The C3 convertase of the alternative pathway of human complement

Enzymic properties of the bimolecular proteinase

Michael K. PANGBURN* and Hans J. MÜLLER-EBERHARD†

Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

The association of Factor B with C3b (the major fragment of complement component C3) in the presence of Mg^{2+} results in the formation of a bimolecular zymogen, C3b,B, which is activated by the serine proteinase Factor D, generating the C3 convertase, C3b,Bb (EC 3.4.21.47). Cleavage of native C3 by the C3 convertase was monitored by recording the increase in fluorescence associated with C3b formation in the presence of the fluorescent probe 8-anilino-1-naphthalene-sulphonate. Measurements of initial rates of C3b formation at various C3 concentrations were analysed in accordance with the Michaelis–Menten equation, yielding $k_{cat.} = 1.78 \pm 0.08 \text{ s}^{-1}$, $K_m = 5.86 \times 10^{-6} \pm 0.44 \times 10^{-6} \text{ M}$ and turnover number = 107 min^{-1} . The assay was used to measure the K_i values of a variety of proteinase inhibitors. The C3 convertase has a short half-life, owing to spontaneous dissociation of the complex. The $t_{1/2}$ and $k_{cat.}/K_m$ of the enzyme were determined by fitting an equation modelling both the kinetic reaction and enzyme decay to the fluorimetrically measured progress curve. The enzyme, C3b,Bb, exhibited a $t_{1/2}$ of $90 \pm 2 \text{ s}$ and a $k_{cat.}/K_m$ of $31.1 \times 10^4 \pm 0.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at physiological pH, ionic strength and temperature. The enzyme that initiates activation of the alternative pathway, C3(H₂O),Bb, was also examined. It was slightly less stable ($t_{1/2} = 77 \pm 3 \text{ s}$) and exhibited only half the activity of C3b,Bb ($k_{cat.}/K_m = 16.3 \times 10^4 \pm 1.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$).

INTRODUCTION

Activation of the alternative pathway of complement initiates a number of proteolytic events, culminating in the formation of a C3 convertase (EC 3.4.21.47). The enzyme is composed of a single Mg^{2+} ion (Fishelson *et al.*, 1983) and two non-covalently bound subunits, C3b and Bb (Müller-Eberhard & Götze, 1972). C3b (M_r 176000) binds the proenzyme Factor B (M_r 93000) in the presence of metal ions to form a bimolecular protein complex, C3b,B. This zymogen is activated by Factor D (EC 3.4.21.46) (Götze & Müller-Eberhard, 1976), a serine proteinase (Johnson *et al.*, 1980) present in plasma in active form (Lesavre *et al.*, 1979), which cleaves Factor B only when it is bound to C3b. The resulting C3 convertase (C3b,Bb) is a serine proteinase, the active site of which resides in the Bb subunit (Medicus *et al.*, 1976a). The enzyme cleaves native C3 (M_r 185000), releasing the anaphylatoxic peptide C3a (M_r 9000) and producing C3b, which can form an additional C3 convertase with a second molecule of Factor B. This positive-feedback process (Müller-Eberhard & Götze, 1972) is a unique feature of the alternative pathway of complement, and results in the deposition of large numbers of C3b molecules on the surface of activating particles (Pangburn *et al.*, 1983a).

In plasma a small number of C3b molecules is continually generated by the initial C3 convertase of the alternative pathway (Pangburn & Müller-Eberhard,

1980; Pangburn *et al.*, 1981). This enzyme contains a form of C3 that arises by spontaneous hydrolysis of the internal thioester of native C3 (Pangburn & Müller-Eberhard, 1980). A well-characterized conformational transition (Isenman *et al.*, 1981) results in formation of a functionally C3b-like molecule capable of binding Factor B and forming the C3 convertase, C3(H₂O),Bb (Pangburn *et al.*, 1981; Fishelson *et al.*, 1984). This enzyme is thought to produce the first metastable C3b molecules.

Characterization of C3 convertases has been hampered by the complexity of their formation, the indirect assay procedures previously employed and the intrinsic lability of the enzymes. The enzyme C3b,Bb has been shown to be short-lived (Müller-Eberhard & Götze, 1972; Medicus *et al.*, 1976b), owing to dissociation of the Bb subunit from C3b, which renders it irreversibly inactive. Decay of enzyme activity is greatly accelerated by interaction with the plasma glycoprotein Factor H (Weiler *et al.*, 1976; Whaley & Ruddy, 1976) as well as by at least two membrane proteins present on human erythrocytes (Fearon, 1979; Nicholson-Weller *et al.*, 1982; Pangburn *et al.*, 1983a,b,c). These proteins control spontaneous activation of the alternative pathway in plasma and on host cells by inhibiting C3 convertase formation and by accelerating the decay of the enzyme.

A previous study of the regulation of C3b by Factor H and the plasma serine proteinase Factor I utilized the change in ANS fluorescence that occurs when C3b is

Abbreviations used: C3b, major fragment of activated C3; C3b,Bb, C3 convertase formed from C3b and Factor B after cleavage of Factor B by Factor D; C3(H₂O), C3 in which the thioester bond has been hydrolysed; C3b,Bb, C3 convertase formed with C3b and Factor B; ANS, 8-anilino-1-naphthalene-sulphonate.

