The kinetic pathway for the Na\(^+\)-induced slow \(\rightarrow\) fast transition of thrombin was characterized. The slow form was shown to consist of two conformers in a 3:1 ratio \((E_{S2}:E_{S1})\) at 5°C, pH 7.4, 1/2 0.3. \(E_{S2}\) binds Na\(^+\) 3 orders of magnitude faster than does \(E_{S1}\). The small molecule active site-directed inhibitor L-371,912, and the exosite I binding ligand hirugen, like Na\(^+\), bind selectively to \(E_{S2}\) and induce the slow \(\rightarrow\) fast conversion of thrombin. The slow \(\rightarrow\) fast transition is limited by the rate of conversion of \(E_{S1}\) to \(E_{S2}\) (k=28 s\(^{-1}\) at 5°C). Replacement of Arg-221a or Lys-224 at the Na\(^+\) binding site with Ala appears to selectively alter the slow form and reduce the apparent affinity of the mutants for Na\(^+\) and L-371,912. This replacement, however, has little effect on the affinity for the inhibitor in the presence of saturating concentrations of Na\(^+\). The kinetically linked ligand binding at the Na\(^+\) binding site, exosite I, and the active site of thrombin characterized in the present study indicates the basis for the plasticity of this important enzyme, and suggests the possibility that the substrate specificity and, therefore, the procoagulant and anticoagulant activities of thrombin may be subject to allosteric regulation by as yet unidentified physiologically important effectors.

The serine protease thrombin plays a central role in the blood coagulation cascade (1, 2). Thrombin catalyzes the conversion of fibrinogen to fibrin monomer and activates factor XIII to factor XIIIa, which in turn cross-links fibrin, thereby mechanically and chemically stabilizing the fibrin blood clot (3). Thrombin also activates factors XI, V, and VIII, important components of the thrombin-generating blood coagulation cascade. In addition, thrombin activates platelets (4). Activated platelets aggregate to form a platelet plug that, together with the fibrin clot, provides hemostasis. Activated platelets also provide the surface for assembly of components of the blood coagulation cascade (5). The ability of thrombin to catalyze the formation of several components involved in its generation is responsible for the explosive formation of thrombin when the blood coagulation cascade is triggered by vascular injury-induced exposure of tissue factor. Thrombin also down-regulates its own formation. Upon binding to endothelial cell membrane-associated thrombomodulin, the specificity of thrombin is altered. Essentially devoid of activity toward fibrinogen, thrombomodulin-bound thrombin efficiently catalyzes the activation of protein C, which in conjunction with activated protein S inactivates factors Va and VIIIa, thereby shutting down the thrombin-generating blood coagulation cascade (6, 7).

Early studies have shown that Na\(^+\) and certain other monovalent ions induce a conformational change in thrombin and alter the substrate specificity of the enzyme (8, 9). Wells and Di Cera (10) were the first to show that thrombin is an allosteric enzyme existing in two forms, designated as slow and fast form thrombin, and that these forms interconvert upon binding of Na\(^+\) to a specific site of the enzyme. The slow \(\rightarrow\) fast transition is also accompanied by a significant increase in the intrinsic fluorescence of the enzyme (10–11). X-ray diffraction analysis of thrombin indicates that Na\(^+\) is coordinated octahedrally by 4 molecules of water and the amide oxygen atoms of Arg-221a and Lys-224 (12–14). Mutation of Arg-221a and Lys-224 to alanine diminishes the ability of thrombin to bind Na\(^+\) (15). The importance of the allosteric regulation mediated by Na\(^+\) is that the Na\(^+\)-bound fast form cleaves fibrinogen with higher specificity, whereas the slow form shows a higher catalytic specificity in the activation of protein C both in the presence and absence of thrombomodulin (16). The existence of two interconvertible conformers provides a means for expanding the substrate specificity of thrombin and regulating the enzyme via interactions with allosteric effectors.

