Thrombin Is a Na$^+$-Activated Enzyme[†]

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ABSTRACT: The amidase activity of human α-thrombin has been studied at steady state as a function of the concentration of several chloride salts, at a constant ionic strength $I = 0.2$ M. All kinetic steps of the catalytic mechanism of the enzyme have been solved by studies conducted as a function of relative viscosity of the solution. Among all monovalent cations, Na$^+$ is the most effective in activating thrombin catalysis. This effect is observed with different amide substrates and also with γ-thrombin, a proteolytic derivative of the native enzyme which has little clotting activity but retains amidase activity toward small synthetic substrates. The specific effects observed as a function of Na$^+$ concentration are indicative of a binding interaction of this monovalent cation with the enzyme. The basis of this interaction has been explored by measurements of substrate hydrolysis collected in a three-dimensional matrix of substrate concentration, relative viscosity, and Na$^+$ concentration, keeping the ionic strength constant with an inert cation such as choline or tetraethylammonium. The data have globally been analyzed in terms of a kinetic linkage scheme where Na$^+$ plays the role of an allosteric effector. The properties of the enzyme change drastically upon binding of Na$^+$, with substrate binding and dissociation, as well as deacylation, occurring on a time scale which is 1 order of magnitude faster. The apparent association constants for Na$^+$ binding to the various intermediate forms of the enzyme have all been resolved from analysis of experimental data and are in the range of 50–100 M^{-1} at 25 °C. Studies conducted at different temperatures, in the range 15–35 °C, have revealed the enthalpic and entropic components of Na$^+$ binding to the enzyme. The results obtained from steady-state measurements are supported by independent measurements of the intrinsic fluorescence of the enzyme as a function of Na$^+$ concentration at a constant ionic strength $I = 0.2$ M, over the temperature range 15–35 °C. These measurements are indicative of a drastic conformational change of the enzyme upon Na$^+$ binding to a single site. The energetics of Na$^+$ binding derived from analysis of fluorescence measurements agree very well with those derived independently from steady-state determinations. It is proposed that thrombin exists in two conformations, slow and fast, and that the slow → fast transition is triggered by binding of a monovalent cation. The high specificity in thrombin activation found in the case of Na$^+$ is the result of its higher affinity compared to all other monovalent cations. The structural domain responsible for Na$^+$ binding controls the energetics of the catalytic pocket of the enzyme in an allosteric fashion and is distinct and seemingly independent from the fibrinogen recognition site.

Thrombin is a trypsin-like enzyme that has developed specificity toward a limited number of macromolecular substrates. Unlike trypsin and chymotrypsin, thrombin has an unusual richness of charged residues that occur in spatially well-defined clusters on its almost spherical surface (Bode et al., 1989, 1992). A patch of positive charges is present on a loop forming a deep canyon adjacent to the catalytic site. This patch is part of the fibrinogen recognition site (FRS), which is the structural domain responsible for thrombin interaction with many macromolecular components and most notably with its natural substrate and inhibitors such as hirudin (Fenton et al., 1988; Rydel et al., 1990). Another patch of positive charges is located almost diametrically to the FRS and is responsible for the interaction with heparin (Bode et al., 1992). The ability of thrombin to interact with negatively charged surfaces and effectors has long been recognized as one of the key aspects of functional and regulatory interactions. However, in recent years a number of important observations have been gathered on the role played by cationic effectors in controlling key functional aspects of the thrombin system. Orthner and Kosow (1980) were the first to show that human

α-thrombin is capable to discriminate among monovalent cations. They also suggested that Na$^+$ would significantly affect the conformation of the enzyme through specific binding interactions. An important role for Na$^+$ was subsequently implied by circular dichroism studies (Villanueva & Perret, 1983) and more extensive studies on amidase, esterase, and clotting activity of the enzyme (Landis et al., 1981). More recently, we have shown that specific ion effects are involved in practically all aspects of thrombin function, from the control of thrombin amidase activity toward synthetic substrates (Di Cera et al., 1991) to the modulation of thrombin interaction with the natural substrate fibrinogen (De Cristofaro & Di Cera, 1992) and the potent competitive inhibitor hirudin (De Cristofaro et al., 1992). The energetics observed in the presence of different salts are specific of the particular cation and anion present in solution. In particular, substrate binding to the catalytic pocket is sensitive to cations, while fibrinogen or hirudin bridge binding to the catalytic pocket and the FRS is sensitive to both cations and anions. Hence, the regulatory properties linked to cationic effectors seem to play an extremely important role in the complex network of interactions of the thrombin system. Indeed a region of quite strongly negative electrostatic potential is sandwiched between the FRS and the heparin-binding site (Bode et al., 1992) and is likely to provide the structural component for cation binding. All these functional and structural observations have set the stage for

the analysis of salt effects in the thrombin system in a more quantitative way.

In this paper, we begin our investigation of the basis of thrombin modulation by monovalent cations. Here we develop a strategy aimed at exploring specific ion effects by minimizing the components arising from nonspecific ionic strength effects due to Debye–Hückel screening and those arising from binding interactions of counterions such as chloride. The combination of theoretical, experimental, and computational approaches provides detail on the energetics of the interaction of monovalent cations with the enzyme under a variety of solution conditions.

MATERIALS AND METHODS

Thrombin Preparation. Human α-thrombin was prepared as described elsewhere (Fenton et al., 1977), and the γ derivative was produced by digestion of the native enzyme with trypsin (Bing et al., 1977). These thrombin forms were carefully purified by cation-exchange chromatography on Bio-Rex 70, using a Pharmacia-LKB XK16 column (1.6 × 40 cm) equilibrated at 4 °C in 0.15 M NaCl, 50 mM Tris,[1] pH 8.00. The γ derivative elutes shortly after the void volume, while α-thrombin elutes after the NaCl concentration is raised to 0.75 M. Purity of the samples was typically >99% as assessed by SDS–PAGE on 4–15% gradient gels. Thrombin concentration was measured using an extinction coefficient $E_{280} = 1.83$ mL/(mg·cm) and a molecular weight of 36 500 (Fenton et al., 1977). Thrombin solutions of 2–4 μM active site concentration were stored in 50-μL vials at –84 °C until use.

Steady-State Measurements. Steady-state measurements of thrombin amidase activity were made using the synthetic chromogenic peptides S-2238 (KabiVitrum, Stockholm, Sweden) and Spectrozyme-TH (American Diagnostica). All assays were performed by following the release of p-nitroaniline at 405 nm using a Cary 3 dual-beam spectrophotometer as described elsewhere (Di Cera et al., 1991). The buffer conditions were 5 mM Tris, 0.1% PEG, pH 8.0, in all cases. The active thrombin concentration was typically 1 nM. All solutions were made using bidistilled, deionized water purified by a Millipore MilliQ-Plus system and were titrated at the desired pH using an Orion Research EA940 expandable ion analyzer equipped with an automatic temperature compensation probe.

