a first order reaction. The apparent first order rate constants, $k_0$, were determined by two methods as described under “Experimental Procedures” and are also listed in Table I.

**Thermodynamic Parameters of the Rate-limiting Step—**

Plotting of $k_0$ values according to the Arrhenius equation resulted in a linear graph (Fig. 5). Linearity of the plot allowed the conclusion that the overall catalytic rate constant, $k_{cat}$, is the rate constant of the rate-limiting, unimolecular, or pseudo-unimolecular step rather than an apparent rate constant resulting from several individual elementary reaction steps. From the slope of the Arrhenius plot, the activation energy, $E_a$, of the rate-limiting step was determined to be 10,000 cal.mol$^{-1}$. This value corresponds to a temperature coefficient, $Q_{10}$, of 1.77 for the temperature range investigated. Since the Arrhenius plot was linear, it was concluded that no change in the rate-limiting step occurred within the temperature range studied. The absence of a sudden decline in the plot at higher temperatures indicates that the apparent temperature optimum of the reaction lies above 37°C.

The transition state theory of absolute reaction rates allowed the determination of the activation enthalpy, $\Delta H^*$, of the rate-limiting step from the slope of a plot of log ($k_{cat}/T$) versus $1/T$ (Fig. 6). A value of 9450 cal.mol$^{-1}$ was found for the activation enthalpy. The relation of activation energy, $E_a$, and activation enthalpy, $\Delta H^*$, is given by $E_a = \Delta H^* + R \cdot T$. Using this expression, an activation energy of 10,000 cal.mol$^{-1}$ was calculated which is consistent with the value derived from the Arrhenius plot. The Gibbs energy of activation, $\Delta G^*$, of the rate-limiting step for the four different temperatures investigated was calculated and the values are shown in Table II. The activation entropy, $S^*$, of the rate-limiting step was determined by several individual experiments.
was determined to be \(-24.82 \text{ cal.mol}^{-1} \cdot \text{K}^{-1}\).

**Thermodynamic Parameters of the Formation of the Enzyme-Substrate Complex**—A plot of the Michaelis constants according to the van’t Hoff law showed a linear graph (Fig. 7). Since the Michaelis constant is composed of at least three rate constants, a linear plot is usually expected only if \(k_{\text{cat}}\) is much smaller than \(k_{-1}\). Therefore, it was possible to conclude that the encounter of C3 and enzyme proceeds under rapid equilibrium conditions. This is consistent with the value of the apparent second order rate constant \(k_{\text{cat}}/K_m\) (Table I), which is too low for a diffusion-controlled reaction, thereby excluding steady state conditions for the formation of the enzyme-substrate complex.

Since the encounter of C3 and enzyme proceeds under rapid equilibrium conditions, the Michaelis constant, \(K_m\), is identical with the substrate constant, \(K_s\), which is the reciprocal of the equilibrium constant \(K\) for the formation of the enzyme-substrate complex and free enzyme and substrate. Therefore, it was possible to calculate the equilibrium constants \(K\) for the four temperatures studied (Table III). The standard enthalpy, \(\Delta H^\circ\), of the formation of the enzyme-substrate complex was calculated from the slope of the linear van’t Hoff plot and a value of 5300 cal.mol\(^{-1}\) was obtained. The standard Gibbs energies, \(\Delta G^\circ\), for formation of the complex are listed in Table IV, and the standard entropy, \(\Delta S^\circ\), was found to be 39.71 cal.mol\(^{-1}\)\cdot K\(^{-1}\).

**Temperature Dependence of the Apparent Second Order Rate Constant—**\(k_{\text{cat}}/K_m\) is a rather complex function of several rate constants (37).

