protozoan and mammalian enzymes might permit the future rational design of selective inhibitors of the trypanosomal dihydroorotate oxidase.

Registry No. Dihydroorotate oxidase, 9029-03-2; L-dihydroorotic acid, 5988-19-2; orotic acid, 65-86-1.

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Kinetic and Thermodynamic Analysis of the Control of C3b by the Complement Regulatory Proteins Factors H and I†

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ABSTRACT: The substrate for the complement serine protease factor I (M_r 88 000) is the C3b portion of the bimolecular complex (M_r 326 000) which is composed of C3b and factor H. This complex is in equilibrium with free C3b and factor H. C3b participates in the recognition function and the positive feedback of the alternative complement pathway, and therefore regulation of C3b activity by factors H and I is crucial for control of this pathway. Conversion of C3b to inactive C3b by factor I (EC 3.4.21.45) was found to be accompanied by a marked decrease in the fluorescence of the probe ANS (8-anilino-1-naphthalenesulfonate), and this change in fluorescence was used to monitor continuously the proteolytic reaction. The initial velocities of the reaction between factor I and various concentrations of the C3b,H complex were analyzed by applying the Michaelis–Menten equation. The analysis indicated that the reaction exhibited simple enzyme–substrate behavior, although the substrate is a bimolecular complex in equilibrium with its subunits. The association constant (K_a) for the complex of C3b and factor H was determined by measuring initial velocities at various concentrations of C3b above and below the estimated dissociation constant for the C3b,H complex. All of the initial velocity measurements were then used simultaneously to refine K_m, V_{max}, and K_a by using an iterative process which yielded the best-fit values of all three constants. The apparent activation energy of the rate-determining step was found to be 24 900 cal·mol^{-1}, reflecting the strong temperature dependence of this proteolytic reaction. The K_a for the interaction between C3b and factor H was 1.6×10^6 M^{-1} at 37 °C and 4.4×10^6 M^{-1} at 20 °C. The K_a for the fluid-phase C3b,H complex was compared to the K_a for the cell-bound C3b,H complex measured in other studies. The comparison suggests that recognition of potentially pathogenic microorganisms by human C3b results in a decrease in affinity between factor H and C3b when C3b is bound to these organisms.

Activation of the complement system leads to the generation of fluid-phase and particle-bound C3b (M_r 176 000) which fulfills various functions in complement reactions (Müller-Eberhard & Schreiber, 1980). Nascent C3b exhibits a metastable binding site (Müller-Eberhard et al., 1966) through which it may form an ester or amide bond with surface constituents of biological particles (Law et al., 1979). C3b initiates the positive feedback system of the alternative pathway (Müller-Eberhard & Götze, 1972) by forming a bimolecular, Mg-dependent complex with the proenzyme factor B (M_r 93 000) and by modulating factor B such that it can be cleaved by its activating enzyme factor D (EC 3.4.21.46). The resulting C3 convertase (C3b,Bb) (EC 3.4.21.47) acts on native C3 (M_r 185 000) and by removing the activation peptide C3a (M_r 9000) produces additional metastable C3b. The first C3b molecules are furnished by the initial C3 convertase of the alternative pathway, C3(H_2O),Bb (Pangburn et al., 1981). Instead of C3b, this fluid-phase enzyme contains uncleaved, functionally C3b-like C3. This form of C3 arises by spontaneous hydrolysis of the internal thioester of native C3 (Law et al., 1980; Pangburn & Müller-Eberhard, 1980; Sim et al., 1981; Tack et al., 1980).

C3b and C3(H_2O) and the respective C3 convertases are

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regulated by factor H[1] (M_r 150000) which acts nonenzymatically (Whaley & Ruddy, 1976; Weiler et al., 1976) and by factor I (M_r 88000) which is a serine protease (EC 3.4.21.45) (Hsiung et al., 1982; Davis, 1981). Factor H regulates amplification of the alternative pathway by binding to C3b and C3(H_2O) and thereby preventing binding of factor B. It also greatly accelerates the rate of decay–dissociation of the C3b,Bb complex and of the initial C3 convertase. Factor I inactivates C3b by cleaving its α' chain into two fragments (M_r 67000 and 40000) which remain bound to the β chain through disulfide bonds (Pangburn et al., 1977). Similarly, it inactivates C3(H_2O) by cleaving its α chain into a 76000- and a 40000-dalton fragment (Pangburn & Müller-Eberhard, 1980). These reactions produce C3bi and C3(H_2O)i, which, while otherwise inactive, can react with specific C3bi receptors on the surface of leukocytes (Dobson et al., 1981; Schmitt et al., 1981; Schreiber et al., 1982). Cleavage of soluble C3b and of C3(H_2O) requires the presence of factor H. Thus, in solution, the actual substrate for factor I is the C3b,H or the C3(H_2O),H complex. Attached to some biological particles, C3b can be cleaved by factor I alone, but the rate of cleavage is 30-fold enhanced by factor H (Pangburn & Müller-Eberhard, 1978). Although factor I has been shown to make at least one additional cleavage, this process is very slow compared to the initial cleavage and would not influence the present study (Harrison & Lachmann, 1980).