Data Analysis. Steady-state measurements of synthetic substrate hydrolysis were analyzed in terms of the widely accepted mechanism of action of serine proteases (Fersht, 1985)

$$E + S \underset{k_{-1}}{\overset{k_1^*}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} EP \overset{k_3}{\rightarrow} E + P' \qquad (1)$$
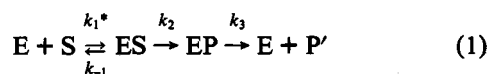
where P′ is the product of the reaction, S is the substrate, E is the free enzyme, ES and EP denote the enzyme–substrate and enzyme–product adducts, while k_1^*, k_{-1}, k_2, and k_3 are the kinetic constants for substrate binding, substrate dissociation, acylation, and deacylation, respectively. The deacylation rate also includes the rate of the product diffusing away from the enzyme. All rate constants in eq 1 are first order except k_1^*, which is second order. The steady-state velocity

of product formation, $v = d[P']/dt$, is given by the Michaelis–Menten equation

$$v = e_T \frac{k_{cat}[S]}{K_m + [S]} \qquad (2)$$

where e_T is thrombin concentration. The Michaelis–Menten parameters are functions of the individual rate constants as follows:

$$k_{cat} = \frac{k_3 k_2}{(k_3 + k_2)} \qquad (3a)$$

$$K_m = \frac{k_3(k_{-1} + k_2)}{k_1^*(k_3 + k_2)} \qquad (3b)$$

The individual kinetic rate constants involved in the scheme in eq 1 can be determined by steady-state measurements run in solutions at different viscosity. This experimental strategy has been applied successfully in other enzyme systems displaying a kinetic mechanism similar to that of serine proteases (Nakatani & Dunford, 1979; Brouwer & Kirsch, 1982; Kurz et al., 1987). It is also the most powerful technique available and is to be preferred to other solvent perturbation methods that usually alter the intrinsic properties of the system (Nakatani & Dunford, 1979). From Einstein's theory of Brownian motion it follows that viscosity must affect all steps involving diffusion, i.e., k_1^*, k_{-1}, and k_3 in eq 1. Any kinetic rate can be expressed as $k' = k/\eta_{rel}$ (Nakatani & Dunford, 1979), where η_{rel} is the viscosity of a given solution with added viscogenic agent relative to the reference solution with no viscogenic agent and k is the value of the rate constant in the reference solution. Straightforward application of the foregoing argument to the analysis of eq 1 yields the exact analytical expressions for the Michaelis–Menten parameters as a function of η_{rel} as

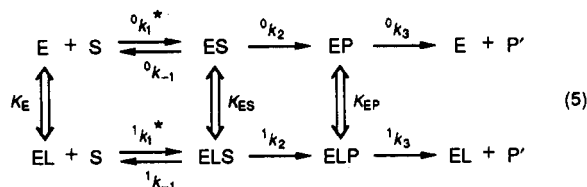$$k_{cat} = \frac{k_3 k_2}{(k_3 + k_2 \eta_{rel})} \qquad (4a)$$

$$K_m = \frac{k_3(k_{-1} + k_2 \eta_{rel})}{k_1^*(k_3 + k_2 \eta_{rel})} \qquad (4b)$$

Here all kinetic rates refer to the solution without viscogenic agent and k_2 is the only kinetic rate not affected by viscosity (see appendix). Studies conducted as a function of relative viscosity allow for solution of the kinetic scheme in eq 1 in terms of all of its components. Experimental data were collected in a matrix fashion (Di Cera et al., 1991; De Cristofaro & Di Cera, 1992) using seven different substrate concentrations, typically in the range 0.5–30 μM, and seven different sucrose concentrations, from 0 to 0.8 M scaled by a factor of 1.25, to change the relative viscosity of the solution. The matrix containing a total of 49 data points was then analyzed by means of eq 2, with K_m and k_{cat} expressed as in eqs 4, to obtain the best-fit values of the kinetic parameters k_1^*, k_{-1}, k_2, and k_3 under all conditions of interest. Minimization was carried out by nonlinear least-squares using the Marquardt method (Bard, 1974), and convergence to a unique minimum was obtained in all cases. Confidence intervals on the parameters were computed by F-testing at the cutoff of one standard deviation (68%).

Linkage Scheme for Na⁺ Binding. The differential effects of monovalent cations on thrombin amidase activity were studied by solving the kinetic scheme in eq 1 over the

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PEG, poly-(ethylene glycol); S-2238, H-D-Phe-pipecolyl-Arg-p-nitroanilide; Spectrozym-TH, H-D-hexahydrotyrosyl-Ala-Arg-p-nitroanilide; TEA, tetraethylammonium.

concentration range 0–0.2 M for each cation of interest. These studies were conducted at a constant ionic strength of 0.2 M, by diluting each salt with choline chloride or tetraethylammonium chloride. Choline and tetraethylammonium are bulky cations and were found to be inert toward thrombin, as reported for many other enzyme systems (Suelter, 1974). Typically 10 matrices of substrate and sucrose concentrations were measured in the presence of NaCl in the concentration range 0–0.2 M, at constant $I = 0.2$ M, using a scaling factor of 2. Under these conditions, the changes observed experimentally in the kinetic rates are due solely to changes in Na$^+$ concentration, since choline is an inert cation and all other thermodynamic variables such as pH, chloride concentration, ionic strength, and temperature are kept constant. The entire set of steady-state velocity measurements containing 490 data points was found to be consistent with the linkage scheme

$$\begin{array}{ccccccc}
E + S & \underset{^0k_{-1}}{\overset{^0k_1{}^*}{\rightleftharpoons}} & ES & \overset{^0k_2}{\longrightarrow} & EP & \overset{^0k_3}{\longrightarrow} & E + P' \\
K_E \Updownarrow & & \Updownarrow K_{ES} & & \Updownarrow K_{EP} & & \\
EL + S & \underset{^1k_{-1}}{\overset{^1k_1{}^*}{\rightleftharpoons}} & ELS & \overset{^1k_2}{\longrightarrow} & ELP & \overset{^1k_3}{\longrightarrow} & EL + P'
\end{array} \quad (5)$$

where L denotes Na$^+$, or equivalently a monovalent cation, while K_E, K_{ES}, and K_{EP} are the equilibrium constants for binding of the monovalent cation to E, ES, and EP. The reactions involving Na$^+$ binding and dissociation are much faster than those involving the synthetic substrate. The time scale for binding and dissociation of a monovalent cation is set by the rate-limiting processes of dehydration and hydration (Diebler et al., 1969). The binding of Na$^+$ occurs with a second-order rate constant of 10^8–10^9 M^{-1} s^{-1} and is practically diffusion-controlled (Diebler et al., 1969). Simple calculations using the values reported in Table IV show that almost all processes pertaining to the synthetic substrate in eq 5 occur on a time scale of milliseconds, while those reflecting Na$^+$ binding and dissociation occur on a time scale of microseconds. The wide separation of time scales allows one to consider the reactions relative to the monovalent cation in a regime of quasiequilibrium (Hill, 1977). Under these conditions, eq 5 contracts into

$$\{E\} + S \underset{k_{-1}}{\overset{k_1{}^*}{\rightleftharpoons}} \{ES\} \overset{k_2}{\rightarrow} \{EP\} \overset{k_3}{\rightarrow} \{E\} + P' \quad (6)$$
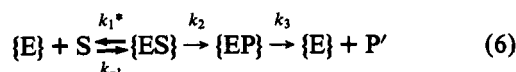
where $\{E\} = [E] + [EL]$, $\{ES\} = [ES] + [ELS]$, and $\{EP\} = [EP] + [ELP]$ are the manifolds of enzyme intermediates in the catalytic cycle. The kinetic constants in eq 6 are average values over each manifold, i.e.

$$k_1{}^* = \frac{^0k_1{}^* + {}^1k_1{}^* K_E[L]}{1 + K_E[L]} \quad (7a)$$

$$k_{-1} = \frac{^0k_{-1} + {}^1k_{-1} K_{ES}[L]}{1 + K_{ES}[L]} \quad (7b)$$

$$k_2 = \frac{^0k_2 + {}^1k_2 K_{ES}[L]}{1 + K_{ES}[L]} \quad (7c)$$

$$k_3 = \frac{^0k_3 + {}^1k_3 K_{EP}[L]}{1 + K_{EP}[L]} \quad (7d)$$

where [L] is the activity of the monovalent cation. Detailed

balancing applied to eq 6 also implies

$$^1k_{-1}\,{}^0K_{ES}\,{}^0k_1{}^* = {}^0k_{-1}\,{}^1K_E\,{}^1k_1{}^* \quad (8)$$

and introduces a constraint among the kinetic constants. The steady-state expression for v in eq 6 is again given by the Michaelis–Menten eq 2, with K_m and k_{cat} expressed as in eqs 4 and the kinetic constants given by eqs 7. Detailed balancing (eq 8) reduces the number of independent parameters to be resolved from analysis of experimental data from 11 to 10. The robustness of the best-fit solution was checked in all cases by extensive search in the parameter space starting with different estimates. A convergence to a unique solution was always found.