Although Na\(^+\)-free thrombin has been designated as the slow form and Na\(^+\)-bound thrombin has been designated as the fast form, the equilibrium distribution of the two interconvertible thrombin conformers in the Na\(^+\)-free and Na\(^+\)-bound states has not been evaluated. Additionally, the reaction pathway for the slow \(\rightarrow\) fast transition has not been established. Elucidation of this pathway is crucial for understanding the mechanism of allosteric regulation of thrombin as a paradigm for serine protease specificity in general. The present study of the interaction of thrombin with Na\(^+\), the small active site-directed ligand L-371,912 (17), and the exosite I binding ligand hirugen (18) was initiated to address these issues and further characterize the effects of Na\(^+\) on the environment at the active site and exosite I.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise specified, all chemicals were purchased from Sigma, Z-Gly-Pro-Arg-7-amino-4-trifluoromethylcoumarin (Z-GPR-AFC) \(^{1}\) was obtained from Enzyme Systems Products. L-371,912 (21) and hirugen (Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr (SO3)-Leu) were prepared by Dr. Terry A. Lyle and Dr. Victor M. Garsky, respectively, of the Medicinal Chemistry Department of Merck

\(^{1}\) The abbreviations used are: Z-GPR-AFC, Z-Gly-Pro-Arg-7-amino-4-trifluoromethylcoumarin; 15-crown-5, 1,4,7,10,13-pentaoxaacyclopenta-decaneter.

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FIG. 1. Fluorescence titration for the binding of Na⁺ to thrombin at 5 °C, pH 7.4, t/2 0.3 (maintained by addition of ChCl), 500 nm thrombin, F₂ - F₁ is the fluorescence of a solution containing the plotted concentration of Na⁺ minus the fluorescence of a solution containing no Na⁺ (in ChCl). Saturation with Na⁺ increased the intrinsic fluorescence of thrombin by ~10%. The solid line is the best least squares fit to Equation 7 with K = 5 m M.

Research Laboratories. Human α-thrombin (in 200 mM NaCl and 50 mM citrate buffer, pH 6.5) was obtained from Enzyme Research Laboratories, Inc. and used without further purification. R221A and K224A thrombin variants were prepared as described previously (15).

Methods—Thrombin concentrations were determined from absorbance measurements (ε₂₈₅ = 1.83 ml/(mg·cm)) and a molecular weight of 36,500 (23). Active site titration indicated that the thrombin was ~95% active (19). Solutions of the slow form were prepared by replacing sodium citrate buffer with Tris or Heps buffer (50 mM, pH 7.4 at 5 °C) containing 300 mM choline chloride (ChCl) by centrifugation using a Centrifor 10 (Amicon Inc.). Thrombin solutions 50–70 μM were stored in 20-μl aliquots at ~70 °C. Unless otherwise specified, studies were performed at 5 °C, pH 7.4, in 50 mM Tris (or 50 mM Heps) buffer containing 0.1% poly(ethylene glycol) 8000 and the indicated concentrations of NaCl and ChCl. Ionic strength was maintained by addition of ChCl. Several rate constants were determined in both Tris and Heps buffers. Rate constants determined in the Tris buffer were indistinguishable from those determined in Heps buffer. The kinetics of the Na⁺ to thrombin were monitored using an Applied Photo- physics SX17 stopped-flow fluorimeter (excitation 285 nm, emission 299 nm). Rate constants for the approach of the fluorescence to its final value or to a final linear decay due to photobleaching were evaluated by fitting the data to Equation 1 using a Kaleidagraph 3.0 least squares fitting routine.

\[ F_f = F_i + (F_f - F_i)e^{(-k_{obs}t)} - kt \] (Eq. 1)

In Equation 1, i, f, and t denote values at the end of fast phase, at the end of slow phase, and at time t, respectively, and k is the apparent rate constant for photobleaching. Similar methods were used to study the kinetics of association of Rb⁺ and K⁺ with thrombin and the dissociation of L-371,912 and hirugen with the slow form of wild-type and mutant thrombins.