* Present address: Department of Biochemistry, University of Texas Health Center, Tyler TX 75710, U.S.A.

† To whom correspondence should be addressed.

proteolytically inactivated (Pangburn & Müller-Eberhard, 1982). The present study (see Pangburn & Müller-Eberhard, 1983) uses the increase in ANS fluorescence associated with proteolytic conversion of C3 into C3b (Isenman & Cooper, 1981; Isenman *et al.*, 1981; Pangburn & Müller-Eberhard, 1982) to study the properties of the bimolecular enzyme complex that is the central enzyme of the alternative complement pathway.

MATERIALS AND METHODS

Materials

Human plasma was purchased from the San Diego Blood Bank (San Diego, CA, U.S.A.). Bovine L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated trypsin and soya-bean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Freeze-dried cobra (*Naja naja kaouthia*) venom was from International Biological Extracts (Sweetwater, TX, U.S.A.). ANS was from Eastman Chemicals (Rochester, NY, U.S.A.). The proteinase inhibitor leupeptin (Ac-Leu-Leu-arginyl form) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.).

Purified proteins

C3 was purified by the method of Hammer *et al.* (1981). C3b was generated from native C3 by limited exposure to trypsin (0.2%, w/w; 8 min; 37 °C) and purified by gel filtration on Bio-Gel A1.5 m (Pangburn & Müller-Eberhard, 1982). C3(H₂O) was generated from native C3 by repeated freeze-thaw cycles (Isenman *et al.*, 1981). Functional activity of C3b and C3(H₂O) was assessed by the ability to bind to Factor B-Sepharose in the presence of 1 mM-NiCl₂. Factor B (Götze & Müller-Eberhard, 1971), Factor D (Lesavre & Müller-Eberhard, 1978) and properdin (Götze *et al.*, 1977) were isolated as described in the references cited. Commercial trypsin was purified by affinity chromatography on soya-bean trypsin inhibitor immobilized on Sepharose as described by Robinson *et al.* (1971). CVF was purified according to published procedures (Ballow & Cochrane, 1969) and was a gift from Dr. Carl-Wilhelm Vogel. All proteins were homogeneous by polyacrylamide-gel electrophoresis in the presence of SDS and by immunoelectrophoresis. Electrophoresis in the presence of SDS was performed in 9% polyacrylamide gels with the Canalco SAGE electrophoretic system (Miles Laboratories, Elkhart, IN, U.S.A.). Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) standardized at 280 nm by using $A_{1\text{cm}}^{1\%} = 6.6$. C3 concentrations were determined by using $A_{1\text{cm}}^{1\%} = 11.0$, obtained by quantitative amino acid analysis of several preparations (Pangburn & Müller-Eberhard, 1982). M_r values employed in the calculations were 185000 for C3, 176000 for C3b, 93000 for Factor B, 150000 for CVF, 60000 for Bb and 24000 for Factor D.

Fluorescence measurements

Fluorescence emission of ANS in the presence and in the absence of proteins was recorded with an Aminco SPF-500 spectrofluorimeter (American Instrument Co., Silver Springs, MD, U.S.A.). Excitation was at 386 nm with a 5 nm band-pass, and emission was measured at

472 nm with a 10 nm band-pass, unless otherwise noted. Recordings were made exclusively in the ratio mode.

Kinetic measurements

Measurements of initial reaction velocity with various concentrations of the substrate C3 were performed in a total volume of 0.4 ml in 5 mm × 5 mm quartz fluorescence cuvettes. Cuvettes containing C3 and 20 μM-ANS in a total volume of 380 μl of phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4) were allowed to equilibrate to the desired temperature in the cell compartment of the spectrofluorimeter. The initial fluorescence intensity was recorded, 20 μl of enzyme in phosphate-buffered saline containing 75 mM-EDTA was added and the change in fluorescence intensity was recorded for 10 min. Trypsin (2 μg) was then added to convert the remaining C3 into C3b, and the reaction was monitored until a maximum in fluorescence intensity was reached 2–4 min after addition of trypsin. This maximum provided a relation between the fluorescence-intensity change and the concentration of native C3 present in the reaction. Time-course studies were performed similarly except that 10 mm × 10 mm cuvettes containing a final reaction volume of 1.6 ml were used and the entire progress curve was analysed to obtain $k_{\text{cat.}}/K_m$ and the half-life of the C3 convertase.