Fluorescence Studies. The intrinsic fluorescence of thrombin solutions was monitored as a function of Na$^+$ and K$^+$ concentration, at a constant ionic strength $I = 0.2$ M, using an SLM Aminco spectrofluorometer thermostated with a Lauda circulating water bath. The excitation and emission wavelengths were 280 and 340 nm, respectively. Thrombin solutions were carefully desalted using a Bio-Rad Econo-Pac 10DG column equilibrated with 5 mM Tris, 0.1% PEG, pH 8.00, at the desired temperature. Sample solutions were prepared by adding NaCl, KCl, or choline chloride to a final concentration of 0.2 M. The solution containing choline chloride was then titrated with aliquots of the solutions containing NaCl or KCl, and the change in intrinsic fluorescence was monitored as a function of Na$^+$ or K$^+$ concentration. At each step, a volume v_D was removed from the sample cuvette containing choline chloride and replaced by an equal volume of titrant solution containing the same concentration of thrombin. This procedure allows for measurements of titration curves at constant enzyme concentration and ionic strength. It is straightforward to prove that the concentration of titrant (Na$^+$, K$^+$), [M$^+$], at each step i is given by

$$[M^+]_i = [M^+]_0 D^i + [M^+]_\infty (1 - D^i) \quad (9)$$

where [M$^+$]$_0$ is the initial concentration of Na$^+$ or K$^+$ in the sample solution, [M$^+$]$_\infty$ = 0.2 M is the concentration of the titrating solution, and $D = (V - v_D)/V$ is the dilution factor defined in terms of the dilution volume v_D and the total volume of the sample $V = 2$ mL. The first 15 points of the titration curve were taken using a dilution volume of 20 μL. For these points $D = 0.99$ and [M$^+$]$_0$ = 0, since the sample cuvette contains initially no Na$^+$ or K$^+$. Starting from the sixteenth point, an additional ten points were taken with $v_D = 100$ μL. For these points $D = 0.95$ and [M$^+$]$_0$ = [M$^+$]$_{15}$ = 28 mM. Each data point was collected in triplicate. The change in intrinsic fluorescence, F, was found to follow the simple expression

$$F = \frac{F_0 + F_\infty K_E[L]}{1 + K_E[L]} \quad (10)$$

where [L] = [M$^+$], while F_0 and F_∞ are the asymptotic values of F. The independent parameters F_0, F_∞, and K_E in eq 10 were estimated by nonlinear least-squares using the Marquardt method as described in the analysis of steady-state measurements.

Control Experiments. A number of control experiments were run to check the reproducibility of our results and some critical steps in the experimental strategy used. Steady-state and fluorescence measurements were run using different preparations of human α-thrombin and were found to be extremely reproducible.

Table I: Best-Fit Values of the Kinetic Constants for Human α-Thrombin Amidase Activity toward S-2238 at pH 8.0 and 25 °C, in the Presence of 0.2 M Cl⁻ or Na⁺ Salts As Indicated

	k_1^* (μM^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Cl⁻ salts						
Li⁺	38 ± 8	173 ± 33	81 ± 5	263 ± 33	5.1 ± 0.4	62 ± 1
Na⁺	103 ± 28	280 ± 29	101 ± 10	315 ± 63	2.8 ± 0.3	77 ± 3
K⁺	82 ± 25	189 ± 67	64 ± 5	119 ± 12	2.0 ± 0.2	42 ± 1
Rb⁺	37 ± 12	99 ± 37	77 ± 8	293 ± 75	3.7 ± 0.4	61 ± 2
Cs⁺	37 ± 9	96 ± 27	77 ± 6	223 ± 34	3.5 ± 0.3	57 ± 2
choline	8 ± 2	26 ± 15	70 ± 12	34 ± 6	3.9 ± 0.6	23 ± 1
TEA	7 ± 1	27 ± 15	59 ± 6	39 ± 5	4.9 ± 0.6	23 ± 1
Na⁺ salts						
F⁻	74 ± 22	175 ± 55	109 ± 9	402 ± 77	3.0 ± 0.3	86 ± 3
Cl⁻	103 ± 28	280 ± 29	101 ± 10	315 ± 63	2.8 ± 0.3	77 ± 3
Br⁻	64 ± 25	199 ± 41	123 ± 15	314 ± 63	3.6 ± 0.4	88 ± 5
I⁻	76 ± 22	262 ± 36	123 ± 10	241 ± 26	3.3 ± 0.3	81 ± 2

The relative viscosity of the solutions used in this study was measured under all conditions of interest with an Ostwald viscometer thermostated with a Forma Scientific circulating water bath. The density of the solutions was measured with an Ohaus GA200D analytical balance. All viscosity measurements were taken in duplicate.

The extinction coefficient of *p*-nitroaniline was measured under all conditions of interest as a function of relative viscosity and temperature, using different salts at a constant ionic strength I = 0.2 M. The extinction coefficient was found to depend solely on ionic strength and relative viscosity, regardless of the particular salt used.

Other control experiments were run to test whether sucrose altered the intrinsic properties of the system by a specific solvent effects. In the absence of such effects, any equilibrium constant must be independent of η_{rel}, since this parameter affects equally the on and off rates for binding and dissociation (Nakatani & Dunford, 1979). Measurements of K_I for competitive inhibitors of thrombin activity, such as hirudin and *p*-aminobenzamidine, yielded the same value (within experimental error) in 0 M (η_{rel} = 1), 0.4 M (η_{rel} = 1.50) and 0.8 M sucrose (η_{rel} = 2.49). Experiments with hirudin were carried out at 37 °C and those with *p*-aminobenzamidine at 25 °C.

The reliability of our experimental strategy in resolving all rate constants in the kinetic scheme in eq 5 from analysis of data collected in a matrix fashion was thoroughly checked by extensive Monte Carlo simulations. Data were simulated in the form taken experimentally using seven values of substrate concentration, seven values of relative viscosity, and ten values of Na⁺ concentration. The steady-state velocity was computed according to the Michaelis–Menten equation with K_m and k_{cat} expressed as in eqs 4 and the catalytic rates given by eqs 7, with the detailed balancing condition in eq 8. A pseudorandom error of the same magnitude as that found experimentally was then added to the data, and the entire set of 490 data points was analyzed by nonlinear least squares. Typically, 10 000 simulations were run under each condition of interest. The average time for simulation and fitting of a single data set of 490 data points to resolve the 10 independent parameters of eq 5 was found to be 1 s on our Hewlett Packard Apollo9000/730 workstation. All parameters were resolved with good accuracy under all conditions of interest.