**TABLE III**

| Temperature (°C) | Equilibrium constant \(K\) |
|------------------|---------------------------|
| 37               | 8.62 × 10^4 M\(^{-1}\) |
| 30               | 6.94 × 10^4 M\(^{-1}\) |
| 20               | 5.56 × 10^4 M\(^{-1}\) |
| 10               | 3.79 × 10^4 M\(^{-1}\) |

**TABLE IV**

| Thermodynamic parameters of the formation of the enzyme-substrate complex |
|-----------------------------|----------------------------------|
| Standard enthalpy, \(\Delta H^\circ\) | 5300 cal.mol\(^{-1}\) (22.18 kJ.mol\(^{-1}\)) |
| Standard entropy, \(\Delta S^\circ\)   | 39.71 cal.mol\(^{-1}\)\cdot K\(^{-1}\) (39.71 e.u.) |
| Standard Gibbs energy            | \(\Delta G^\circ\) |
| \(\Delta G^\circ_{373}^\circ\)      | \(-5530 \text{ cal.mol}^{-1}\) \((-24.81 \text{ kJ.mol}^{-1})\) |
| \(\Delta G^\circ_{298}^\circ\)      | \(-6370 \text{ cal.mol}^{-1}\) \((-26.65 \text{ kJ.mol}^{-1})\) |
| \(\Delta G^\circ_{293}^\circ\)      | \(-6710 \text{ cal.mol}^{-1}\) \((-28.07 \text{ kJ.mol}^{-1})\) |
| \(\Delta G^\circ_{292}^\circ\)      | \(-7000 \text{ cal.mol}^{-1}\) \((-29.29 \text{ kJ.mol}^{-1})\) |

**FIG. 7** (left). Temperature dependence of the Michaelis constant \(K_m\) (van’t Hoff plots). **FIG. 8** (right). Temperature dependence of the apparent second order rate constant \(k_{\text{cat}}/K_m\).

The CVF-dependent C3 convertase rather than the C3b-dependent enzyme was chosen for these studies because of its greater stability. The C3b-dependent enzyme, C3b,Bb, exhibits a spontaneous decay-dissociation into its subunits, with a half-life of approximately 1.5 min at 37 °C (21), whereas a half-life of 7 h was found for the CVF-dependent enzyme. At lower temperatures, the decay-dissociation was slower and freezing to ~70 °C inactivated only 30% of the enzyme. A rather high stability of the CVF-dependent C3 convertase has also been reported by other investigators (38–40). The enzyme was also stable enough to perform ultrastructural studies by high resolution transmission electron microscopy (41).

Using the natural substrate C3, we determined the Michaelis constant, \(K_m\), the turnover number, the overall catalytic rate constant, \(k_{\text{cat}}\), the specific activity, the apparent second order rate constant, \(k_{\text{cat}}/K_m\), the apparent first order rate constant for the low substrate concentration range, \(k_0\), and the catalytic cycle time. Despite the importance of enzymatic reactions for activation of complement, kinetic parameters of complement enzymes have been little investigated. Michaelis constants for low molecular weight substrates, like esters and oligopeptides, have been reported for C1s (EC 3.4.21.42) (42–44), for the classical C3 convertase (EC 3.4.21.43) generated with \(^{125}\text{I}^2\text{C2} (45), and recently for the CVF-dependent C3 convertase (46). Michaelis constants for natural substrates have been reported in hemolytic units, which is of rather limited value, for C1s (47), for the classical C3 convertase generated with \(^{125}\text{I}^2\text{C2} (48), and for the CVF-dependent C3 convertase (38). The Michaelis constant determined with the natural
substrate and expressed in molar concentration has been reported for the classical C3 convertase generated with \(^{125}\text{I} \text{C2} \left( 1.8 \times 10^{-8} \text{ M} \right) \) and for C1a acting on C4 \( \left( 9.6 \times 10^{-7} \text{ M} \right) \). Except for the recent report of C1 acting on C4 \( \left( 49 \right) \), the values have not been determined for complement enzymes. None of the other kinetic parameters described here for the CVF-dependent C3 convertase has been reported for any of the other complement enzymes.

All well investigated proteases like trypsin or chymotrypsin show substrate specificity for a type of peptide bond rather than for a defined molecule and, consequently, hydrolyze substrate proteins at multiple sites into numerous split products. Therefore, all detailed kinetic analyses of these proteases have been performed with artificial substrates. Many kinetic parameters were reported for these substrates \( \left( 8, 50-53 \right) \), but these are not comparable with the data presented here. Because of the high substrate specificity of the CVF-dependent C3 convertase, it was possible to determine many kinetic parameters for this protease acting on its natural high molecular weight protein substrate. The results demonstrate, in addition, that an enzyme-substrate system where enzyme and substrate are both proteases of high molecular weight \( \left( \approx 200,000 \right) \) can be described by Michaelis-Menten kinetics.