The kinetics of dissociation of Na⁺ from the thrombin-Na⁺ complex were monitored by an Applied Photophysics SX17 stopped-flow fluorimeter (excitation 285 nm, emission 299 nm) using an asymmetric mixing method. Dissociation of Na⁺ was initiated by a 1:6 dilution of the solution containing thrombin (3 μM) and Na⁺ (100 mM or 30 mM) with 300 mM ChCl solution while maintaining the ionic strength at 0.3. Dissociation of Na⁺ (5 mM) from the thrombin-Na⁺ complex in the presence of the crown ether, 1,4,7,10,13-pentaaoxacyclopentadecanether, 15-crown-5 (25 μM), in ChCl was conducted by the dilution procedure described above. Rate constants for the approach of the fluorescence to its final value or to a final linear decay due to photobleaching were evaluated from fits of the data to Equation 1, using a Kaleidagraph 3.0 least squares fitting routine.

The full time course of Na⁺ or L-371,912 binding to thrombin was simulated using Runge-Kutta digital integration methods, and rate constants for the approach of the fluorescence to its final value were evaluated from fits of the data to Equation 2 using a Kaleidagraph 3.0 least squares fitting routine.

FIG. 2. Biphasic binding of Na⁺ to thrombin at 5 °C, pH 7.4, t/2 0.3, 5 mM Na⁺, 500 mM thrombin as monitored by stopped-flow fluorimetry. F₁ and F₂ are defined in the text. The upper trace was obtained by mixing a Na⁺-containing solution (t/2 0.3) with a Na⁺ free thrombin solution (t/2 0.3). The lower trace was obtained by mixing a ChCl solution with the thrombin solution used to obtain the upper trace.

\[ F_f = F_i + (F_f - F_i)e^{(-k_{obs}t)} \] (Eq. 2)

Inhibition constants for L-371,912 were determined from studies of the dependence of the initial velocity for the release of AFC from the substrate on the inhibitor concentration as described previously (20, 21) at 5 °C, t/2 0.3, pH 7.4. The final concentrations were as follows: thrombin or thrombin mutants (10 mM), Z-GPR-AFC (12 μM or 100 μM), and inhibitor (0.1–60 μM). Since the inhibitor concentration is in large excess of the total enzyme concentration, the dependence of the initial rate of substrate hydrolysis (V₁) on the inhibitor concentration is described by Equation 3, where V₀ and Kₐpp denote the initial rate of substrate hydrolysis in the absence of inhibitor and apparent inhibition constant, respectively.

\[ V_0/V_1 = 1 + [I]/K_{app} \] (Eq. 3)

The inhibition constant (Kᵢ) is related to Kₐpp by Equation 4.

\[ K_i = K_{app}/(1 + [S]/K_a) \] (Eq. 4)

The value of the observed pseudo first order rate constants (kobs) for the binding of L-371,912 to thrombin was derived from nonlinear regression fits of progress curves to Equation 5 as described by Morrison and Walsh (22).

\[ F_f = F_i + (F_f - F_i)e^{(-k_{obs}t)} \] (Eq. 5)

where F₀, F₁, V₀, and V₁ represent the initial fluorescence, the fluorescence at time t, and the initial and final rate of fluorescence change, respectively. The dependence of the first order rate constant (kobs) on the inhibitor concentration is depicted in Equation 6.

\[ k_{obs} = k_{-1} + k_1[I]/(1 + [S]/K_a) \] (Eq. 6)

The association rate constant (k₁) was determined from the slope of a linear plot of kobs versus [I] at constant substrate concentration.

The total change in the fluorescence of thrombin (0.5 μM) upon binding to Na⁺ was monitored as a function of the Na⁺ concentration at 5 °C, t/2 0.3, pH 7.4. Static fluorescence measurements were performed on an SLM8000 fluorimeter (excitation 285 nm, emission 335 nm) using a thermostatted cell that was flushed with nitrogen and maintained at 5 °C with a circulating water bath. Each measurement was made after thermal equilibration. Thermal equilibration was confirmed by measurement of the temperature of the solution in the cuvette using a thermocouple.