RESULTS

The results of solving the kinetic scheme for the hydrolysis of synthetic substrates by thrombin are summarized in Table I. The values of the kinetic rates are the best-fit estimates derived from analysis of a 7 × 7 matrix of steady-state measurements obtained as a function of substrate concentration and relative viscosity. The four kinetic constants

involved in eq 1 show a drastic dependence on the particular cation present in solution, and are maximal in the presence of Na⁺. The second-order rate constant k_1^* is about the same for Na⁺ and K⁺, decreases by a factor of 3 with Li⁺, Rb⁺, and Cs⁺, and is drastically reduced in the presence of the inert cations choline and tetraethylammonium. In the case of the kinetic constants k_2 and k_3, acylation is rate-limiting in the hydrolysis of S-2238 in the presence of Na⁺, K⁺, or any other monovalent cation of group I, but deacylation becomes rate-limiting in the presence of inert cations. The results obtained in the presence of Na⁺ are consistent with those obtained in previous studies of thrombin catalysis (Stone et al., 1991). It should also be noticed that the values of the kinetic rates in eq 3b obtained in the presence of Na⁺ are such to make K_m practically identical to $K_d = k_{-1}/k_1^*$, so that the substrate behaves as nonsticky as reported before (Di Cera et al., 1991) although its dissociation is not much faster than acylation. The particular anion present in solution does not seem to affect the activating properties of Na⁺, as also shown in Table I. The differences between the physiological cations Na⁺ and K⁺ can further be illustrated in a plot of K_m/k_{cat} and $1/k_{cat}$ versus the relative viscosity, as shown in Figures 1 and 2. The results obtained in the presence of the inert cation choline are also given for the sake of comparison. The results shown in Figures 1 and 2 are readily understood in terms of eqs 4 rewritten as

$$K_m/k_{cat} = \frac{(k_{-1} + k_2\eta_{rel})}{k_1^*k_2} = K_d/k_2 + \eta_{rel}/k_1^* \quad (11a)$$

$$1/k_{cat} = \frac{(k_3 + k_2\eta_{rel})}{k_3k_2} = 1/k_2 + \eta_{rel}/k_3 \quad (11b)$$

The slope in Figure 1 increases with decreasing k_1^* and is drastically different in the case of the inert cation. The slope in Figure 2 increases with decreasing k_3 and again is more pronounced in the case of the inert cation. The intercept is a measure of $1/k_2$ and is minimum in the presence of Na⁺. The data depicted in Figures 1 and 2 also provide unequivocal evidence that all steps but acylation in the catalytic mechanism in eq 1 are affected by diffusion, as shown in the appendix. These data, along with the results in Table I, draw attention to the fact that all steps of the catalytic cycle are sensitive to the particular cation present in solution. A strong preference is shown for Na⁺ which optimizes substrate binding, substrate dissociation, acylation, and deacylation. The presence of an inert cation significantly slows down all kinetic steps. Substrate binding and dissociation decrease by 1 order of magnitude, and the same applies to deacylation which becomes rate limiting. All these differences are observed at a constant ionic strength I = 0.2 M and therefore cannot be accounted
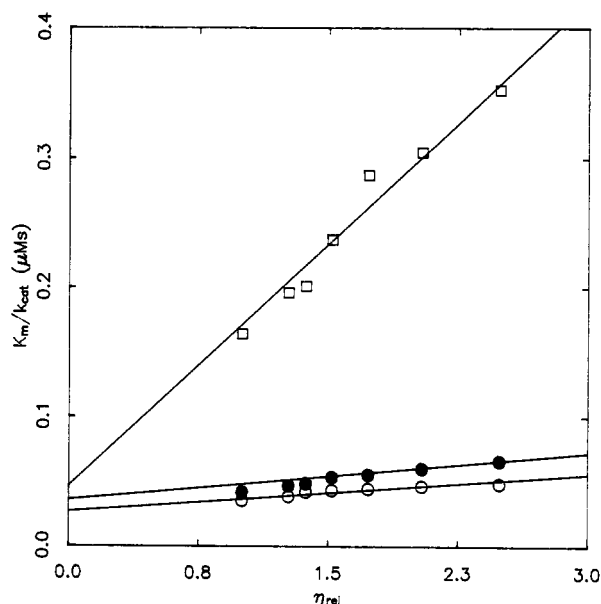
FIGURE 1: Effect of relative viscosity on K_m/k_{cat} for the hydrolysis of S-2238 by human α-thrombin. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and 0.2 M NaCl (O), KCl (●), or choline chloride (□). The curves were drawn from eq 11a in the text with the parameter values listed in Table I. Notice that the data points are the best-fit values of steady-state determinations (seven points at different substrate concentration) analyzed independently at each relative viscosity value, while the continuous lines depict the best fit to the entire 7 × 7 matrix of steady-state determinations as a function of substrate concentration and relative viscosity. The significance of these data in connection with the kinetic mechanism in eq 1 is further discussed in the appendix.
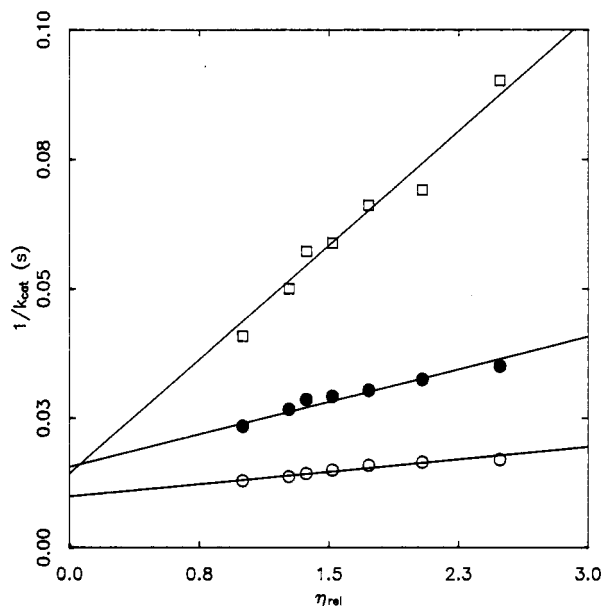

FIGURE 2: Effect of relative viscosity on $1/k_{cat}$ for the hydrolysis of S-2238 by human α-thrombin. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and 0.2 M NaCl (O), KCl (●), or choline chloride (□). The curves were drawn from eq 11b in the text with the parameter values listed in Table I. Notice that the data points are the best-fit values of steady-state determinations (seven points at different substrate concentration) analyzed independently at each relative viscosity value, while the continuous lines depict the best fit to the entire 7 × 7 matrix of steady-state determinations as a function of substrate concentration and relative viscosity. The significance of these data in connection with the kinetic mechanism in eq 1 is further discussed in the appendix.

for by nonspecific Debye–Hückel screening effects. Furthermore, the effects observed do not follow the lyotropic series $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ and therefore cannot be

accounted for by preferential hydration phenomena. The control of thrombin catalytic mechanism by monovalent cations is only consistent with a specific activation of the enzyme by Na^+.

The differences observed between Na^+ and K^+, and especially the drastic changes in the properties of the enzyme in the presence of inert cations, strongly support the idea that Na^+ may play a key role in the control of thrombin function by specifically binding to the enzyme. In the presence of inert cations such as choline and tetraethylammonium, thrombin assumes a conformation where the physical steps of substrate binding and dissociation are slow and so are the catalytic steps of acylation and deacylation. When Na^+ is added to the solution, a conformational transition is observed to a state where all rate constants are faster. This picture is simple enough to be explored along a number of functional coordinates. It is important to exclude beforehand that the differential effects of cations are due to interaction with the substrate rather than the enzyme. This point can be addressed by using either a different substrate or an experimental strategy where the properties of the enzyme are measured in the absence of substrate. The results of solving the kinetic scheme in eq 1 in the presence of a different amide substrate are summarized in Table II. In the case of Spectrozym-TH deacylation is found to be rate-limiting under all conditions, which provides an interesting case to be compared to S-2238, notwithstanding the structural similarity of the two substrates. Again, a preferential activation of the enzyme is observed in the presence of Na^+ and the value of the second-order rate constant k_1^* increases drastically in the presence of Na^+ as seen in the case of S-2238. Furthermore, all other kinetic rates significantly increase upon addition of Na^+, just as seen in the case of S-2238, thereby indicating that binding of Na^+ to the enzyme affects its conformation in a global way. From the data listed in Tables I and II it can be concluded that Na^+ interacts with the enzyme and not the substrate, in agreement with the results obtained with almost all other enzymes activated by monovalent cations (Evans & Sorger, 1966; Suelter, 1974). Additional and unequivocal evidence of a direct interaction of Na^+ with thrombin comes from measurements of the intrinsic fluorescence of the free enzyme (see below).