In recent years, an important function of the alternative pathway of complement has been attributed to C3b: the ability to distinguish between different cells (Schreiber et al., 1978). For instance, human C3b bound to human erythrocytes binds factor H and can be degraded by factor I. In contrast, human C3b bound to yeast cell walls or certain bacteria has a low affinity for factor H (Kazatchkine et al., 1979; DiScipio, 1981) and is relatively resistant to degradation by factor I (Pangburn & Müller-Eberhard, 1978; Pangburn, 1982). As a result of this discriminatory ability of C3b, the human alternative pathway will be activated by yeast cell walls and certain bacteria, but not by human erythrocytes. Thus, recognition is expressed on the molecular level by a change in the affinity of bound C3b for factor H and on the physiological level by activation of the alternative complement pathway which leads to cytolysis and phagocytosis. The present study was performed to determine the association constant for the interaction between soluble C3b and factor H as well as the kinetic and thermodynamic parameters of the proteolytic reaction between the C3b,H complex and factor I. The kinetic analysis of the conversion of C3b to C3bi has been facilitated by the development of a fluorometric assay which allows continuous monitoring of the reaction. The emission intensity of the fluorescent probe ANS shows a pronounced increase when C3 is converted to C3b in its presence (Isenman & Cooper, 1981; Isenman et al., 1981). This increase has been shown (Stryer, 1965) to be due to the interaction of the dye with hydrophobic regions of proteins. Subsequent cleavage of C3b to C3bi causes an almost equal decrease in fluorescence (Isenman, 1982; Pangburn, 1982), and this change has been employed to monitor the conversion of C3b to C3bi.

[1] Abbreviations: factor I, a human plasma protein also referred to as C3b/C4b inactivator; factor H, a human plasma protein previously referred to as β1H; ANS, 8-anilino-1-naphthalenesulfonate; K_a(C3b + H), association constant between C3b and factor H; K_a(I + C3b,H), association constant between factor I and the complex containing C3b and factor H; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)-aminomethane; TBS, Tris-buffered saline; VBS, veronal-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

Materials and Methods

Materials. Bovine TPCK-treated trypsin and soybean trypsin inhibitor (SBTI) were obtained from Worthington (Freehold, NJ); 8-anilino-1-naphthalenesulfonate (ANS) was from Eastman (Rochester, NY). Frozen human plasma was purchased from the San Diego Blood Blank.

Purified Components. C3 was purified according to the method of Hammer et al. (1981). C3b was generated from native C3 by limited tryptic digestion (0.2% w/w, 8 min, 37 °C). The reaction was followed fluorometrically in the presence of ANS, and a 2-fold excess of SBTI added when the conversion was complete (Isenman et al., 1981). Factors H and I were purified as previously described (Pangburn et al., 1977). All protein components were found to be homogeneous by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and by immunoelectrophoresis. They were stored at −70 °C. The concentration of the proteins was determined spectrophotometrically at 280 nm by using an $E^{1\%}_{280nm}$ of 11.0 for C3 and C3b, 12.4 for factor H, and 14.3 for factor I. Calculations of molar concentration assumed molecular weights of 185000 for C3, 176000 for C3b, 150000 for factor H, and 88000 for factor I.

Buffers. Veronal-buffered saline (VBS), Tris-buffered saline (TBS), and phosphate-buffered saline (PBS) contained 150 mM NaCl and 10 mM of the respective buffer. All buffers were adjusted to pH 7.4. Although no significant change in kinetic parameters occurred between pH 6.9 and 7.4, adjustments were made for the change of pH of TBS with temperature. No correction was made for temperature when PBS was used.

Electrophoresis. Electrophoresis in the presence of $NaDodSO_4$ was performed in 9% polyacrylamide gels by using the Canalco SAGE electrophoretic system (Miles Laboratories, Inc., Elkhart, IN).

Fluorescence Measurements. Fluorescence emission of ANS in the presence or absence of proteins was recorded with an Aminco SPF-500 spectrofluorometer (American Instrument Co., Silver Springs, MD). Excitation was at 386 nm with a 5-nm band-pass, and emission was measured at 472 nm with a 10-nm band-pass. Recordings were made exclusively in the ratio mode.

Temperature Measurements. The cell compartment was thermostated 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 thermostated, four-cell carrousel of the spectrofluorometer. Temperature variation and reproducibility were within ±0.2 °C.

Measurement of Equilibrium Constants. So that concentrations of C3b and factor H appropriate for kinetic measurements could be chosen, an estimate of the affinity between these proteins was needed. Values of K_a reported for factor H with cell-bound C3b at low ionic strength varied between 10^7 and 10^9 M^{-1} (Conrad et al., 1978; Kazatchkine et al., 1979; DiScipio, 1981). An estimate of this value for fluid-phase C3 at physiological ionic strength was made by diluting a mixture of C3b (3×10^{-6} M) and factor H (0.4×10^{-6} M) and measuring the initial velocity of C3b cleavage by factor I at each dilution. For a nondissociating substrate, a 1:2 dilution will produce a 2-fold decrease in the reaction velocity unless the substrate concentration is above the K_m in which case the decrease would be less than 2-fold. Any decrease in velocity greater than the dilution factor (e.g., 3-fold after a 1:2 dilution) was taken to reflect dissociation of the C3b,H complex. The degree of dissociation was then used to estimate the association

constant. The final association constants were measured experimentally in a similar way, but the mathematical treatment employed V_{max} and K_m values as described under Results.

Kinetic Measurements. Measurements of the initial reaction velocity were performed in 10 × 10 mm quartz fluorescence cuvettes in a total volume of 1.6 mL. A typical assay was performed as follows: 112 μL of C3 (52.9×10^{-6} M), 80 μL of ANS (0.4×10^{-3} M), and 2 μL of trypsin (1 mg/mL) were added to 1323 μL of TBS in the cuvette. The fluorescence increase due to the conversion of C3 to C3b (Isenman & Cooper, 1981; Isenman et al., 1981) was monitored, and 2 μL of SBTI (2 mg/mL) was added when conversion was complete. factor H (55 μL at 2.86×10^{-6} M) was then added. The cell was capped to prevent evaporation and remained in the thermostated cell compartment until the temperature equilibrated. A base line was recorded, and 26 μL of factor I (2.95×10^{-6} M) was added to start the assay. The initial reaction rate was determined from the recorder tracing.