The turnover number, or \( k_{\text{cat}} \), for C3 hydrolysis by the CVF-dependent C3 convertase is rather low in comparison with many other enzymatic reactions. In order to evaluate the catalytic efficiency of an enzyme, the appropriate parameter is neither \( k_{\text{cat}} \) nor \( K_m \), but the apparent second order rate constant \( k_{\text{cat}}/K_m \), which describes the reaction as a function of the concentration of substrate and free enzyme.

\[
v = \frac{k_{\text{cat}}}{K_m} [S] [E] \tag{1}
\]

A high \( k_{\text{cat}}/K_m \) at a \( K_m \) that is greater than the physiological substrate concentration results in most efficient catalysis and is indicative of a well evolved enzyme \( \left( 8 \right) \). At 37 °C, the \( k_{\text{cat}}/K_m \) for the CVF-dependent C3 convertase was found to be 4.05 \( \times 10^4 \text{ s}^{-1} \text{ M}^{-1} \). This value excludes the diffusion-controlled encounter of enzyme and substrate as the rate-limiting step of the overall reaction as in the case of extremely fast acting enzymes like catalase \( \left( 54 \right) \) or carbonic anhydrase \( \left( 55 \right) \). But the \( k_{\text{cat}}/K_m \) for C3 hydrolysis by the CVF-dependent C3 convertase is higher than most \( k_{\text{cat}}/K_m \) values reported for proteases like trypsin \( \left( 50 \right) \), chymotrypsin \( \left( 8 \right) \), elastase \( \left( 51 \right) \), papain \( \left( 52 \right) \), and pepsin \( \left( 53 \right) \) acting on low molecular weight substrates. The rather high \( k_{\text{cat}}/K_m \) found for the CVF-dependent C3 convertase suggests a better complementarity between the substrate C3 and the enzyme in the transition state rather than in the enzyme-substrate complex. This conclusion implies that the maximum binding energy is present in the transition state, which is advantageous for the catalytic function of the enzyme \( \left( 8 \right) \). In addition, a high binding energy suggests multiple site interactions between enzyme and substrate and may explain the high specificity of the CVF-dependent C3 convertase for its natural substrate C3. The \( K_m \) for C3, which is \( 1.16 \times 10^{-4} \text{ M} \) at 37 °C, is approximately twice the physiological C3 concentration in human plasma, which is \( 6.5 \times 10^{-5} \text{ M} \). Consequently, when the C3 convertase is acting at physiological C3 concentration, the majority of the enzyme is present in free form, which contributes to a high velocity of C3 hydrolysis according to Equation 1.

This analysis of the kinetic data indicates that the C3 convertase is a rather highly evolved enzyme and well suited for its biological function. This conclusion is consistent with \( \text{in vivo} \) studies \( \left( 56 \right) \) which show that this enzyme introduced into the vasculature of an animal effectively hydrolyzes circulating C3.