RESULTS

Fig. 1 depicts the dependence of the difference in thrombin fluorescence (arbitrary units) in the presence of Na⁺ (F₁) and absence (F₀) of Na⁺ on the Na⁺ concentration at 5 °C. A value of 5.0 mM for the apparent dissociation constant Kᵢ was obtained from fits of the dependence of the Na⁺-induced change in fluorescence (ΔF) on the Na⁺ concentration ([Na⁺]) to the absorption isotherm defined by Equation 7 where ΔF is the
A stopped-flow fluorescence study of the association of Na$^+$ to E$_{S1}$ at 5 °C with [Na$^+$] = 5 mM is shown in Fig. 2. Inspection of Fig. 2 reveals that the binding of Na$^+$ to thrombin in a rapid reversible fashion is a biphasic process with a fast phase that occurs within the dead time of the stopped-flow fluorimeter and with a slow phase defined by a pseudo first order rate constant ($k_{obs}$) of 32 s$^{-1}$. Such biphasic binding of a ligand to a protein has usually been interpreted in terms of one of two limiting kinetic pathways analogous to those depicted in Scheme 1, A and B (23). In Scheme 1A, Na$^+$ binds to thrombin in a rapid reversible fashion to produce an initial thrombin-Na$^+$ complex (E$_S$Na$^+$) with an increased fluorescence followed by a slow conformational change to produce a second thrombin-Na$^+$ (E$_F$Na$^+$) complex. This scheme predicts that the rate constant for the slow phase should increase to a limiting value ($k_1 + k_-2$) as the Na$^+$ concentration is increased (Equation 8).

$$k_{obs} = \frac{k_f[Na^+]}{K_s + [Na^+]} + k_-2$$

(Eq. 8)

In Scheme 1B, Na$^+$ rapidly binds to the thrombin conformer E$_{S2}$ to produce an E$_F$Na$^+$ complex concomitant with a fluorescence increase. This process perturbs the equilibrium between free E$_{S2}$ and E$_{S1}$. In this pathway, the slow phase reflects conversion of E$_{S1}$ to E$_{S2}$. If Scheme 1B were operating, the limiting value of the Na$^+$-induced fluorescence change and $K$ is equivalent to $K_s$ (Fig. 1).

$$A = \frac{\Delta F}{\Delta F_f[Na^+]}$$

(Eq. 7)

A stopped-flow fluorescence study of the association of Na$^+$ with thrombin at 5 °C with [Na$^+$] = 5 mM is shown in Fig. 2. Inspection of Fig. 2 reveals that the binding of Na$^+$ to thrombin is a biphasic process with a fast phase that occurs within the dead time of the stopped-flow fluorimeter and with a slow phase defined by a pseudo first order rate constant ($k_{obs}$) of 32 s$^{-1}$. Such biphonic binding of a ligand to a protein has usually been interpreted in terms of one of two limiting kinetic pathways analogous to those depicted in Scheme 1, A and B (23). In Scheme 1A, Na$^+$ binds to thrombin in a rapid reversible fashion to produce an initial thrombin-Na$^+$ complex (E$_S$Na$^+$) with an increased fluorescence followed by a slow conformational change to produce a second thrombin-Na$^+$ (E$_F$Na$^+$) complex. This scheme predicts that the rate constant for the slow phase should increase to a limiting value ($k_1 + k_-2$) as the Na$^+$ concentration is increased (Equation 8).

$$k_{obs} = \frac{k_f[Na^+]}{K_s + [Na^+]} + k_-2$$

(Eq. 8)

In Scheme 1B, Na$^+$ rapidly binds to the thrombin conformer E$_{S2}$ to produce an E$_F$Na$^+$ complex concomitant with a fluorescence increase. This process perturbs the equilibrium between free E$_{S2}$ and E$_{S1}$. In this pathway, the slow phase reflects conversion of E$_{S1}$ to E$_{S2}$. If Scheme 1B were operating, the
FIG. 4. Fit of the time dependence of the fluorescence upon mixing thrombin (500 mM) with Na\(^+\) at 5 °C, pH 7.4, \(1/2\) 0.3. Trace a, no Na\(^+\); trace b, 5 mM Na\(^+\); trace c, 17 mM Na\(^+\); trace d, 150 mM Na\(^+\). Open circles represent observed fluorescence values. Filled circles represent the best fit of the data obtained by Runge-Kutta integration of Equation 22a–22d using values of \(k_1 = 28\) s\(^{-1}\), \(k_2 = 8.8\) s\(^{-1}\), \(k_3 = 1.2 \times 10^5\) M\(^{-1}\) s\(^{-1}\), \(k_4 = 456\) s\(^{-1}\), \(k_5 = 40\) M\(^{-1}\) s\(^{-1}\), and \(k_6 = 0.053\) s\(^{-1}\) and Equations 15–18.