Thermodynamic Calculations. The activation energy, E_a, was calculated from the slope of a plot of k_{cat} vs. $1/T$ by using the Arrhenius equation. The activation enthalpy, ΔH^*, was calculated from the relationship $E_a = \Delta H^* + RT$. The Gibbs energy of activation, ΔG^*, was calculated from $\Delta G^* = -2.3RT \log [k_{cat}h/(k_BT)]$, and the activation entropy, ΔS^*, was calculated by using $\Delta G^* = \Delta H^* - T\Delta S^*$. The standard enthalpy, $\Delta H°$, was obtained for K_m from the slope of a plot of $-\log K_m$ vs. $1/T$ by applying the van't Hoff equation. The $\Delta H°$ for K_a for the interaction between C3b and factor H was similarly determined from a plot of $\log K_a$ vs. $1/T$. The standard Gibbs energy, $\Delta G°$, was calculated from $\Delta G° = -2.3RT \log (1/K_m)$ or $\Delta G° = -2.3RT \log K_a$. The standard entropy, $\Delta S°$, was calculated from $\Delta G° = \Delta H° - T\Delta S°$.

Measurement of the Association Constant between Factor I and Cell-Bound C3b,H. Sheep erythrocytes bearing approximately 40 000 C3b molecules per cell were prepared as previously described (Pangburn et al., 1980). Factor I was labeled with ^{125}I by using insolubilized lactoperoxidase at 0 °C (David & Reisfeld, 1974). Binding assays were performed as described (Pangburn & Müller-Eberhard, 1978), except that the entire procedure was performed at 0 °C by keeping all reagents and reaction mixtures on ice.

Statistical Analyses. All data were analyzed 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 Hewlett Packard Model 9815A computer.

Results

Characteristics of the ANS Fluorometric Assay. It is known that conversion of C3 to C3b by trypsin causes an increase in ANS fluorescence. Figure 1 shows that this change can be almost entirely reversed upon cleavage of C3b to C3bi by factors H and I. Addition of sufficient trypsin to produce the fragments C3c (M_r 140 000) and C3d (M_r 35 000) from C3bi results in a further decrease in fluorescence.

The initial rate of the cleavage reaction was measured by continuously recording the decrease in ANS fluorescence with time as C3b was converted to C3bi (Figure 2). The concentration of substrate C3b,H could be varied by changing the factor H concentration since it is known that factor I only cleaves fluid-phase C3b when it is bound to factor H:

$$C3b + H \rightleftarrows C3b,H \quad (1)$$

$$C3b,H + I \rightleftarrows I,C3b,H \rightarrow C3bi + H + I \quad (2)$$

The initial C3b concentration was chosen to be sufficiently high so that over 90% of the factor H was in complex with C3b. As Figure 2 shows, the initial portion of the reaction was linear for an extended period of time because the excess C3b continuously re-formed substrate with factor H freed by C3bi formation as shown in eq 2. It is known that neither factor H nor factor I binds significantly to C3bi (Pangburn

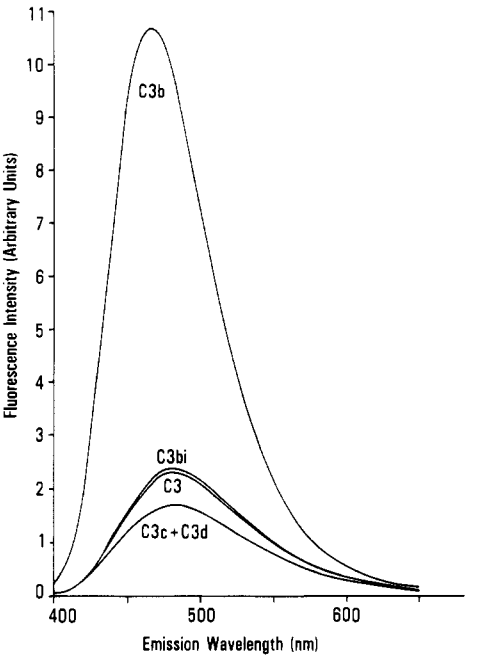

FIGURE 1: Decrease of ANS fluorescence upon cleavage of fluid-phase C3b to C3bi by factor I in the presence of factor H. Native C3 (2.12×10^{-6} M final concentration) in the presence of 0.01 M ANS in TBS was converted to C3b by the addition of 8 μg of trypsin. The increase in ANS fluorescence accompanying this change is shown. Trypsin was inactivated by the addition of 16 μg of SBTI, and factors H (1.43×10^{-7} M) and I (1.48×10^{-7} M) were added. Complete conversion of C3b to C3bi resulted in an 80% decrease in fluorescence at 472 nm. The excitation wavelength was 386 nm. Subsequent addition of 32 μg of trypsin produced the spectra indicated for the resulting mixture of C3c and C3d.

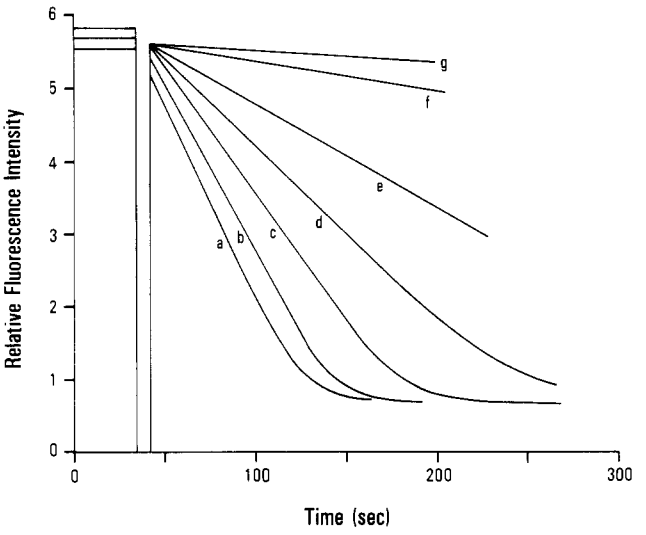

FIGURE 2: Change of ANS fluorescence intensity at 472 nm during cleavage of C3b to C3bi by factor I at 30 °C. Assays were performed as described under Materials and Methods. Each assay contained identical concentrations of C3b (3.70×10^{-6} M), ANS (20×10^{-6} M), and factor I (4.8×10^{-8} M) in TBS, pH 7.4, at 30 °C. Curves a–g represent seven individual assays containing the following factor H concentrations (nM): 790 (a), 393 (b), 197 (c), 98.5 (d), 49.3 (e), 12.4 (f), and 4.50 (g). After temperature equilibration was complete and a base line established, factor I was added (at 35 s in this figure). Initial rates were determined as described in the text.