If Na^+ binds to the enzyme and alters its binding and catalytic properties toward the substrate, then it is extremely important to characterize the energetics of this interaction in quantitative terms. Measurements of substrate hydrolysis conducted as a function of relative viscosity were carried out over the Na^+ concentration range 0–0.2 M, in order to resolve the binding parameters relative to Na^+. The Na^+ concentration was changed by changing the concentration of NaCl at an ionic strength $I = 0.2$ M kept constant with choline chloride. This eliminates any complications arising from changes in the ionic strength or the chloride concentration, so that the effects observed can fully be ascribed to changes in the chemical potential of Na^+. The whole set of experimental data collected as a function of substrate (S-2238) concentration (7 values), relative viscosity (7 values), and Na^+ concentration (10–15 values) typically contains 500–700 data points. The entire set of data points was analyzed using the linkage scheme in eq 5, and the results are given in Table III. All parameters are satisfactorily resolved, which demonstrates the validity of the experimental strategy introduced in this study. The results are perfectly consistent with those listed in Table I. The binding of Na^+ triggers a conformational transition of the enzyme toward a state where substrate binding and dissociation are about 1 order of

Table II: Best-Fit Values of the Kinetic Constants for Human α-Thrombin Amidase Activity toward Spectrozym-TH at pH 8.0 and 25 °C, in the Presence of 0.2 M Chloride Salt As Indicated

	k_1^* ($\mu M^{-1} s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Na$^+$	95 ± 14	833 ± 123	253 ± 55	101 ± 12	3.3 ± 0.5	72 ± 6
K$^+$	63 ± 22	758 ± 69	179 ± 55	51 ± 3	3.3 ± 0.3	40 ± 2
choline	7 ± 2	7 ± 5	32 ± 12	13 ± 2	1.6 ± 0.3	9 ± 1

Table III: Best-Fit Values of the Parameters Involved in the Linkage Scheme in Equation 5 for the Amidase Activity of Human α- and γ-Thrombin toward S-2238 at pH 8.00, 25 °C, and $I = 0.2$ M Kept Constant with Choline Chloride[a]

	α-thrombin		γ-thrombin	
	Na$^+$	K$^+$	Na$^+$	K$^+$
$^0k_1^*$ ($\mu M^{-1} s^{-1}$)	8 ± 1	8 ± 1	18 ± 2	18 ± 2
$^1k_1^*$ ($\mu M^{-1} s^{-1}$)	122 ± 10	84 ± 7	73 ± 9	67 ± 3
$^0k_{-1}$ (s^{-1})	39 ± 2	41 ± 4	98 ± 15	88 ± 13
$^1k_{-1}$ (s^{-1})	263 ± 29	77 ± 7	275 ± 28	31 ± 4
$^0k_2$ (s^{-1})	62 ± 3	65 ± 4	31 ± 4	33 ± 2
$^1k_2$ (s^{-1})	94 ± 1	60 ± 1	126 ± 4	79 ± 2
$^0k_3$ (s^{-1})	26 ± 2	32 ± 1	51 ± 13	58 ± 15
$^1k_3$ (s^{-1})	541 ± 22	179 ± 7	603 ± 60	319 ± 30
K_E (M^{-1})	46 ± 3	8 ± 1	79 ± 9	3 ± 1
K_{ES} (M^{-1})	104 ± 6	45 ± 6	114 ± 12	32 ± 1
K_{EP} (M^{-1})	58 ± 4	17 ± 2	76 ± 18	3 ± 1
σ (s^{-1})	2.4	2.4	2.8	2.7

[a] Data refer to the chloride salt as indicated.

magnitude faster. Also, a drastic increase in the value of k_3 is observed upon Na$^+$ binding, whereby deacylation becomes faster than acylation for S-2238. The Na$^+$-induced transition between the two states occurs with different affinity depending upon which step of the catalytic cycle is taken into account. The Na$^+$ affinity of the free enzyme is about 50 M^{-1}. It increases upon substrate binding and decreases upon formation of the acyl intermediate. The association constants for Na$^+$ binding are in the same range as those found for Na$^+$ or K$^+$ binding to other enzyme systems (Evans & Sorger, 1966; Suelter, 1974). Analogous measurements were carried out in the presence of K$^+$ over the concentration range 0–0.2 M, at constant $I = 0.2$ M, and the results are also given in Table III. All rate constants reflecting the properties of the enzyme in the absence of monovalent cation (i.e., $^0k_1^*$, $^0k_{-1}$, $^0k_2$, and $^0k_3$) derived from studies with K$^+$ are identical, within experimental error, to those derived from studies with Na$^+$. This fact demonstrates the self-consistency of our results. The kinetic rates reflecting the properties of the K$^+$-bound enzyme are slightly different from the rate constants for the Na$^+$-bound enzyme. However, the most striking difference between Na$^+$ and K$^+$ is seen in the values of the association constants for cation binding that differ by a factor of about 2–6. This result indicates that both Na$^+$ and K$^+$ are capable of interacting with the enzyme, but they do so with different affinity. It seems as though there are two limiting conformations for thrombin, slow and fast, and that the transition between the two conformations is triggered allosterically by the binding of monovalent cations. Sodium is the most effective cation since it binds to the enzyme with the highest affinity. Higher K$^+$ concentrations are needed to induce the slow → fast transition of the enzyme, since K$^+$ has lower affinity than Na$^+$. Consequently, measurements conducted in the presence of 0.2 M Na$^+$ are expected to show a faster enzyme than those conducted at 0.2 M K$^+$, as it is indeed seen experimentally (see Table I). The allosteric picture outlined above is also consistent with the data obtained for the derivative γ thrombin, which is structurally perturbed at the level of the FRS. The results shown in Table III for this derivative indicate that the

mechanism of thrombin activation by Na$^+$ is not abolished or drastically affected by the structural perturbation of the FRS.

Additional support for the idea that Na$^+$ activates thrombin by specifically binding to the enzyme comes from studies conducted at different temperatures. The entire set of steady-state velocity measurements as function of substrate concentration, viscosity, and Na$^+$ concentration, at constant $I = 0.2$ M, was collected over the temperature range 15–35 °C. The best-fit values of the parameters involved in the linkage scheme in eq 5 are listed in Table IV. The parameters reflecting Na$^+$ binding to the various intermediates of the catalytic cycle show a significant dependence on temperature, as expected for a binding reaction involving dehydration (Diebler et al., 1969), with associated enthalpy changes on the order of –20 kcal/mol as summarized in Table V. The remaining parameters reflecting the catalytic properties of the enzyme and substrate binding and dissociation all increase with temperature. The relevant thermodynamic quantities based on Eyring's theory of transition state are summarized in Table V for all catalytic steps. For each step of the kinetic mechanism in eq 1, the activation enthalpies differ by about 8 kcal/mol between the two conformations (slow and fast) of the enzyme. The difference is particularly significant in the case of the rates of substrate binding and acylation and reveals the thermodynamic basis for the structural changes that must occur upon Na$^+$ binding to the enzyme.