Progress-curve simulation

The basic Michaelis-Menten equation simplifies when the substrate concentration, [S], is much smaller than K_m to:

$$v = (k_{\text{cat.}}/K_m)[E][S]$$

where [E] is the enzyme concentration. This equation expresses the rate of product formation as a function of the enzyme and substrate concentrations. Since, in the present case, the bimolecular enzyme is subject to rapid inactivation owing to first order decay-dissociation (Medicus *et al.*, 1976b; Müller-Eberhard & Götze, 1972), its concentration at any point in time, t , is:

$$[E_t] = [E_0]e^{-k_0 t}$$

where $[E_0]$ is the enzyme concentration at zero time and k_0 is related to the half-life by $k_0 = (\ln 2)/t_{1/2}$. Representing v as $d[\text{C3b}]/dt$ yields the equation:

$$d[\text{C3b}] = (k_{\text{cat.}}/K_m)[E_0]e^{-k_0 t}[\text{C3}_t]dt \quad (1)$$

which can be integrated to yield:

$$\ln [\text{C3}_t] = (k_{\text{cat.}}/K_m)[E_0](1/k_0)(e^{-k_0 t} - 1) + \ln [\text{C3}_0] \quad (2)$$

where $[\text{C3}_t]$ represents the concentration of substrate C3 at time t . Calculations using the integrated equation and numerical integration of the differential equation were performed with a Hewlett-Packard HP-87 computer. Numerical integration of eq. (1) by using $dt = 1$ s gave progress-curve values for C3b concentration within 0.02% of values calculated with the integrated equation at all points of the progress curve. The program utilized a data-fitting routine that varied $k_{\text{cat.}}/K_m$ and $t_{1/2}$ in order to minimize the summed variance between the model progress curve and the data.

Temperature measurements

The cell compartment was thermostatically controlled and the temperature of the samples monitored continuously by using a digital thermistor thermometer with an immersible probe. The probe was placed in a cuvette containing only buffer in the thermostatically controlled

four-cell carousel of the spectrofluorimeter. Temperature variation and reproducibility were within $\pm 0.2^\circ\text{C}$.

Statistical analyses

All data except double-reciprocal plots were analysed by using linear-regression analysis, and the results of an analysis of variance are reported as the standard errors. These computations were performed with a statistical program written for the HP 87 computer.

RESULTS

C3 convertase generation and measurement of enzymic activity

The formation, enzymic activity and decay of the C3 convertase are described by the following reactions:

Formation:

C3 cleavage:

Enzyme decay:

Because of rapid decay of the enzyme C3b,Bb, preparation of a known concentration of C3 convertase required the use of excess Factor B to drive reaction (3) to the right. Fig. 1 shows a time course of enzyme formation demonstrating that more than 98% of C3b was in complex with Bb between 1 and 4 min after Factor D was added. Enzyme formation was stopped by the addition of excess EDTA, and the enzyme was added to the cuvette containing C3. Identical mixtures to which EDTA was added before addition of Factor D yielded no enzymic activity. This control indicates that under the formation occurred in the assay mixtures that contained EDTA. Use of 3-fold higher concentrations of Factor B or Factor D gave no increase in observed activity, suggesting that the concentrations used were sufficient to convert C3b quantitatively into C3b,Bb. C3 cleavage was monitored in the presence of ANS, as shown in Fig. 2.

Fig. 1. Time course of C3b,Bb formation

The enzyme C3b,Bb was prepared with purified C3b (14 μg) in the presence of Mg^{2+} (5 mM) and excess Factor B (48 μg) and Factor D (0.5 μg) in a total volume of 25 μl at 22°C . Further enzyme formation was blocked at various times with 75 μl of 0.1 M-EDTA, and the enzyme was added to the cuvette containing 1 μM -C3 and 20 μM -ANS in phosphate-buffered saline at 37°C .

Fig. 2. Fluorimetric assay of C3 convertase activity with the natural substrate C3

Proteolytic conversion of C3 into C3b causes a 6-fold increase in ANS fluorescence intensity. After establishment of the initial fluorescence of a cuvette containing 20 μM -ANS and 1 μM -C3 in phosphate-buffered saline at 37°C , the C3 convertase C3b,Bb prepared as described in Fig. 1 was added. The change in fluorescence intensity was recorded for 600 s, at which time 10 μg of trypsin (T) was added in order to determine the maximum fluorescence intensity change due to complete conversion of C3 into C3b.

The assay takes advantage of the rapid 6-fold increase in fluorescence intensity of ANS that reports the conversion of substrate C3 into the product C3b (Isenman & Cooper, 1981; Isenman *et al.*, 1981; Pangburn & Müller-Eberhard, 1982) as shown in reaction (4) above. The initial rate of conversion of C3 into C3b was calculated from the slope of the fluorescence change. The maximum fluorescence intensity obtained when all the C3 was converted into C3b with trypsin was used to relate fluorescence change to the C3b concentration. The fluorescence of ANS increased 6.0-fold on conversion of C3 into C3b and 1.2-fold on conversion of C3(H_2O) into C3b. Since trypsin converts C3(H_2O) into C3b, but C3 convertases do so at an extremely low rate (Janatova *et al.*, 1980; Pangburn & Müller-Eberhard, 1980; Parkes *et al.*, 1981; VonZabern *et al.*, 1981), the total fluorescence change was corrected for the C3(H_2O) content of the C3 and the maximum fluorescence change due to C3 alone was used. This correction was based on evidence from gels that C3 preparations contained only C3 and C3(H_2O). The percentage native C3 could then be calculated from the initial fluorescence intensity (F_i) and the final fluorescence intensity (F_f) after trypsin cleavage by using the formula:

$$\% \text{ C3} = 100 \times 6(F_f - 1.2F_i) / F_i(6 - 1.2)$$

It was found that the initial rate increased linearly with increases of either C3 or C3b,Bb concentration when C3 concentrations below 2 μM were used. Under these conditions the initial velocity was related to the $k_{\text{cat.}}/K_m$ of the C3 convertase according to:

$$v = (k_{\text{cat.}}/K_m)[\text{C3b,Bb}][\text{C3}]$$

(see the Materials and methods section). Since C3 preparations invariably contain some C3(H_2O), the possibility of C3(H_2O) being a competitive inhibitor was considered. Product inhibition by C3b was also a potential problem in these assays and in progress-curve measurements. Assays containing 2 μM -C3 and up to 15.7 μM -C3(H_2O) or 14 μM -C3b showed no significant

change in the rate of C3 cleavage compared with assays containing only C3. C3a has been shown not to be an inhibitor of this enzyme (Strunk & Webster, 1985).

Measurement of kinetic parameters

Initial-rate measurements were made at various C3 concentrations to determine the apparent kinetic parameters. The plot of initial velocity versus C3 concentration showed a hyperbolic dependence of rate on concentration, in accordance with the Michaelis–Menten model shown in reaction (4). The data were plotted (Fig. 3) according to a rearrangement of the Michaelis–Menten equation:

$$v = -K_m(v/[S]) + V_{\max.}$$

which allows determination of K_m and $V_{\max.}$ from a plot of v versus $v/[S]$. The kinetic parameters obtained are given in Table 1. The catalytic constant $k_{\text{cat.}}$ and the turnover number were calculated according to:

$$k_{\text{cat.}} = V_{\max.}/[E_t]$$

The enzyme concentration was calculated from the concentration of C3b used to prepare the enzyme,

Fig. 3. Initial velocities measured as shown in Fig. 2 plotted according to the Michaelis–Menten equation

Cleavage of C3 by the C3 convertase C3b,Bb (Mg^{2+}) was measured at different concentrations of C3 and the average initial velocity of triplicate measurements plotted. The enzyme concentration was 63 nM and the reaction was performed in phosphate-buffered saline at 37 °C.

Table 1. Kinetic parameters of the alternative-pathway C3 convertase C3b,Bb acting on native C3 at 37 °C

Parameter	Value
K_m	$5.86 \times 10^{-6} \pm 0.44 \times 10^{-6} \text{ M}$
$V_{\max.}$	$1.12 \times 10^{-7} \pm 0.05 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$
$k_{\text{cat.}}$	$1.78 \pm 0.08 \text{ s}^{-1}$
Turnover number	107 min^{-1}
$k_{\text{cat.}}/K_m$	$30.4 \times 10^4 \pm 0.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$
Specific activity	$7.6 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$
Enzyme half-life	$90 \pm 2 \text{ s}$
k_0 (enzyme decay)	$7.70 \times 10^{-3} \text{ s}^{-1}$

Fig. 4. Apparent competitive inhibition of C3 convertase activity by the substrate analogue guanidine

Initial rates were measured at different C3 concentrations in the presence of 30 mM- ($\blacktriangle$), 90 mM- ($\blacksquare$) or no ($\bullet$) guanidine at 37 °C. The apparent K_i was $27 \times 10^{-3} \text{ M}$. The presence of guanidine at these concentrations had no effect on the half-life of the C3b,Bb(Mg^{2+}) complex.

assuming that all of the C3b was present as C3b,Bb at the time of addition (Fig. 1).

Inhibitors of C3 convertase activity

The fluorimetric assay (Fig. 2) was used to examine inhibitors of trypsin-like enzymes for their ability to inhibit C3b,Bb (Fig. 4). The C3 concentration was 1 μM , approximately one-sixth the K_m of the enzyme. Very few inhibitors of complement C3 convertases have been reported. Soya-bean trypsin inhibitor did not inhibit C3 cleavage, and leupeptin, although reported to have a K_i of 40 μM for C3b,Bb (Caporale, 1981), was found to have no detectable inhibitor activity in the present assay when used at concentrations as high as 363 μM . At 93 μM leupeptin completely inhibited the trypsin added to convert the remaining C3. Both benzamidine and guanidine were found to inhibit the proteolysis of C3 by C3b,Bb. Benzamidine had an apparent K_i of 10 mM. Fig. 4 shows data in the form of Lineweaver–Burk plots for guanidine inhibition at 0, 32 and 96 mM. The three lines intersect very close to the vertical axis, suggesting that the inhibition by guanidine is competitive with the natural substrate C3. Linear-regression analysis of a replot of the slopes versus inhibitor concentration yielded an apparent K_i for guanidine of 27 mM.