Table I: Steps in the Iterative Procedure Used To Refine the Values of K_a, K_m, and V_{max} at 30 °C[a]

				first iteration[c]		second iteration[d]		third iteration[d]		fourth iteration[d]	
assay	$[C3b]_o$ ($\times 10^6$ M)	$[H]_o$ ($\times 10^6$ M)	v ($\times 10^9$ M·s^{-1})	$[S]_t$ ($\times 10^6$ M)	K_a ($\times 10^{-6}$ M^{-1})	$[S]_t$ ($\times 10^6$ M)	K_a ($\times 10^{-6}$ M^{-1})	$[S]_t$ ($\times 10^6$ M)	K_a ($\times 10^{-6}$ M^{-1})	$[S]_t$ ($\times 10^6$ M)	K_a ($\times 10^{-6}$ M^{-1})
1	3.34	0.500	18.4[b]	0.454	3.42	0.440	2.53	0.426	1.99	0.421	1.83
2	1.07	0.160	9.72	0.125	3.78	0.121	3.27	0.118	2.93	0.117	2.86
3	0.334	0.050	2.90	0.0280	4.16	0.0272	3.89	0.0265	3.77	0.0264	3.64
4	0.107	0.016	0.318	0.00282	2.05	0.00275	1.99	0.00268	1.93	0.00267	1.92
5	0.0334	0.005	0.042	0.00037	2.42	0.00036	2.35	0.00035	2.28	0.00035	2.27
					av 3.17		av 2.81		av 2.58		av 2.50

[a] The iterative procedure and the equations used are explained in the text. [b] The initial reaction velocity, v, was measured by using the C3b and factor H concentrations shown for each assay and a factor I concentration, $[E]_t$, of 4.80×10^{-8} M. [c] The C3b,H complex concentration, $[S]_t$, was calculated by using eq 4. The initial K_m and V_{max} values were determined by using the K_a at 20 °C which was refined in a similar manner by beginning with the estimated K_a (see Materials and Methods). Using $K_a = 4.4 \times 10^6$ M^{-1} to calculate S, the first Eadie-Hofstee plot yielded $K_m = 1.85 \times 10^{-7}$ M and $V_{max} = 26.5 \times 10^{-9}$ M·s^{-1}. [d] The average K_a from each iteration was used to recalculate S which was used in turn to determine new values for K_m and V_{max} as shown in Figure 3. The values for K_m and V_{max}, respectively, at each iteration were the following: second, 1.79×10^{-7} M, 26.5×10^{-9} M·s^{-1}; third, 1.73×10^{-7} M, 26.5×10^{-9} M·s^{-1}; fourth, 1.73×10^{-7} M, 26.6×10^{-9} M·s^{-1}.

& Müller-Eberhard, 1978). Thus, in curve a of Figure 2, the reaction rate remained linear until more than half of the C3b was cleaved. Because binding of factor H to C3b slightly decreases binding of ANS to C3b, a 5% decrease in fluorescence was observed at the highest factor H concentration as evident at zero time in Figure 2. For this reason, the initial rates were calculated by dividing the total C3b concentration by the time required to convert all of the C3b to C3bi, assuming that the reaction proceeded to completion at the initial rate. The rate of the reaction was not affected by varying the ANS concentration from 10 to 100 μM. At given concentrations of C3b and factor H, the initial rates were directly proportional to the concentration of factor I. Complete conversion of the two-chain structure of C3b to the three-chain structure of C3bi was confirmed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and a reducing agent.

Analysis of Kinetic Measurements. The initial reaction velocities, v, were measured at various concentrations of factor H as shown in Figure 2. The substrate concentrations, S, were estimated from the initial concentrations of C3b and factor H by using an estimate of the association constant for the complex C3b,H (3×10^6 M^{-1}). The initial velocities and substrate concentrations were plotted according to a rearrangement of the Michaelis–Menten equation:

$$v = -K_m(v/S) + V_{max} \quad (3)$$

which allowed determination of K_m and V_{max} from a plot of v vs. v/S (Figure 3). This method of plotting allows a statistical analysis of the linear regression lines and eliminates the uneven weighting of assays at low S values found with Lineweaver–Burk plots. In these experiments, the C3b concentration was sufficiently high to nearly saturate factor H. Thus, changes in the K_a for the interaction of C3b with factor H as large as an order of magnitude in either direction had only a small effect on S and subsequently on K_m and V_{max}. Over a narrow range of K_a close to the real K_a, K_m and V_{max} could be considered independent of K_a. For determination of the K_a and for refinement of the initial values of K_m and V_{max}, a separate set of assays was performed as detailed in Table I. Concentrations of C3b were varied 100-fold such that the range was centered on the estimated dissociation constant, $1/K_a$ (Figure 4). The initial rate of cleavage of C3b by factor I in these experiments depended upon K_m and V_{max} as before, but at these concentrations of C3b, the rate was also very sensitive to K_a. By estimation of K_m and V_{max} with eq 3 (Figure 3) and by measurement of the initial rate of cleavage, v, the concentration of the C3b,H complex, S, could be calculated