The substantial amount of information provided by steady-state measurements of substrate hydrolysis strongly supports the hypothesis of a major conformational transition occurring upon Na$^+$ binding to the enzyme. Of particular importance is the observation of a drastic change in the value of k_1^* induced by Na$^+$ or K$^+$ binding, as shown in Figure 3. The change in the second-order rate constant depends solely on K_E and provides information on the interaction of Na$^+$ or K$^+$ with the free enzyme. Hence, any process linked to k_1^* reflects the energetics of the enzyme alone, independent of any substrate. Alternative measurements are necessary to check the predictions drawn from steady-state measurements and to verify that Na$^+$ binding to the enzyme indeed brings about a conformational transition. A powerful approach is provided by the study of the intrinsic fluorescence of the enzyme as a function of Na$^+$ or K$^+$ concentration, at constant $I = 0.2$ M. The intrinsic fluorescence of the enzyme increases significantly (>20%) with increasing concentrations of Na$^+$ or K$^+$. The results of the fluorescence measurements are shown in Figure 4 along with those derived from steady-state measurements. The fractional change in intrinsic fluorescence parallels the fractional change in k_1^* as a function of the concentration of monovalent cation. This indicates that the values of K_E obtained independently with the two experimental techniques are in very good agreement. In fact, from eq 7a one has that the fractional change in k_1^* is given by

$$\theta_k = \frac{k_1^* - {}^0k_1^*}{{}^1k_1^* - {}^0k_1^*} = \frac{K_E[L]}{1 + K_E[L]} \qquad (12)$$

Likewise, the fractional change in intrinsic fluorescence is

Table IV: Best-Fit Values of the Parameters Involved in the Linkage Scheme in Equation 5 for the Effect of Na⁺ on the Amidase Activity of Human α-Thrombin at pH 8.00, I = 0.2 M Kept Constant with Choline Chloride, and Temperature As Indicated

	15 °C	20 °C	25 °C	30 °C	35 °C
$^0k_1^*$ (μM^{-1} s^{-1})	5 ± 1	6 ± 1	8 ± 1	8 ± 1	9 ± 1
$^1k_1^*$ (μM^{-1} s^{-1})	33 ± 1	58 ± 5	122 ± 10	138 ± 21	153 ± 14
$^0k_{-1}$ (s^{-1})	9 ± 1	13 ± 4	39 ± 2	56 ± 7	62 ± 4
$^1k_{-1}$ (s^{-1})	46 ± 4	51 ± 6	263 ± 29	414 ± 48	527 ± 53
$^0k_2$ (s^{-1})	15 ± 1	36 ± 3	62 ± 3	150 ± 8	197 ± 7
$^1k_2$ (s^{-1})	77 ± 1	88 ± 3	94 ± 1	180 ± 6	284 ± 6
$^0k_3$ (s^{-1})	12 ± 2	14 ± 3	26 ± 2	167 ± 13	232 ± 7
$^1k_3$ (s^{-1})	140 ± 20	301 ± 23	541 ± 22	2290 ± 700	1030 ± 239
K_E (M^{-1})	146 ± 9	58 ± 6	46 ± 3	30 ± 3	8 ± 2
K_{ES} (M^{-1})	188 ± 6	143 ± 30	104 ± 6	70 ± 5	20 ± 3
K_{EP} (M^{-1})	91 ± 12	70 ± 5	58 ± 4	8 ± 3	7 ± 2
σ (s^{-1})	2.0	2.2	2.4	2.4	2.8

Table V

Values[a] of Kinetic Steps in the Linkage Scheme in Equation 5

kinetic rate	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/(mol·deg))
$^0k_1^*$	8.05 ± 0.12	5 ± 1	−12 ± 3
$^1k_1^*$	6.43 ± 0.08	13 ± 3	23 ± 9
$^0k_{-1}$	15.30 ± 0.05	18 ± 3	10 ± 8
$^1k_{-1}$	14.17 ± 0.11	24 ± 5	33 ± 16
$^0k_2$	15.03 ± 0.05	23 ± 2	26 ± 6
$^1k_2$	14.78 ± 0.01	11 ± 2	−12 ± 8
$^0k_3$	15.54 ± 0.08	29 ± 5	45 ± 19
$^1k_3$	13.74 ± 0.04	21 ± 5	24 ± 21

Values[b] of Na⁺ Binding to Thrombin

binding constant	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/(mol·deg))
K_E	−2.27 ± 0.06	−23 ± 4	−69 ± 13
K_{ES}	−2.76 ± 0.06	−18 ± 4	−52 ± 14
K_{EP}	−2.41 ± 0.07	−26 ± 5	−78 ± 21

[a] Values given are the transition-state free energy, enthalpy, and entropy changes associated to each of the kinetic steps involved in the linkage scheme in eq 5. [b] Values given are the standard free energy, enthalpy, and entropy changes associated with Na⁺ binding to thrombin, as derived from the linkage scheme in eq 5.

derived from eq 10 as

$$\theta_F = \frac{F - F_0}{F_\infty - F_0} = \frac{K_E[L]}{1 + K_E[L]} \qquad (13)$$

Equations 12 and 13 represent two equivalent ways of probing the interaction of monovalent cations with the free enzyme, and hence the same result should be expected from analysis of intrinsic fluorescence or steady-state data on k_1^*, as it is indeed seen in Figure 4. The self-consistency of these results supports the idea that the conformational transition monitored as a change in intrinsic fluorescence coincides with the slow → fast transition triggering the change in k_1^* observed experimentally upon Na⁺ or K⁺ binding. The fluoresence measurements also confirm the conclusion drawn independently from steady-state determinations regarding the number of Na⁺ binding sites. The data are perfectly consistent with the existence of a single binding site responsible for the effects observed experimentally and show no departure from the condition of rotational symmetry expected for ligand binding to a single site (Di Cera et al., 1992). Another important conclusion drawn from the data shown in Figure 4 is the difference in binding affinity seen between Na⁺ and K⁺, just as implied by steady-state measurements. This finding supports the idea that K⁺ is capable of inducing the same conformational transition as Na⁺ but requires higher concentrations. Fluorescence measurements were also carried out as a function of Na⁺ concentration at constant I = 0.2 M, over the temperature range 15–35 °C, under the same
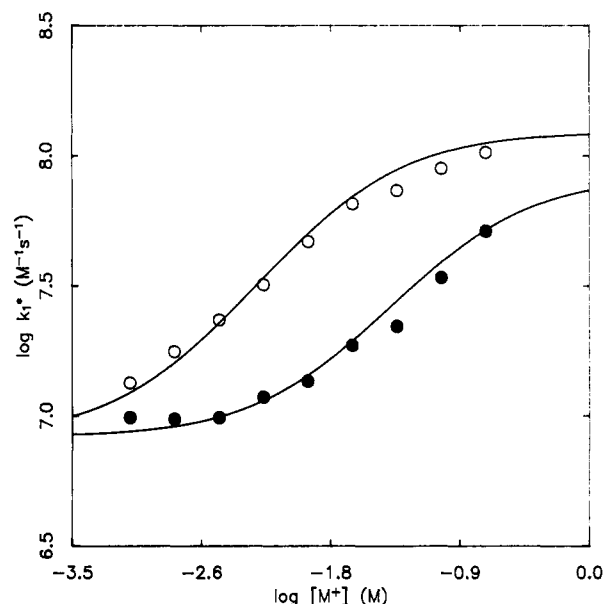

FIGURE 3: Effect of Na⁺ (O) and K⁺ (●) concentration on the second-order rate constant k_1^* for the interaction of human α-thrombin with S-2238. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The ionic strength was kept constant at 0.2 M with choline chloride. Notice how the data converge for [M⁺] → 0, as expected. The curves were drawn from eq 7a in the text with the parameter values listed in Table III, as derived from the global analysis of 490 data points obtained as a function of substrate concentration, relative viscosity, and Na⁺ or K⁺ concentration. Notice also that the data points are the best-fit values derived from analysis of a 7 × 7 matrix of steady-state determinations as a function of substrate concentration and relative viscosity obtained independently at each Na⁺ concentration, while the continuous lines depict the best fit to the entire 7 × 7 × 10 matrix of steady-state determinations as a function of substrate concentration, relative viscosity, and Na⁺ concentration.

conditions as those employed in steady-state measurements. The resulting titration curves are shown in Figure 5. Again, the transitions observed at all temperatures are consistent with the existence of a single Na⁺ binding site. The values of K_E obtained from fluorescence measurements as a function of temperature are shown by open circles in Figure 6 in the form of a van't Hoff plot. These values agree very well with those derived independently from steady-state measurements (filled circles) as summarized in Table IV.