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Analyzing the dependence of the amplitude of the fast phase on the Na\(^+\) concentration (at [Na\(^+\)]<150 mM) in terms of the absorption isotherm defined by Equation 7. A value of 3.8 mM was obtained from the fit of the data in Fig. 3 to Equation 7, where \(K\) is equivalent to \(k_2\) of Scheme 2. Analysis of the sum of the amplitudes of the fast phase \((F' - F)\) and slow phase \((F - F')\) in terms of Equation 7 yields a value of \(K = 5.0\) mM, which as expected is equivalent to the dissociation constant determined from the static fluorescence measurements. Equations 10–14 relate the equilibrium constant \(K_d\) to the equilibrium constants that define the processes depicted in Scheme 2.

\[
K_d = ([E_o] + [E_{o2}][Na^+]/[E_F \cdot Na^+])
\]  
(Eq. 10)

\[
K_{-2} = [E_{o2}][Na^+]/[E_F \cdot Na^+]
\]  
(Eq. 11)

\[
K_{-3} = [E_{o1}][Na^+]/[E_F \cdot Na^+]
\]  
(Eq. 12)

\[
K_0 = K_{-2} + K_{-3}
\]  
(Eq. 13)

\[
K_1 = K_{-2}/K_0 = K_{-3}/(K_0 - K_{-2})
\]  
(Eq. 14)

Thus, the experimentally determined values of \(K_d\) and \(K_{-2}\) of 5.0 mM and 3.8 mM yield values of 1.2 mM and 3.2 for \(K_{-3}\) and \(K_1\). As the Na\(^+\) concentration is increased, \(k_2[Na^+]\) becomes comparable to \(k_1\), formation of \(E_F[Na^+]\) directly from \(E_{o1}\) becomes important. Equations 15–18 relate fluorescence values observed in the stopped-flow fluorimeter to the concentration of the species in Scheme 2, where the subscripts \(o, i, f\), and \(t\) denote values in the absence of Na\(^+\), at the end of the fast phase, at the end of the slow phase, and at time \(t\) after mixing, respectively, and \(e\) represents fluorescence response factors for the subscripted species.

\[
F_o = e_{o}E_{o1} + e_{o2}E_{o2}
\]  
(Eq. 15)

\[
F_i = e_{i}E_{i1} + e_{i2}E_{i2} + e_{i3}[E_F Na^+]\]

(Eq. 16)

\[
F_t = e_{t}E_{t1} + e_{t2}E_{t2} + e_{t3}[E_F Na^+]\]

(Eq. 17)

\[
F_f = e_{f}E_{f1} + e_{f2}E_{f2} + e_{f3}[E_F Na^+]\]

(Eq. 18)

The observed values of \(F_o, F_i, F_t\), and \(F_f\) in experiments with 150 mM [Na\(^+\)] and 0.5 \(\mu\)M thrombin, and Equations 19–21 were used to calculate values for the fluorescence response factors \(e_{Eo1}, e_{Eo2}, e_{Ei1}, e_{Ei2}, e_{Ei3}, e_{Et1}, e_{Et2}, e_{Et3}, e_{Ef1}, e_{Ef2}, e_{Ef3}\) of 11.4 \(\mu\)M\(^{-1}\), 14.5 \(\mu\)M\(^{-1}\), and 15.9 \(\mu\)M\(^{-1}\), respectively.  

\[
e_{Eo1} = \frac{F_k[K_3 + [Na^+]] - K_{-1}F_o}{[Na^+][E_k]}
\]  
(Eq. 19)

\[
e_{Eo2} = \frac{e_{Eo1}[E_F]K_0}{[Na^+][E_k]}\]

(Eq. 20)

\[
e_{Ef3} = \frac{e_{Ef2}[E_F]K_{-2}}{[Na^+][E_k]}\]