Estimation of $k_{\text{cat.}}/K_m$ and $t_{1/2}$ by analysis of progress curves

Measurement of initial rates provides no information about enzyme stability. This information can be obtained from progress curves provided that a suitable model for the overall reaction, including enzyme decay, can be derived. As shown in Figs. 3 and 4, the cleavage of C3 by C3b,Bb appears to conform to the Michaelis–Menten mechanism shown in reaction (4). The equation describing this reaction can be simplified when low substrate concentrations are used, and the equation describing first-order enzyme decay was incorporated into the equation as described in the Materials and methods section. The resulting equation (eqn. 1) was integrated, and a computer was used to generate a series of theoretical progress curves (Fig. 5) representing the cleavage of C3 by any C3 convertase. The shape of each

Fig. 5. Progress curves calculated according to eqn. (1), which models the catalytic and enzyme-decay reactions of C3 convertases under conditions of low substrate concentration

(a) Time courses of C3 conversion with enzymes having different half-lives (as indicated), but the same $k_{\text{cat.}}/K_m$ ($4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; enzyme concentration was set at $0.1 \mu\text{M}$). (b) Time courses of C3 cleavage with enzymes exhibiting 2-fold variations of $k_{\text{cat.}}/K_m$ (2×10^4 , 4×10^4 , 8×10^4 , $16 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; enzyme concentration $0.1 \mu\text{M}$) but identical half-life (120 s). The Figures illustrate the characteristic changes in the shape of the progress curves that accompany changes in $k_{\text{cat.}}/K_m$ or $t_{1/2}$. These characteristics allow accurate and independent determination of both constants from each progress curve by computer-assisted curve-fitting.

curve is a unique result of the values of the two constants $k_{\text{cat.}}/K_m$ and $t_{1/2}$, which characterize the enzyme. As shown in Fig. 5, even small changes in either of these constants make significant and characteristic changes in the resulting curves. Fitting of model progress curves to experimentally obtained data (Fig. 6) yielded best-fit $k_{\text{cat.}}/K_m$ and $t_{1/2}$ values for each reaction. Assays were performed with C3 concentrations between 0.1 and $1.0 \mu\text{M}$, but the relationship between initial velocity and C3 concentration appeared to be linear up to $2.0 \mu\text{M}$ -C3. A linear relationship also existed between the initial rate and C3b,Bb concentration (2.5 to 20 nM). The variation between the constants ($k_{\text{cat.}}/K_m$ and $t_{1/2}$) from assay to

Fig. 6. Comparison of fluorimetrically measured progress-curve data (points) with the computer-derived best-fit theoretical curves (lines)

The assays were performed as shown in Fig. 2 with C3b,Bb at 7.87 nM (▲) and 15.7 nM (●). Best-fit curves yielded values of $k_{\text{cat.}}/K_m = 28.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $t_{1/2} = 93 \text{ s}$ (▲) and of $k_{\text{cat.}}/K_m = 31.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $t_{1/2} = 89 \text{ s}$ (●).

assay was small and typically similar to the variation indicated in the legend to Fig. 6. Theoretical progress curves generated according to eqn. (1) or eqn. (2) could be fitted with the same statistical variation to experimental progress curves of enzymes of vastly differing half-lives (77 to 25200 s) and $k_{\text{cat.}}/K_m$ (4.7×10^4 to $31.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) (Table 2).

The $k_{\text{cat.}}/K_m$ and $t_{1/2}$ of C3b,Bb were determined at various temperatures between 18 and 40°C by fitting the experimental progress curve as described above. A plot of the rate constant for the dissociation reaction (k_0 in eqns. 1 and 2) versus temperature according to the Arrhenius equation:

$$\log k_0 = -(E_a/2.3R)(1/T) + \log A$$

was linear (Fig. 7). The temperature-dependence of the apparent second-order rate constant ($k_{\text{cat.}}/K_m$) did not show a linear relationship when plotted as $\log(k_{\text{cat.}}/K_m)$ versus $1/T$, suggesting that a change in the rate-limiting reaction may occur within the temperature range studied. The activation energy obtained from the slope of the curve in Fig. 7 was 81.6 kJ/mol (19.5 kcal/mol). This represents the energy barrier for dissociation of the C3b,Bb(Mg^{2+}) complex and is not unusually high for an uncatalysed reaction. It corresponds to a Q_{10} of 2.9. The other thermodynamic parameters characterizing enzyme dissociation were determined (Pangburn & Müller-Eberhard, 1982) on the basis of the transition-state theory of absolute reaction rates. From a plot of $\log(k_0/T)$ versus $1/T$ (not shown) $\Delta H^\ddagger$ was found to be 79.5 kJ/mol (19.0 kcal/mol), $\Delta G_{37}^\ddagger$ was -88.3 kJ/mol (21.1 kcal/mol) and $\Delta S_{37}^\ddagger$ was $-27.9 \text{ kJ/mol per degree}$ ($-6.67 \text{ cal/mol per degree}$).

The $k_{\text{cat.}}/K_m$ and $t_{1/2}$ of any enzyme that generates C3b by cleavage of native C3 could be determined by progress-curve analysis. Table 2 lists the characteristics of a number of C3 convertases. The $k_{\text{cat.}}/K_m$ determined for C3b,Bb ($31.1 \times 10^4 \pm 0.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) was similar to the value derived from initial-rate data ($30.4 \times 10^4 \pm 0.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$). The $k_{\text{cat.}}/K_m$ of the stable C3 convertase

Fig. 7. Temperature-dependence of the first-order rate constant of enzyme dissociation, k_0

The half-life of the C3 convertase was determined at different temperatures by computer fitting of progress curves as shown in Fig. 6. The activation energy of dissociation of the trimolecular complex C3b,Bb (Mg^{2+}) was calculated from the slope to be 81.6 kJ/mol (19.5 kcal/mol).