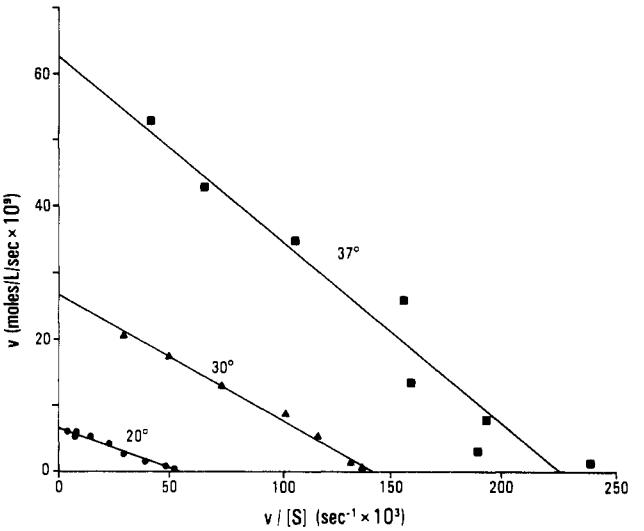

FIGURE 3: Analysis according to the Michaelis–Menten equation (eq 3) of kinetic measurements at three temperatures. Initial velocities, v, of the cleavage of C3b to C3bi were determined as described in Figure 2. The initial substrate concentration, S, is the concentration of C3b,H complex which was calculated by using the K_a determined for C3b and factor H at each temperature.

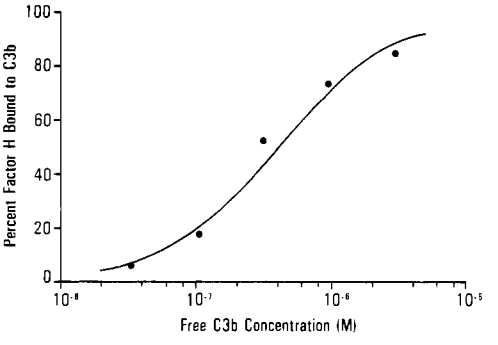

FIGURE 4: Comparison of the kinetically determined concentration of the C3b,H complex at 30 °C (data points) with the theoretical curve (line) for a simple association–dissociation reaction between C3b and factor H ($K_a = 2.5 \times 10^6$ M^{-1}). The concentration of C3b,H complex was calculated by measuring the velocity of cleavage by factor I and by applying eq 4 as described in the text. The K_a was determined as shown in Table I.

according to the following equation [rearranged from that of Henderson (1973)]:

$$S_t = \frac{K_S v}{V_{max} - v} + \frac{E_t v}{V_{max}} \quad (4)$$

Table II: Kinetic and Equilibrium Parameters of C3b Cleavage by Factors H and I

parameter	temp (°C) 37	30	20
K_m (M)	(2.52 ± 0.26) × 10^{-7}	(1.73 ± 0.06) × 10^{-7}	(1.21 ± 0.06) × 10^{-7}
k_{cat} (s^{-1})	1.32 ± 0.10	0.55 ± 0.01	0.137 ± 0.004
k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)	(5.2 ± 0.28) × 10^6	(3.20 ± 0.12) × 10^6	(1.13 ± 0.07) × 10^6
V_{max} ($M\cdot s^{-1}$)	(63 ± 4.6) × 10^{-9}	(26.6 ± 0.6) × 10^{-9}	(6.59 ± 0.18) × 10^{-9}
turnover no. (min^{-1})	79	33	8
sp act. ($nmol\cdot s^{-1}\cdot mg^{-1}$)	15	6.3	1.5
K_a(C3b,H + I) (M^{-1})	(4.0 ± 0.4) × 10^6	(5.8 ± 0.2) × 10^6	(8.3 ± 0.4) × 10^6
K_a(C3b + H) (M^{-1})	(1.6 ± 0.37) × 10^6	(2.5 ± 0.75) × 10^6	(4.4 ± 2.2) × 10^6

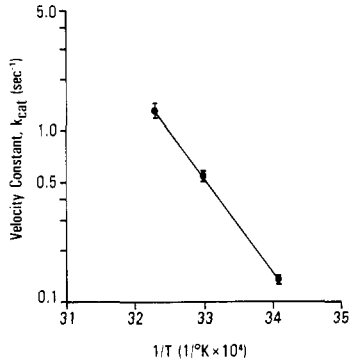

FIGURE 5: Temperature dependence of the velocity constant, k_{cat}, of the rate-limiting step. The data were plotted according to the Arrhenius equation.

where S_t and E_t are the total substrate and enzyme concentrations, respectively. This equation is valid for any enzyme:substrate ratio and assumes that $K_S = K_m$. This relationship is true when the catalytic rate constant k_2 is smaller than the rate constant of the dissociation of the enzyme–substrate complex, k_{-1}. It is probably true in the present case as will be shown below. In an iterative best-fit process, a complete example of which is given in Table I, the initial values for K_m and V_{max} were used to calculate K_a. This refined value was then used to recalculate the C3b,H complex concentration employed in the determination of K_m and V_{max}, and these values were used to again refine K_a. The iteration was stopped when no value was changed by more than 1% by the process. The relative insensitivity of K_m and V_{max} to K_a is responsible for the rapid convergence of the iterative process to the final values which required only four cycles for the data at 30 °C. The kinetic and equilibrium parameters obtained in this manner are given in Table II. The catalytic constant, k_{cat}, and the turnover number were calculated according to $k_{cat} = V_{max}/E_t$. The K_a for the interaction between factor I and the C3b,H complex given in Table II was calculated by assuming $K_m = K_S = 1/K_a$.

It should be noted that in order to generate sufficiently rapid initial velocities for accurate measurements, a relatively high concentration of factor I was used in these studies. At low substrate concentrations, the Michaelis–Menten assumption that the total enzyme concentration was small compared to S_t was not always met. Therefore, the data were analyzed by the method of Dixon (1972) which is a mathematical and graphical treatment valid for any enzyme to substrate ratio. No change greater than the standard error of either K_m or V_{max} was found at any temperature (Table II).