(Eq. 21)

\[\text{Fluorescence response factors are in arbitrary units that are a function of signal amplification. The posted values, however, reflect the relative molar fluorescence responses of each species. (At [Na\(^+\)] = 150 mM, the process defined by \(k_2\) is negligible so that at the end of the fast phase only \(E_{o1}\) has been converted to \(E_F Na^+)\).}\]
E_{S2} + Na^+ \xrightarrow{k_2/k_2} E_{S2}Na^+ \xrightarrow{k_4} E_FNa^+ \\
k_1 \xrightarrow{k_1} E_S1 + Na^+ \xrightarrow{k_3/k_3} E_{S1}Na^+

**Scheme 3.**

\[ e_{E_0} = \frac{F_0 - F_{E_0}}{K_f + 1}[E_0] \]  

(Eq. 21)

Runge-Kutta digital integration of the set of differential equations (Equations 22a–22d) for Scheme 2, with \( k_1 = 28 \text{ s}^{-1}, k_2 = 8.8 \text{ s}^{-1}, k_2 = 1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}, k_3 = 456 \text{ M}^{-1} \text{ s}^{-1}, k_4 = 40 \text{ M}^{-1} \text{ s}^{-1}, k_5 = 0.053 \text{ s}^{-1}, \) yielded the time dependence of [\( E_{S1} \)], [\( E_{S2} \)], and [\( E_FNa^+ \)], which, when substituted into Equation 18, provided the best fit for the time-dependent changes in fluorescence observed upon mixing Na\(^+\) with thrombin in the stopped-flow spectrometer (Fig. 4). Additionally, the apparent first order rate constants for the approach of the calculated fluorescence to its final value agreed with the corresponding first order rate constants obtained from the observed time dependence of fluorescence over the entire range of Na\(^+\) concentrations studied (Table I). Primes in Equations 22a–22d denote the derivative of the designated concentrations with respect to time.

\[ [E_{E_0}] = -([E_{S2}][Na^+]k_2 + [E_FNa^+]k_2 - [E_{S1}]k_1 - [E_{E_0}]k_1) \]  

(Eq. 22a)

\[ [E_{E_0}] = -([E_{S2}][Na^+]k_2 + [E_FNa^+]k_2 - [E_{S1}]k_1 - [E_{E_0}]k_1) \]  

(Eq. 22b)

\[ [E_{E_0}] = -([E_{S2}][Na^+]k_2 + [E_FNa^+]k_2 - [E_{S1}]k_1 - [E_{E_0}]k_1) \]  

(Eq. 22c)

\[ [Na^+] = -[E_{E_0}][Na^+] \]  

(Eq. 22d)

Consistent with Scheme 2, digital integration of Equations 22a–22d using the rate constants listed above accounts for the observed time dependence of fluorescence in experiments where the thrombin-Na\(^+\) complex is dissociated by dilution with Na\(^+\)-free buffer (Fig. 5). Fig. 6 depicts the effect of diluting the thrombin-Na\(^+\) complex with Na\(^+\)-free buffer containing a crown ether (15-crown-5) that sequesters free Na\(^+\) and thereby promotes further dissociation of Na\(^+\) from thrombin. Additionally, the alkali metal ions Rb\(^+\) and K\(^+\) that bind to thrombin and result in the slow → fast conversion do so with a limiting rate constant of 33 \text{ s}^{-1} (data not shown). This rate constant is close to the value for the rate constant for conversion of \( E_{S1} \) to \( E_{S2} \) observed in studies of Na\(^+\) binding to thrombin, as expected for the pathway depicted in Scheme 2.

Although Scheme 2 depicts the simplest kinetic pathway for the binding of Na\(^+\) to thrombin that accounts for our experimental observations, Na\(^+\) probably binds to thrombin via a more complex pathway involving formation of Na\(^+\)-complexes of \( E_{S1} \) and \( E_{S2} \) that undergo conformational rearrangements to \( E_FNa^+ \) (Scheme 3). Rapid rates of conversion of \( E_{S2}Na^+ \) and \( E_{S2}Na^+ \) to \( E_FNa^+ \), or the failure to saturate a weak \( E_{S1}Na^+ \) interaction for our inability to detect \( E_{S1}Na^+ \) and \( E_{S2}Na^+ \).