formed between human Factor B and CVF, i.e. CVF,Bb, was determined by progress-curve fitting ($4.70 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) and found to be very similar to that reported by Vogel & Müller-Eberhard (1982) ($4.05 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) from the use of initial-rate measurements. The initial enzyme of the alternative pathway, C3(H_2O),Bb, was found to be less active and less stable than C3b,Bb (Table 2) (Fishelson *et al.*, 1984). The C3 convertase, C3b,Bb, containing a single Ni^{2+} ion is known to be more stable than the complex containing Mg^{2+} (Fishelson & Müller-Eberhard, 1982). Analysis of progress curves indicated that the Ni^{2+} -containing enzyme was less efficient ($k_{\text{cat.}}/K_m = 24.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) but more stable ($t_{1/2} = 674 \text{ s}$) than the Mg^{2+} -containing enzyme. Trypsin was shown to be less efficient than C3b,Bb but more efficient than CVF,Bb in converting C3 into C3b (Table 2).

DISCUSSION

The central enzyme of the alternative pathway of complement has been difficult to characterize enzymically because it exhibits rapid spontaneous decay-dissociation into its subunits C3b and Bb (Medicus *et al.*, 1976b; Müller-Eberhard & Götze, 1972). The enzyme is also unusual in that the cleavage of its natural substrate C3 produces C3b, which is one of the subunits of the enzyme (Müller-Eberhard & Götze, 1972). This characteristic provides the alternative pathway with the unique ability to amplify C3b formation. The regulation of C3 convertase decay provides a means of modulating the amplification process to enhance amplification of C3b on activators and to prevent inappropriate amplification on host cells. Two plasma proteins alter the decay-dissociation rate of C3b,Bb. Properdin stabilizes the complex, whereas Factor H greatly accelerates its dissociation (Pangburn & Müller-Eberhard, 1984). In order to understand quantitatively the kinetic behaviour of the activation and regulation processes (Reeve & Woo, 1982) it was first necessary to characterize the central enzyme of the pathway. The present paper describes a fluid-phase fluorimetric assay for C3 convertases and reports the kinetic properties of C3b,Bb acting on its natural substrate.

The measurement of C3 convertase activity took advantage of the 6-fold increase in fluorescence intensity that occurs when C3 is cleaved in the presence of ANS (Isenman & Cooper, 1981; Isenman *et al.*, 1981; Pangburn & Müller-Eberhard, 1982). C3b has been shown to possess two binding sites for ANS with 5-fold higher affinity than the single site on native C3 (Isenman, 1983). ANS concentrations from 10 to 100 μM (4% to 31% saturation of the ANS-binding site on C3) had no effect on the rate of C3 cleavage by C3b,Bb, suggesting that ANS does not interfere with the cleavage reaction. This conclusion is further supported by the similarity in the $k_{\text{cat.}}/K_m$ value for CVF,Bb found in the absence of ANS (Vogel & Müller-Eberhard, 1982) and that found in the present study (Table 2). Preparation of known concentrations of C3b,Bb was accomplished by using saturating concentrations of Factor B and excess Factor D. Addition of EDTA blocked further production of enzyme, which would have occurred in the cuvette in the absence of EDTA once C3b production began. EDTA dissociates Factor B bound to C3b, but the chelator has no effect on the activated complex C3b,Bb (Fishelson

Table 2. Kinetic parameters of different C3 convertases at 37 °C

C3 convertase	$t_{1/2}$ (s)	$k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_m (M)	$k_{\text{cat.}}$ (s^{-1})
C3b,Bb	90	31.1×10^4	5.86×10^{-6}	1.78
CVF,Bb*	(23800)§	4.70×10^4	—	—
CVF,Bb†	25200	4.05×10^4	11.6×10^{-6}	0.47
C3(H_2O),Bb	77	16.3×10^4	—	—
C3b,Bb(Ni^{2+})‡	674	24.6×10^4	—	—
Trypsin	(21250)§	10.9×10^4	—	—

* Present study. CVF is an analogue of human C3b.

† From Vogel & Müller-Eberhard (1982).

‡ C3 convertase containing Ni^{2+} instead of Mg^{2+} .

§ Since progress curves were monitored for a relatively short period of time, the analysis of very-long-lived enzymes yields half-lives that represent lower limits.

et al., 1983). It was estimated that less than 3% of the enzyme decayed between the time of EDTA addition and the time of enzyme addition to the assay. The ability to monitor the course of the cleavage reaction continuously was essential for accurate measurement of initial rates, owing to the rapid decay of enzyme activity. This method was superior in accuracy and simplicity to secondary assays of remaining substrate or SDS-polyacrylamide-gel-electrophoretic analysis of cleavage products.