Table III: Thermodynamic Parameters of the Rate-Limiting Reaction

parameter	value
activation energy, E_a ($cal\cdot mol^{-1}$)	24 900
activation enthalpy, ΔH^* ($cal\cdot mol^{-1}$)	24 300
activation entropy, ΔS^* ($cal\cdot mol^{-1}\cdot deg^{-1}$)	20.6
Gibbs energy of activation, ΔG^*, at 37 °C ($cal\cdot mol^{-1}$)	17 900

Table IV: Thermodynamic Parameters of the Formation of the Substrate Complex (C3b,H) and the Enzyme–Substrate Complex (I,C3b,H)

parameter	complex C3b,H	I,C3b,H
standard enthalpy, ΔH° ($cal\cdot mol^{-1}$)	−10642	−7641
standard entropy, ΔS° ($cal\cdot mol^{-1}\cdot deg^{-1}$)	−6.04	5.44
standard Gibbs energy, ΔG°, at 37 °C ($cal\cdot mol^{-1}$)	−8770	−9320

Thermodynamic Parameters of the Rate-Limiting Step. Figure 5 shows the effect of temperature on the catalytic rate constant, k_{cat}, plotted according to the Arrhenius equation. The plot was linear, suggesting that the same reaction step was rate limiting throughout this temperature range. From the slope of the Arrhenius plot, the apparent activation energy, E_a, of the rate-limiting step was determined to be 24 900 $cal\cdot mol^{-1}$. This value corresponds to a temperature coefficient, Q_{10}, of 3.9. The apparent activation enthalpy, activation entropy, and Gibbs energy of inactivation were also determined as described under Materials and Methods for the rate-limiting step and are given in Table III.

Thermodynamic Parameters of the Association Constants. The equilibrium constant for the interaction of C3b and factor H was measured at three temperatures, and the values are given in Table II. The plot of log K_a vs. $1/T$ was linear (not shown). The apparent standard enthalpy, standard entropy, and Gibbs energy for this interaction were calculated (Table IV) by applying the van't Hoff equation and other thermodynamic relationships as described under Materials and Methods. A plot of $-\log K_m$ vs. $1/T$ according to van't Hoff law was also linear, and the apparent thermodynamic parameters are listed in Table IV. The Michaelis constant is normally composed of at least three rate constants ($K_m = k_{-1} + k_2/k_1$) and can be equated with the equilibrium constant for the enzyme–substrate complex when k_2 is much smaller than k_{-1}. In the present case, however, K_m is more complex than suggested by eq 2, since the possible modes of formation and decay of the enzyme–substrate complex (I,C3b,H) are numerous. A detailed analysis of the underlying rate constants comprising K_m is beyond the scope of this study. However, the linearity of the van't Hoff plot suggested that the dominant constants in K_m remain dominant throughout the temperature range studied. Furthermore, the temperature dependence of K_m was much less pronounced than that of k_{cat}, suggesing that the catalytic rate constant did not dominate K_m. This observation suggests that K_m approximates the reciprocal of the association constant for the enzyme–substrate complex. This possibility was tested by comparing the kinetic data with K_a measured directly.

Measurement of the Association Constant for the Interaction of Factor I and Cell-Bound C3b,H. Evidence supporting the conclusion that the measured K_m approximates the equilibrium constant was provided by direct measurement of the interaction at 0 °C. The catalytic rate constant k_{cat} was estimated to be 0.007 s^{-1} at 0 °C by extrapolation of the data

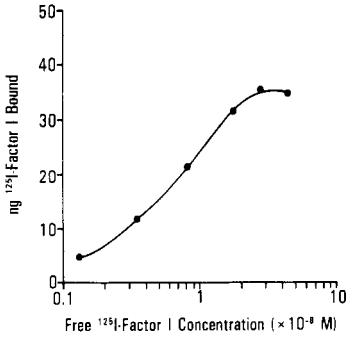

FIGURE 6: Determination of the association constant between factor I and cell-bound C3b,H at 0 °C. Sheep erythrocytes bearing specifically bound C3b were incubated with factor H and various concentrations of ^{125}I-labeled factor I. Bound components were separated rapidly from free components as described in the text.

in Figure 5. This value corresponded to a turnover number of 0.4 C3b min^{-1} $enzyme^{-1}$ and suggested that rapid separation of bound from free factor I would allow a direct estimation of K_a without significant interference from proteolysis. The procedure employed sheep erythrocytes with surface-bound C3b which were incubated with a constant concentration of factor H and varying concentrations of ^{125}I-labeled factor I. Cells previously treated with factors H and I at 37 °C to convert cell-bound C3b to C3bi lost over 95% of the ability to bind factors H and I. The erythrocytes bearing I,C3b,H complexes were rapidly separated (<15 s) from free factor I by centrifugation through a solution of 20% sucrose at 0 °C. The concentrations of free and bound factor I were determined from the distribution of radioactivity between the pellets and supernatants. The results are shown in Figure 6. The binding was saturable, and the apparent K_a was 1×10^8 M^{-1}. An extrapolation of the plot of log K_m vs. $1/T$ to 0 °C predicted a K_a at this temperature of 0.23×10^8 M^{-1}. The similarity between these values supports the conclusion that K_m approximates K_a for the enzyme–substrate complex.

Discussion

The present study utilized a fluorometric assay which allows continuous monitoring of the conversion of soluble C3b to C3bi by factors H and I. This assay was used to determine the equilibrium constant for the C3b and factor H interaction under physiological conditions as well as the kinetic and thermodynamic parameters of the enzymatic reaction. A recent study (Vogel & Müller-Eberhard, 1982) described the kinetic and thermodynamic properties of a C3 convertase prepared with cobra venom factor and human factor B. The results demonstrated that the action of a bimolecular protease of high molecular weight (M_r 210000) on its macromolecular substrate, C3 (M_r 185000), could be described by the Michaelis–Menten equation. The present study has shown that the action of factor I (M_r 88000) on its bimolecular substrate, C3b,H (M_r 326000), which is in equilibrium with free C3b and factor H, also follows Michaelis–Menten kinetics.