At 150 mM Na\(^+\) (37 °C), ~50% of the thrombin contains no bound Na\(^+\) (9). The Na\(^+\)-free thrombin (slow form thrombin) appears predominantly \( E_{S2} \) as judged by the ratio of the amplitudes between fast and slow phases (−7:1), assuming that the relative fluorescence response factors for the different forms of thrombin are independent of temperature. Pseudo first order rate constants of 184 ± 6 \text{ s}^{-1} and 463 ± 20 \text{ s}^{-1} were determined for the binding of 300 and 700 mM Na\(^+\) to thrombin at 25 °C and 37 °C, respectively. The observation of biphasic Na\(^+\) binding at 25 °C and 37 °C similar to that observed at 5 °C, suggests that the Na\(^+\)-induced slow to fast transition is not qualitatively altered in the temperature range 5–37 °C. Consistent with this view, we found no evidence for temperature-induced changes in thrombin structure in the temperature range (5–37 °C), as judged by the temperature dependence of affinity for the active site-directed inhibitor and catalytic activity (data not shown).

Recent studies have shown that the mutants R221Aa and R221aA (panel B) as monitored by stopped-flow fluorimetry. Trace a was obtained in an experiment wherein a Na\(^+\)-containing solution (I/2 0.3) was mixed with a Na\(^+\)-free thrombin solution (I/2 0.3). Trace b obtained in an experiment wherein a solution of ChCl (I/2 0.3) was mixed with the thrombin solution used to obtain trace a.  

**FIG. 7.** Binding of Na\(^+\) to thrombin mutants at 5 °C, pH 7.4, I/2 0.3, 150 mM Na\(^+\), 500 mM K224A (panel A) and R221aA (panel B) as monitored by stopped-flow fluorimetry.
The effect of Na<sup>+</sup> on affinity and rate of binding of the active site-directed inhibitor L-371,912 to thrombin was a biphasic process, as in the case of the binding of Na<sup>+</sup> to thrombin. The failure to observe a biphasic reaction at low concentrations of L-371,912 (1 μM) and 150 mM NaCl, 5 °C, suggests that Scheme 3 is operative with K224A, but no fluorescence change is associated with the transition of E<sub>S2</sub> to E<sub>F</sub>·Na<sup>+</sup> with the K224A mutant. This conclusion implies that the integrity of the Lys-224–Glu-217 ion pair is important for the fluorescence change induced by Na<sup>+</sup> binding (15).

To determine how the binding of Na<sup>+</sup> to thrombin might allosterically affect the environment at the active site, we studied the effect of Na<sup>+</sup> on affinity and rate of binding of the active site-directed inhibitor L-371,912 (Structure 1). Equilibrium and rate constants for the interaction of L-371,912 with thrombin are listed in Table II. These thermodynamic and kinetic parameters were determined from studies of the time dependent inhibition of thrombin catalyzed hydrolysis of Z-GPR-AFC by L-371,912 (Figs. 8–10). Inhibition constants were deduced from the dependence of the limiting velocity on inhibitor concentration (obtained from plots such as those illustrated in Fig. 8). Pseudo first order rate constants for the binding of the active site-directed inhibitor to thrombin were obtained from an analysis of the time dependent approach of the velocity to its final value using previously described methods (20, 21). The slopes of linear plots of pseudo first order rate constants versus inhibitor concentration (Fig. 10) yielded the second order rate constants listed in Table II for the binding of L-371,912 to thrombin. In each case, the inhibitor appears to bind the fast form tighter and more rapidly than the slow form. Interestingly, although the inhibitor binds the fast form of wild-type and mutant thrombins with similar affinities and rate constants, the inhibitor shows a >10-fold reduction in affinity for the slow form of the K224A and R221A thrombin mutants.