The kinetic parameters have been determined for the CVF-dependent C3 convertase at four different temperatures. Consequently, numerous thermodynamic parameters could be calculated. The Arrhenius plot of the \( k_{\text{cat}} \) values was linear and the activation energy could be determined. The linear Arrhenius plot suggests that \( k_{\text{cat}} \) depends only on a single rate constant, namely that of the rate-limiting elementary step of the overall reaction. Applying the transition state theory of absolute reaction rates, the activation enthalpy, activation entropy, and Gibbs energy of activation of this rate-limiting step were calculated. The Michaelis constants determined at different temperatures obeyed the van’t Hoff law. The linear van’t Hoff plot allowed the conclusion that \( k_{\text{cat}} \) is \( 1.16 \) \( \times 10^{-4} \text{ M}^{-1} \) for C3, which is \( 1.8 \times 10^{-8} \text{ M} \), and the activation energy could be determined. The linear Arrhenius plot suggests that \( k_{\text{cat}} \) depends only on a single rate constant, namely that of the rate-limiting elementary step of the overall reaction. Applying the transition state theory of absolute reaction rates, the activation enthalpy, activation entropy, and Gibbs energy of activation of this rate-limiting step were calculated. The Michaelis constants determined at different temperatures obeyed the van’t Hoff law. The linear van’t Hoff plot allowed the conclusion that \( k_{\text{cat}} \) is much smaller than \( k_1 \), the rate constant of the dissociation of the enzyme-substrate complex into free enzyme and substrate. Therefore, the encounter of enzyme and substrate proceeds under rapid equilibrium conditions. This finding is consistent with the order of magnitude of \( K_m \) (see above) and its temperature dependence (see Fig. 8). It was possible to calculate the equilibrium constant of the equilibrium between the enzyme-substrate complex and free enzyme and substrate and the standard entropy, and standard Gibbs energy for the formation of the enzyme-substrate complex. Despite the positive standard enthalpy, \( \Delta H^o \), the formation of the enzyme-substrate complex is favored due to the increase in entropy. However, the equilibrium constant is not very high \( \left( K = 8.62 \times 10^4 \text{ M}^{-1} \right) \) indicating a rather weak affinity of the substrate for the enzyme. The positive standard enthalpy and the rather low equilibrium constant suggest, in accordance with conclusions derived above from kinetic data, that in proceeding from the enzyme-substrate complex to the transition state, no major binding energy has to be overcome, but that the maximum binding energy occurs in the transition state.

The values of the standard entropy, \( \Delta S^o \), and the activation entropy, \( \Delta S^a \), provide information on the nature of the changes in structure and solvation which accompany the formation of the enzyme-substrate complex and the transition state. Formation of the enzyme-substrate complex is accompanied by an increase in entropy. Since two molecules of about identical size form this complex, a decrease of total surface area must occur and therefore it is very likely that the increase in entropy is primarily due to solvation effects such as the release of water molecules. The subsequent conversion of the enzyme-substrate complex to the transition state should not be accompanied by major changes in solvation; and the decrease found in entropy is most likely due to tightening of the structure of the complex. This structural change suggests, in addition, a better complementarity between substrate and enzyme in the transition state than in the enzyme-substrate complex.

The CVF-dependent C3 convertase has been proposed to constitute a serine protease. This hypothesis was first advanced on the basis of inhibition of the proenzyme Factor B as well as the activated enzyme by diisopropylfluorophosphate \( \left( 57 \right) \). But the inhibition needed higher diisopropylfluorophosphate concentrations than are required for the inhibition of other serine proteases and other authors reported that Factor B-derived enzymes cannot be inactivated by diisopropylfluorophosphate \( \left( 36, 39, 58 \right) \). In addition, the molecular weight of the Bb polypeptide chain is approximately twice that of other serine proteases. And, no homology of the amino acid sequence in the NH2-terminal region between Bb and other serine proteases was found \( \left( 59 \right) \). While the present work was in progress, two reports \( \left( 60, 61 \right) \) demonstrated an extensive sequence homology of Bb with other serine proteases in the COOH-terminal region of the molecule and showed the pres-
Fig. 9. Thermodynamic reaction profiles of C3 hydrolysis by the CVF-dependent C3 convertase. $E_n$, enzyme = CVF-dependent C3 convertase; $S$, substrate = C3; ($E + S$)*, transition state for the formation of the enzyme-substrate complex; $ES^*$, transition state for the formation of the acylenzyme-product complex; the minor dip following $ES^*$ represents the tetrahydral intermediate.

Activation entropy, activation entropy, and Gibbs energy of activation for the enzyme-substrate complex have not been determined. Values shown are hypothetical, but obey the Gibbs-Helmholtz law.
If the second substrate is water, as in hydrolytic reactions of serine proteases, it is present in saturating concentrations. Under these conditions, the velocity equation reduces to the normal Michaelis-Menten equation.

The velocity equations of two other common kinetic reaction mechanisms for two-substrate reactions (rapid equilibrium random sequential Bi Bi and steady state ordered sequential Bi Bi) reduce also to the Michaelis-Menten equation if one substrate is water. However, a rapid equilibrium ordered sequential Bi Bi mechanism, with the first substrate being C3 and the second substrate being water, can be excluded for the CVF-dependent C3 convertase because our results show a dependence of the initial velocity on the C3 concentration.

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