The simplest kinetic model possible was initially chosen in order to test the applicability of the Michaelis–Menten equation. Equations 1 and 2 describe this model and provide the basis for the mathematical treatment used, but they clearly do not represent all the intermediate complexes which are typically found in proteolytic reactions. A description of the complete reaction mechanism would require measurement of individual rate constants which was beyond the scope of the present study. The mathematical treatment used was based on a number of assumptions and predictions. It was necessary to assume that the rate of ANS signal change and the rate of factor H release from C3bi were fast compared to the catalytic rate. The conformational change which causes the change in ANS binding following hydrolysis must be fast for these conditions to be met. It has been shown (Isenman et al., 1981) that a fast conformational change occurs following proteolysis of C3, forming C3b. The rate of association of C3b and factor H was fast ($k_1 \geq 5.2 \times 10^6$ $s^{-1} \cdot M^{-1}$), since k_{cat}/K_m must be equal to or less than any second-order rate constant on the forward reaction pathway (Fersht, 1977). Product inhibition by C3bi was unlikely since an almost complete loss of binding of factor H and of factor I was observed when C3b was converted to C3bi (Pangburn & Müller-Eberhard, 1978). No cleavage of soluble C3b by factor I occurs in the absence of factor H (Pangburn et al., 1977), but substrate inhibition by free C3b was considered. Kinetic data gathered at each of the three temperatures studied were linear when plotted according to either the Lineweaver–Burk (Lineweaver & Burk, 1934) or the Eadie–Hofstee (Hofstee, 1959) form of the Michaelis–Menten equation, and these plots gave no suggestion of substrate or product inhibition (Cleland, 1970). The results indicate that treatment of C3b,H formation as a fast reaction governed by a simple equilibrium constant was valid under the conditions used in this study. Subsequent treatment of this complex as the substrate in a typical enzyme–substrate reaction appears to be valid since calculated substrate concentrations and measured initial rates obeyed the Michaelis–Menten relationship over a wide range of experimental conditions.

The association constant for the interaction between fluid-phase C3b and factor H was determined at physiological pH and ionic strength to be 4.4×10^6 M^{-1} at 20 °C and 1.6×10^6 M^{-1} at 37 °C. Other studies have reported association constants for cell-bound C3b and radiolabeled factor H under a variety of conditions. Kazatchkine et al. (1979) found a K_a of 1×10^7 M^{-1} at 30 °C and physiological ionic strength, whereas Conrad et al. (1978) found the K_a to be between 2×10^9 and 5×10^9 M^{-1} at half physiological ionic strength. Kazatchkine et al. (1979) also reported a K_a of 5.7×10^7 M^{-1} for the interaction between factor H and zymosan-bound C3b at 30 °C. This affinity applied to only 16% of the bound C3b molecules. The remainder of C3b sites had considerably lower affinities. DiScipio (1981) made a smilar measurement at 37 °C but found nonlinear, nonsaturable binding of factor H and estimated the high-affinity sites to have a K_a of 2×10^7 M^{-1}. Similar experiments (unpublished) in our laboratory confirm the finding of DiScipio that bound C3b is heterogeneous with respect to factor H binding. Since the interaction of C3b and factor H in the fluid phase was homogeneous, the heterogeneity seen with cell-bound C3b is not due to intrinsic heterogeneity of C3b or factor H. It is most probable that membrane-associated C3b molecules are located in heterogeneous environments on the surface of biological particles. Microenvironmental differences may in part be due to the ability of metastable C3b to attach nonspecifically to many different cell-surface constituents.

The major cause of the heterogeneity of affinities between bound C3b and factor H is, in all probability, related to recognition of surface structures by bound C3b through a site distinct from the attachment site (Müller-Eberhard & Schreiber, 1980; Reid & Porter, 1981). It has been proposed that biological particles which activate the alternative complement pathway do so because C3b bound to their surface interacts with specific structures, some of which have been identified as carbohydrates (Pangburn & Müller-Eberhard, 1978; Pangburn et al., 1980). Interaction of C3b with such surface markers has been proposed to lower the affinity of C3b

for factor H and thereby to allow activation of the alternative pathway. This mechanism may be operative in host resistance to infectious agents. Cell-surface neuraminic acid has been proposed to have the opposite effect on the C3b–factor H interaction and thereby to prevent activation of the alternative pathway (Fearon, 1978). An examination of the data published by Kazatchkine et al. (1979) reveals that at least 70% of the C3b bound to sheep erythrocytes have $K_a = 1 \times 10^7$ M^{-1}. Removal of sialic acid from these cells with neuraminidase resulted in 80% of the cell-bound C3b binding factor H with a K_a of less than 6×10^5 M^{-1}. DiScipio (1981) showed that while 20% of the C3b bound to zymosan exhibited an average K_a of 2×10^7 M^{-1}, 80% had association constants of less than 3×10^5 M^{-1}. Since the association constant measured in the fluid phase in the present study was 2.5×10^6 M^{-1} at 30 °C, it appears that surface structures can both increase and decrease the binding affinity of surface-bound C3b for factor H. The most important consideration in this comparison is that 80% of the C3b on zymosan had a K_a at least 10-fold lower than that of soluble C3b. This appears to confirm the proposal that biological particles that activate the human alternative pathway restrict control of bound C3b (Pangburn et al., 1980).