The kinetic parameters describing the enzymic properties of C3b,Bb and other C3 convertases are listed in Tables 1 and 2. The K_m of C3b,Bb for C3 was 5.86×10^{-6} M, whereas the K_m reported for CVF,Bb was twice this value (Vogel & Müller-Eberhard, 1982). The K_m of the classical-pathway C3 convertase C4b,2a for C3 has been reported to be 1.8×10^{-6} M (Cooper, 1975). The k_{cat} of C3b,Bb was almost 4 times that of CVF,Bb. These comparisons are of interest because the catalytic subunit of C3b,Bb and CVF,Bb is the same. These results suggest that the interaction of Bb with C3b or CVF alters the catalytic efficiency of the active site. The apparent second-order rate constant k_{cat}/K_m provides the best measure of catalytic efficiency, and this constant differs by more than 7-fold for the two enzymes. High-resolution transmission electron microscopy (Smith *et al.*, 1984) has revealed two distinct domains in the Bb molecule. Only one of these interacts with C3b. If, as has been suggested, the catalytic site resides on the domain distant from C3b, then the effect of the cofactor on the catalytic site may be transmitted through the binding domain of Bb. The C-terminal half of Bb is homologous with other serine proteinases, whereas the N-terminal portion is structurally unrelated (Mole *et al.*, 1984).

The kinetic parameters of C3 convertase can be compared with the parameters determined for the cleavage of C3b by the serine proteinase Factor I (Pangburn & Müller-Eberhard, 1982). The K_m for the reaction of Factor I with the bimolecular complex of C3b and Factor H (M_r 326000), which is the substrate, was found to be 0.252×10^{-6} M. This value is 20-fold lower than that characterizing the interaction of C3b,Bb and C3, and probably reflects the differing biological roles of the two enzymes. Factor I must inactivate very low concentrations of C3b generated by C3 convertases during complement activation. The substrate concentration for C3 convertases, on the other hand, is that of C3 in plasma ($6.5 \mu\text{M}$). C3b,Bb, with the considerably higher K_m of 5.86×10^{-6} M, would therefore be half-saturated and operate at half-maximal velocity under physiological conditions.

Few inhibitors of the active site of complement C3 convertases have been found. This is probably due to their extreme specificity and reflects their resistance to inhibition by any of the proteinase inhibitors of plasma. The only high- M_r inhibitors known are those that inactivate the enzyme by accelerating the dissociation of the complex. Leupeptin, a low- M_r proteinase inhibitor (M_r 427), failed to inhibit C3 convertase activity in the present study, although it has been reported to inhibit (K_i 40×10^{-6} M) cleavage of a peptide substrate by CVF,Bb (Caporale, 1981). Recent work in this laboratory (V. Ganu, personal communication) has demonstrated that neither CVF,Bb nor Factor B cleaves the peptide substrate (t-butyloxycarbonyl-Leu-Gly-Arg-7-amino-4-methylcoumarin) used in that study. The lack of inhibition of C3b,Bb by leupeptin would also explain the failure of

this inhibitor to block alternative-pathway activation in serum at a concentration of 1 mM (Takada *et al.*, 1978). Benzamidine (M_r 120) and guanidine (M_r 59) were the only inhibitors found for C3b,Bb. The K_i of benzamidine for C3b,Bb was 10^3 -fold higher than its K_i for trypsin (Gutierrez *et al.*, 1983).

The half-life of C3b,Bb due to dissociation was determined to be 90 ± 2 s at physiological pH, temperature and ionic strength. Other studies, with erythrocyte-bound enzymes, have reported half-lives ranging from 90 s to 135 s under similar conditions (Medicus *et al.*, 1976b,c,d). The agreement between these measurements suggests that the microenvironment of membrane-bound enzymes has little or no effect on stability of the complex. The decay rate of the complex is unaffected by the presence of EDTA. This suggests that on cleavage of the Factor B subunit of C3b,B(Mg^{2+}) by Factor D the metal ion either becomes inaccessible to EDTA or it is completely co-ordinated by ligands provided by the proteins. The temperature-dependence of the half-life (Fig. 7) showed that dissociation requires attainment of an activation energy of 81.6 kJ/mol (19.5 kcal/mol). Part of this energy requirement may be the result of the necessity to dissociate one or more of these ligands.

Note added in proof (received 17 February 1986)

A recent report (Prydzial & Isenman, 1986) suggested that EDTA does not completely prevent C3 convertase formation. Controls (see the Results section) indicated that addition of EDTA before the addition of Factor D prevented formation of detectable C3 convertase activity. More stringent analyses confirm that enzyme formation in the presence of EDTA under the conditions used in the present study was below detectable levels and had no effect on the results.

This is Publication no. 3178IMM from the Research Institute of Scripps Clinic. This work was supported by U.S. Public Health Service Grant AI-17354. The work was done during the tenure by M.K.P. of an Established Investigatorship from the American Heart Association with funds contributed in part by the American Heart Association, California Affiliate. H.J.M.-E. is Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic. We thank Lisa B. McGuire and Beverly A. Johnson for excellent technical assistance and Dr. Carl-Wilhelm Vogel, Georgetown University, Washington, DC, U.S.A., for samples of CVF.