Like the binding of Na<sup>+</sup> to the slow form, the binding of inhibitor to the slow form is associated with a significant increase in fluorescence. Upon binding of L-371,912 to the fast form, however, no significant change in fluorescence is observed (data not shown). This observation suggests that, upon binding to thrombin, L-371,912 induces a conformational change similar to that induced by Na<sup>+</sup>. The change in intrinsic protein fluorescence accompanying the binding of L-371,912 to thrombin allowed us to study the binding of this inhibitor at higher inhibitor concentrations and thereby enabled us to determine whether L-371,912, like Na<sup>+</sup>, binds selectively to E<sub>S2</sub>

### Table II: Values of \( K_b \) and \( k_{on} \) for the binding of L-371,912 to slow and fast form thrombin at 5 °C, pH 7.4

|        | Fast form \( K_b \) (\( M \)) | Slow form \( K_b \) (\( M \)) | \( k_{on} \) (\( 10^6 M^{-1} s^{-1} \)) | \( k_{off} \) (\( 10^{-3} s^{-1} \)) |
|--------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|
| Wild-type | 5.1                           | 23.9                          | 1.3                             | 0.17                            |
| K224A   | 2.7                           | 319                           | 118.1                           | 0.052                           |
| R221A   | 9.3                           | 380                           | 40.9                            | 0.12                            |

\( K_b \), the equilibrium constant for dissociation of L-371,912 from the thrombin-Na<sup>+</sup>L-371,912 ternary complex, was calculated using the relationship \( K_b = K_{obs} - K_a \cdot [Na<sup>+</sup>] \), where \( K_{obs} \) is the apparent equilibrium constant for dissociation of L-371,912 from thrombin at the Na<sup>+</sup> concentration (300 mM) used for the measurement, \( K_a \) is the equilibrium constant for dissociation of Na<sup>+</sup> from thrombin and \( K_{obs} \) is the equilibrium constant for dissociation of L-371,912 from thrombin in the absence of Na<sup>+</sup>.
obtained from the slope of the plot (Fig. 11, inset) yields a value of 0.09 mM$^{-1}$ s$^{-1}$, which was as expected similar to the value obtained for this rate constant (0.1 mM$^{-1}$ s$^{-1}$) by monitoring the time dependent effect of L-371,912 on substrate hydrolysis (Fig. 10, Table II). Fig. 11 illustrates the fit of the observed data to Scheme 4. The open circles plot rate constants for the first order approach of the observed fluorescence to its final value. The filled circles plot rate constants obtained by simulating the time dependence of fluorescence accompanying the binding of L-371,912 to thrombin using the kinetic parameters stipulated in Scheme 4 and determining the apparent first order rate constant for the approach of the simulated time dependence of fluorescence to its final value.

To assess the effect of the slow → fast transition of thrombin on the environment of exosite I, we investigated the effect of Na$^+$ on the binding of hirugen to exosite I of thrombin. Fluor-
Recognition that thrombin undergoes a conformational change upon binding Na\(^{+}\) resulted in the designation of Na\(^{+}\)-free thrombin as slow form thrombin and Na\(^{+}\)-bound thrombin as fast form thrombin. The kinetic pathway (Scheme 3) for the binding of Na\(^{+}\) to thrombin delineated in the present study indicates that slow form thrombin is an equilibrium mixture of two conformers with the more abundant conformer \(E_{S2}\) binding Na\(^{+}\) faster than the less abundant one. Fast form thrombin appears to be an equilibrium mixture of three thrombin conformers. All observations regarding the alterations in the properties of thrombin induced by the binding of Na\(^{+}\) are consistent with \(E_{P}\) being the predominant species and conformationally distinct from \(E_{S1}\), Na\(^{+}\) and \(E_{S2}\), Na\(^{+}\).

It is important to note that the Ala substitution at Lys-221a resulted in the designation of Na\(^{+}\)-free thrombin as slow form thrombin L-371,912 and in the presence of saturating Na\(^{+}\), whereas in the Na\(^{+}\)-free state these substitutions reduced the affinity of thrombin for L-371,912 by ~15-fold. This observation is consistent with the view that the Ala substitutions selectively perturb the slow form. The observation that binding of hirugen to thrombin exosite I, like the binding of Na\(^{+}\) to the alkali metal binding site and the binding of L-371,912 to the active site, suggests the slow fast transition, indicates that the equilibrium between slow and fast forms can be altered by interactions at three structurally distinct domains of thrombin. The occurrence of concerted alterations in the allosteric sites of the enzyme reported here suggests the possible existence of physiologically important allosteric effectors of thrombin that selectively stabilize the slow or fast form, and thereby regulate the anticoagulant and procoagulant activities of the enzyme.

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