The kinetic parameters of the reactions studied are given in Table II. The apparent second-order rate constant, k_{cat}/K_m, suggests that factor I is a very effective protease. Compared to other proteases, factor I cleaved C3b almost as efficiently as most serine proteases cleave synthetic ester substrates (Zerner et al., 1964). Both terms in k_{cat}/K_m contributed to its high value. The k_{cat} of factor I was approximately 10-fold higher than that of chymotrypsin acting on most peptide substrates, and K_m was low compared to many enzyme–substrate interactions. Reactions with very low K_m values often exhibit product inhibition or very low turnover numbers. In the present study, factor H may function to temporarily increase the affinity between C3b and factor I. Cleavage of the peptide bond may then release factor H which lowers the stability of the enzyme–product complex and prevents product inhibition. It is interesting to speculate that the low K_m (2.5×10^{-7} M), which is similar to the serum concentration of factor I (3.9×10^{-7} M), is necessary to inactivate C3b, small amounts of which are continuously generated in serum (Pangburn et al., 1981). Low concentrations of C3b in serum will be 85% saturated with factor H since its serum concentration (3.7×10^{-6} M) is greater than its dissociation constant (0.63×10^{-6} M). Only when C3b becomes attached to foreign particles which activate the alternative pathway does the effectiveness of the regulatory system become impaired.

Measurement of the temperature dependence allowed the calculation of the thermodynamic properties of the catalytic reaction. The Arrhenius plot of k_{cat} was linear, and the apparent activation energy of the rate-limiting step was 24 900 cal·mol^{-1}. This value was high for enzyme-catalyzed reactions (White et al., 1973) but not unusual. Pepsin cleavage of ester and peptide substrates exhibits apparent activation energies of 23 100 and 20 200 cal·mol^{-1}, respectively (Casey & Laidler, 1950), and trypsin cleavage of chymotrypsinogen exhibits an apparent activation energy of 16 300 cal·mol^{-1} (Butler, 1941). The rate of enzymatic reactions, on the average, increases 2-fold with a 10 °C rise in temperature. Cleavage of C3b by factors H and I, however, exhibits a 3.9-fold rise which reflects the high activation energy of the rate-limiting step. The apparent activation enthalpy, activation entropy, and Gibbs free energy were determined for the change of the I,C3b,H complex to the transition-state complex of the rate-determining step. All three parameters were positive, and the large ΔH^* indicated that this was a strongly endothermic reaction.

Thermodynamic parameters were also determined for the association reactions which form the C3b,H and I,C3b,H complexes (Table IV). The parameters for I,C3b,H formation were determined from K_m with the assumption that this constant was dominated by the rate constants for factor I association with and dissociation from the the C3b,H complex. This assumption was confirmed by measuring the equilibrium constant directly at 0 °C. The change in enthalpy (ΔH) for the two association reactions was a net −18 283 cal·mol^{-1} while the change in enthalpy for the transition-state complex was 24 300 cal·mol^{-1}. Thus, the net heat of activation required for the change from free C3b, factor H, and factor I to the transition-state complex was 6017 cal·mol^{-1}. The net entropy was positive (20.0 cal·mol^{-1}·deg^{-1}), which probably contributes to overcoming the activation energy barrier. Entropy increases often reflect solvation effects such as the release of bound water and electrostatic neutralization, but many factors may contribute to the overall entropy change. The Gibbs free energy reflects the energy available to do work. The change in free energy due to the two association reactions is almost completely balanced by that of transition-state formation, leaving a net of only −190 cal·mol^{-1}.

Acknowledgments

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Registry No. C3b, 80295-43-8; factor H, 80295-65-4; factor I, 80295-66-5.

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N-Terminal Amino Acid Sequences of the α and β Chains of HLA-DR1 and HLA-DR2 Antigens†

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ABSTRACT: The N-terminal amino acid sequences of the α and β chains of HLA-DR1 and HLA-DR2 antigens were obtained with subnanomole quantities of material by using a gas–liquid solid-phase sequencer. A comparison of the N-terminal amino acid sequences of HLA-DR1 and HLA-DR2 α chains revealed no differences. However, in the first 35 N-terminal residues of the β chains from HLA-DR1 and HLA-DR2 antigens, two regions of variability are readily apparent, each comprising about six amino acids. Conceivably one or both of these variability regions may be responsible for the serologically defined polymorphism of HLA-DR alloantigens.

HLA-DR antigens constitute a set of highly polymorphic glycoproteins which are expressed mainly by B lymphocytes and have been implicated as functional components in graft rejection and cell–cell interactions between T and B cells necessary for immune responsiveness [for a recent review, see Amos & Kostyu (1980)]. These antigens are composed to two chains designated α and β with molecular weights of 34 000 and 29 000, respectively. Peptide maps of the β chains indicate that they express the majority of the detectable structural polymorphism whereas the α chains appear to be more conserved in structure (Silver and Ferrone, 1979; Walker et al., 1980). Recently, Kratzin et al. (1981) reported the amino acid sequence of the β chains of HLA-DR2 antigen and demonstrated the existence of a family of seven such chains whose structures were highly homologous except for one small region of five amino acids located between residues 65 and 69. In this report, we present and compare the amino acid sequences of the N-terminal 71 residues of the HLA-DR1 α chain, the N-terminal 40 residues of the HLA-DR2 α chain, and the N-terminal 35 amino acids of HLA-DR1 and HLA-DR2 β chains.

Materials and Methods

Cells. The homozygous B lymphoid cell lines LG-2 (HLA-DR1) and GM3107 (HLA-DR2) were cultured in RPMI 1640 media supplemented with 10% fetal calf serum and 25 μg/mL gentamycin.

Isolation of HLA-DR Antigens. The procedure used to isolate HLA-DR antigens has been described in detail in a separate report (Walker & Reisfeld, 1982). Briefly, 10 g of LG-2 or GM3107 cells was extracted with 20 mL of 0.01 M Tris buffer, pH 8.0, containing 1% Renex-30 (Accurate Chemical Co., Hicksville, NY) and 0.5 mM phenylmethanesulfonyl fluoride. Following centrifugation (30 min at 15000 *g*), the supernatant was applied to a 10-mL column of *Lens culinaris* lectin–Sepharose at a flow rate of 5 mL/h. After thorough washing of the column to remove unbound

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