REFERENCES

- Ballow, M. & Cochrane, C. G. (1969) *J. Immunol.* **103**, 944–952
- Caporale, L. H. (1981) *Biochim. Biophys. Acta* **660**, 151–153
- Cooper, N. R. (1975) *Biochemistry* **14**, 4245–4251
- Fearon, D. T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5867–5871
- Fishelson, Z. & Müller-Eberhard, H. J. (1982) *J. Immunol.* **129**, 2603–2607
- Fishelson, Z., Pangburn, M. K. & Müller-Eberhard, H. J. (1983) *J. Biol. Chem.* **258**, 7411–7415.
- Fishelson, Z., Pangburn, M. K. & Müller-Eberhard, H. J. (1984) *J. Immunol.* **132**, 1430–1434
- Götze, O. & Müller-Eberhard, H. J. (1971) *J. Exp. Med.* **134**, 90s (abstr.)
- Götze, O. & Müller-Eberhard, H. J. (1976) *Adv. Immunol.* **24**, 1–35
- Götze, O., Medicus, R. G. & Müller-Eberhard, H. J. (1977) *J. Immunol.* **118**, 525–528
- Gutierrez, J. C., Götze, O. & Caporale, L. H. (1983) *Biochim. Biophys. Acta* **744**, 276–280

- Hammer, C. H., Wirtz, G. H., Renfer, L., Gresham, H. D. & Tack, B. F. (1981) *J. Biol. Chem.* **256**, 3995–4006
- Isenman, D. E. (1983) *J. Biol. Chem.* **258**, 4238–4244
- Isenman, D. E. & Cooper, N. R. (1981) *Mol. Immunol.* **18**, 331–339
- Isenman, D. E., Kells, D. I. C., Cooper, N. R., Müller-Eberhard, H. J. & Pangburn, M. K. (1981) *Biochemistry* **20**, 4458–4467
- Janatova, J., Tack, B. F. & Prahl, J. W. (1980) *Biochemistry* **19**, 4479–4485
- Johnson, M. S., Gagnon, J. & Reid, K. B. M. (1980) *Biochem. J.* **187**, 863–874
- Lesavre, P. H. & Müller-Eberhard, H. J. (1978) *J. Exp. Med.* **148**, 1498–1509
- Lesavre, P. H., Hugli, T. E., Esser, A. F. & Müller-Eberhard, H. J. (1979) *J. Immunol.* **123**, 529–534
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Medicus, R. G., Götze, O. & Müller-Eberhard, H. J. (1976a) *Scand. J. Immunol.* **5**, 1049–1055
- Medicus, R. G., Götze, O. & Müller-Eberhard, H. J. (1976b) *J. Exp. Med.* **144**, 1076–1093
- Medicus, R. G., Götze, O. & Müller-Eberhard, H. J. (1976c) *J. Immunol.* **116**, 1741 (abstr.)
- Medicus, R. G., Schreiber, R. D., Götze, O. & Müller-Eberhard, H. J. (1976d) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 612–616
- Mole, J. E., Anderson, J. K., Davison, E. A. & Woods, D. E. (1984) *J. Biol. Chem.* **259**, 3407–3412
- Müller-Eberhard, H. J. & Götze, O. (1972) *J. Exp. Med.* **135**, 1003–1008
- Nicholson-Weller, A., Burge, J., Fearon, D. T., Weller, P. F. & Austen, K. F. (1982) *J. Immunol.* **129**, 184–189
- Pangburn, M. K. & Müller-Eberhard, H. J. (1980) *J. Exp. Med.* **152**, 1102–1114
- Pangburn, M. K. & Müller-Eberhard, H. J. (1982) *Biochemistry* **22**, 178–185
- Pangburn, M. K. & Müller-Eberhard, H. J. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2075 (abstr.)
- Pangburn, M. K. & Müller-Eberhard, H. J. (1984) *Springer Semin. Immunopathol.* **7**, 163–192
- Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. (1981) *J. Exp. Med.* **154**, 856–867
- Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. (1983a) *J. Immunol.* **130**, 1930–1935
- Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. (1983b) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5430–5434
- Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. (1983c) *Blood* **62**, 105a (abstr.)
- Parkes, C., DiScipio, R. G., Kerr, M. A. & Prohaska, R. (1981) *Biochem. J.* **193**, 963–970
- Prydzial, E. L. G. & Isenman, D. E. (1986) *Mol. Immunol.* **23**, 87–96
- Reeve, J. & Woo, M. M. (1982) *Immunology* **46**, 859–866
- Robinson, N. C., Tye, R. W., Neurath, H. & Walsh, K. A. (1971) *Biochemistry* **10**, 2743–2747
- Smith, C. A., Vogel, C.-W. & Müller-Eberhard, H. J. (1984) *J. Exp. Med.* **159**, 324–329
- Strunk, R. C. & Webster, R. O. (1985) *Mol. Immunol.* **22**, 37–44
- Takada, Y., Arimoto, Y., Minoda, H. & Takada, A. (1978) *Immunology* **34**, 509–515
- Vogel, C.-W. & Müller-Eberhard, H. J. (1982) *J. Biol. Chem.* **257**, 8292–8299
- VonZabern, I., Nolte, R. & Vogt, W. (1981) *Scand. J. Immunol.* **13**, 413–431
- Weiler, J. M., Daha, M. R., Austen, K. F. & Fearon, D. T. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3268–3272
- Whaley, K. & Ruddy, S. (1976) *J. Exp. Med.* **144**, 1147–1163

Received 13 August 1985/10 December 1985; accepted 30 December 1985