DISCUSSION

Starting with the important discovery of the activating effect of K⁺ on pyruvate kinase (Boyer et al., 1942, 1943), more than 60 enzymes have been reported to be activated by monovalent cations (Evans & Sorger, 1966; Suelter, 1970, 1974). Almost all of them are activated by K⁺, and only a
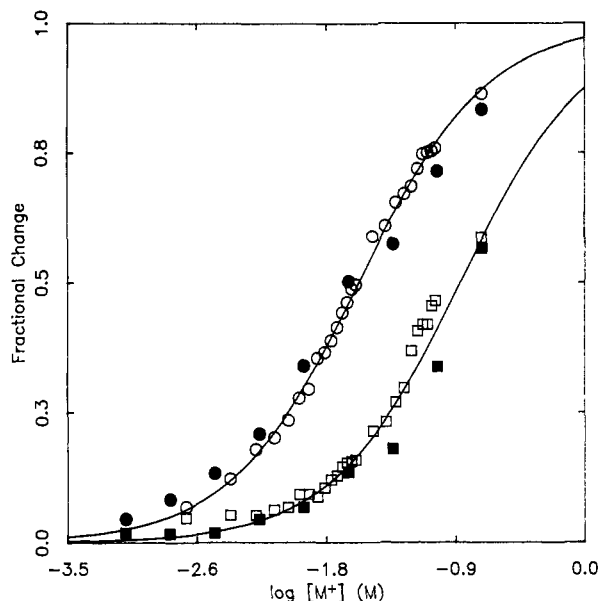
FIGURE 4: Fractional change in the intrinsic fluorescence of human α-thrombin (O, □) obtained as a function of Na^+ (O) or K^+ (□) concentration. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0, 25 °C, and 0.125 μM thrombin. The ionic strength was kept constant at 0.2 M with choline chloride. The data were first analyzed according to eq 10 in the text and then expressed as fractional change using eq 13. The curves were drawn according to eq 13 in the text with values of K_E equal to 36 ± 2 for Na^+ and 8 ± 1 for K^+. The fractional change in k_1^* (●, ■) obtained as a function of Na^+ (●) or K^+ (■) concentration under solution conditions identical to those of fluorescence studies is also shown for comparison. The data are the same as those shown in Figure 3 but are expressed in fractional form using eq 12 in the text with the parameter values listed in Table III.
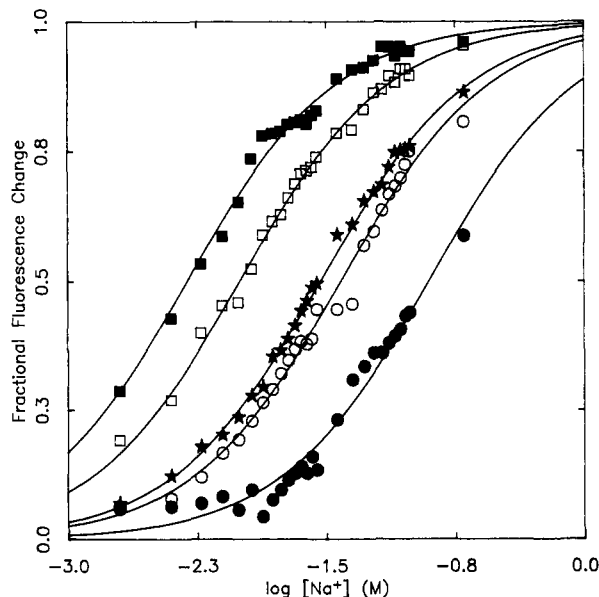

FIGURE 5: Fractional change in the intrinsic fluorescence of human α-thrombin obtained as a function of Na^+ concentration over the temperature range 15–35 °C. Experimental conditions are 5 mM Tris, 0.1% PEG, pH 8.0. The temperatures are 15 (■), 20 (□), 25 (★), 30 (O), and 25 °C (●). The thrombin concentration is 0.125 μM, except for the data at 30 and 35 °C, where 0.15 μM was used. The ionic strength was kept constant at 0.2 M with choline chloride. The data were first analyzed according to eq 10 in the text and then expressed as fractional change using eq 13. The curves were drawn according to eq 13 in the text, and the values of K_E are given in the van't Hoff plot shown in Figure 6.

few use Na^+ to optimize their efficiency. This important observation must reflect a fundamental mechanism of evo-
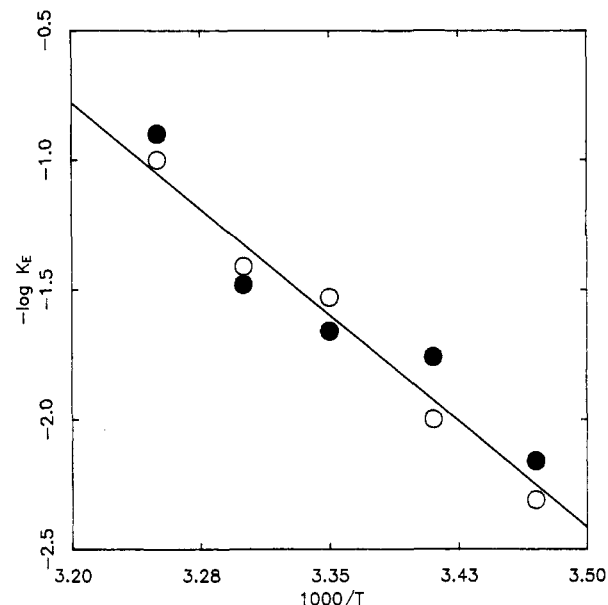

FIGURE 6: van't Hoff plot for the association constant K_E for Na^+ binding to the free form of human α-thrombin under experimental conditions of 5 mM Tris, 0.1% PEG, and pH 8.0 and a constant ionic strength $I = 0.2$ M. The data depict the best-fit values of $-\log K_E$ derived independently from fluorescence measurements (O) shown in Figure 5 and steady-state determinations (●) as summarized in Table IV. The continuous curve is the best fit to all data. The values of the enthalpic and entropic components of Na^+ binding to the free enzyme are $\Delta H = -25 \pm 2$ kcal/mol and $\Delta S = -76 \pm 7$ cal/(mol·deg) and are in very good agreement with the results drawn from steady-state measurements summarized in Table IV.

lutionary adaptation, for the majority of enzymes work inside the cell where K^+ is more concentrated than Na^+. In view of this fact, our finding that thrombin is a Na^+-activated enzyme is certainly quite unusual, but not surprising. All the physiologically important functions of this protease take place in extracellular environments such as the blood stream where Na^+ is more concentrated than K^+. Factor Xa, which is another enzyme involved in the coagulation cascade, is also activated by Na^+ (Orthner & Kosow, 1978), and so are oxalacetate decarboxylase (Sachan & Stern, 1971) and the E conformation of the Na^+/K^+ ATPase (Whittam & Wheeler, 1970) that both face extracellular environments. Since optimal catalytic efficiency has apparently been tailored to best fit environmental conditions, the analysis of regulatory interactions involving ubiquitous effectors such as small ions may play a fundamental role in our understanding of the physicochemical basis of catalytic optimization.

The exact mechanism by which enzymes discriminate between physiologically important cations such as Na^+ and K^+ is presently unknown. Eisenman (1961) postulated that the binding of a monovalent cation is set by the balance of two opposite forces. One is the attractive electrostatic force provided by negative charges or carbonyl dipoles on the surface of the protein, while the other is the hydration free energy that opposes the cation from being stripped of water molecules. Although very similar in many respects, monovalent cations differ substantially in their ability to interact with water molecules. Due to its higher charge density, the hydration free energy of Na^+ is about 20 kcal/mol larger than that of K^+. A weak electrostatic field on the surface of the enzyme will cause K^+ to dehydrate preferentially, so that this cation will be bound with higher affinity. On the other hand, a strong electrostatic field will dehydrate both cations significantly and will make Na^+ bind with higher affinity due to its smaller ionic radius (Eisenman, 1961). The "anionic field" hypothesis

is very attractive since it provides some clues as to the macromolecular components involved in the binding of monovalent cations. The strongly negative electrostatic field generated by the residues in the region containing the catalytic triad (Bode et al., 1992) might be responsible for the preferential binding of Na⁺. Additional support to this hypothesis comes from the observation that γ-thrombin, a proteolytic derivative of α-thrombin where the FRS is drastically perturbed, retains the activation by Na⁺ as seen for the native enzyme. This derivative shows no perturbation of the region giving rise to the strongly negative electrostatic potential.

Substantial support to the idea of a major conformational transition induced by Na⁺ binding comes from the results of intrinsic fluorescence measurements. Our observation is consistent with a number of previous spectroscopic findings obtained by titrating solutions of thrombin with different monovalent cations. Increasing concentrations of NaCl cause a positive ultraviolet difference spectrum of the enzyme indicative of a decreased polarity of the environment of tyrosine residues (Orthner & Kosow, 1980; Villanueva & Perret, 1983). Similar changes are implied by measurements of the circular dichroism of the enzyme as a function of NaCl concentration (Villanueva & Perret, 1983). These findings are supportive of our conclusions on the specific binding interaction of Na⁺ with the enzyme. However, previous spectroscopic measurements cannot be used to quantitatively assess the energetics of Na⁺ binding, since they have been collected by changing the NaCl concentration. In this case the effects observed can in principle be due not only to Na⁺ binding but also to chloride binding and nonspecific contributions arising from changes in the ionic strength of the solution. The possible complications arising from additional binding interactions due to counterions and nonspecific ionic strength effects have been eliminated in our studies by using an inert chloride salt at constant ionic strength. This feature has allowed us to quantitatively address the energetics of Na⁺ binding to the enzyme using both spectroscopic and kinetic methods. In addition to the unequivocal evidence of a major conformational transition of the enzyme induced by the binding of Na⁺, as proved by intrinsic fluorescence measurements, the results of our kinetic linkage studies provide the functional counterpart underlying the structural changes. All the rate constants involved in the kinetic scheme for the hydrolysis of amide substrates are affected by Na⁺ concentration. These changes are specific, since they occur at constant ionic strength. Furthermore, they are not due to changes in the hydration of the enzyme, since in that case they would be small, almost independent of temperature, and consistent with the lyotropic series Li > Na⁺ > K⁺ > Rb⁺ > Cs⁺ (von Hippel & Schleich, 1969). The changes observed are due to specific binding of Na⁺ to the enzyme. The effect on k_1* is particularly important since this parameter gives the rate for a productive collision between the substrate and the active site of the enzyme and provides information on the structure of the free enzyme in solution. The capture-window model provides an approximate value of the area on the protein surface leading to the active site. If R_s is the radius of the substrate and R_0 is the radius of access to the active site, the second-order rate constant k_1* is proportional to the difference $R_0 - R_s$ (Hill, 1975, 1976; Nakatani & Dunford, 1979). If it is reasonably assumed that the radius of the substrate is not affected by the presence of Na⁺, then a change in k_1* can only occur if the access to the active site has been affected. This change seems to provide a rationale for the significant structural transition observed

by spectroscopic techniques. The conformational change slow → fast must be such to drastically enhance the rate of substrate binding to the enzyme by widening the access to the catalytic pocket. Recent structural findings have pointed out the peculiar position of three tryptophan residues in the B chain, Trp60D, Trp215, and Trp148, that shape the access to the catalytic pocket (Bode et al., 1992). It is quite tempting to speculate that the slow → fast transition might be linked to the displacement of one or more of these residues with a resulting widening of the access to the catalytic pocket. The displacement would be responsible for the change in intrinsic fluorescence measured experimentally, as well as for the substantial increase in k_1*. The substantial mobility of the Trp148 loop can be brought out in this correction. According to this molecular picture, the entire kinetic mechanism of the enzyme would be controlled by the physical steps of diffusion from and to the protein moiety. Access to the catalytic pocket opens up when Na⁺ is bound and remains in a wide open configuration during all steps of catalysis. As a result of this, substrate binding occurs on a faster time scale, and so do substrate dissociation and deacylation, which involve escape from the active site to the solution. In the slow conformation, all these steps are significantly slowed down with a resulting decrease in the catalytic efficiency of the enzyme.

Although further structural and functional investigation is necessary to support the molecular picture outlined above, the existence of substantial conformational transitions triggered by Na⁺ binding to the enzyme is now well established. The slow and fast conformations of thrombin are likely to play an important role in vivo. In fact, although the dissociation constant for Na⁺ is about 30 mM at 25 °C, it increases significantly with temperature and exceeds 100 mM at 37 °C, thereby approaching the physiological concentration of Na⁺ in the blood.

ACKNOWLEDGMENT

APPENDIX

In this appendix we show that the data depicted in Figures 1 and 2 provide unequivocal evidence that all steps but acylation in the kinetic mechanism in eq 1 are affected by diffusion. Since the physical steps of substrate binding and dissociation are certainly affected by diffusion, it remains to be established whether it is possible to uniquely solve the effect on k_2 and k_3 from measurements of K_m and k_{cat} as a function of η_{rel}. There are four different cases to be considered.

Case 1: k_2 and k_3 Are Not Affected by Diffusion. In this case the expression for K_m/k_{cat} and $1/k_{cat}$ as a function of η_{rel} are

$$K_m/k_{cat} = K_d/k_2 + \eta_{rel}/k_1* \qquad (A1a)$$

$$1/k_{cat} = 1/k_2 + 1/k_3 \qquad (A1b)$$

Although the expression for K_m/k_{cat} is in agreement with the data shown in Figure 1, a plot of $1/k_{cat}$ versus η_{rel} is expected to yield a straight line with zero slope, contrary to what is seen in Figure 2.

Case 2: k_2 and k_3 Are Affected by Diffusion. In this case, the expressions for K_m/k_{cat} and $1/k_{cat}$ as a function of η_{rel} are

$$K_m/k_{cat} = (k_d/k_2 + 1/k_1{}^*)\eta_{rel} \qquad (A2a)$$

$$1/k_{cat} = (1/k_2 + 1/k_3)\eta_{rel} \qquad (A2b)$$

Both expressions predict a linear dependence of K_m/k_{cat} and $1/k_{cat}$ on η_{rel}. However, both K_m/k_{cat} and $1/k_{cat}$ should vanish as $\delta_{rel} \rightarrow 0$, contrary to what is seen in Figures 1 and 2.

Case 3: k_2 Is Affected by Diffusion and k_3 Is Not. In this case, the expressions for K_m/k_{cat} and $1/k_{cat}$ as a function of η_{rel} are

$$K_m/k_{cat} = (K_d/k_2 + 1/k_1{}^*)\eta_{rel} \qquad (A3a)$$

$$1/k_{cat} = 1/k_3 + \eta_{rel}/k_2 \qquad (A3b)$$

Although the expression for $1/k_{cat}$ may be consistent with the data shown in Figure 2, the expression for k_m/k_{cat} predicts a linear relationship with η_{rel} with an intercept of zero, contrary to what is seen in Figure 1.

Case 4: k_3 Is Affected by Diffusion and k_2 Is Not. In this case, the expressions for K_m/k_{cat} and $1/k_{cat}$ as a function of η_{rel} coincide with eqs 4, i.e.

$$K_m/k_{cat} = K_d/k_2 + \eta_{rel}/k_1{}^* \qquad (A4a)$$

$$1/k_{cat} = 1/k_2 + \eta_{rel}/k_3 \qquad (A4b)$$

Both expressions predict a linear relationship with η_{rel} with a nonvanishing intercept for $\eta_{rel} \rightarrow 0$, as indeed found experimentally for the data shown in Figures 1 and 2.

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