Thrombin–Fibrinogen Interaction: pH Dependence and Effects of the Slow→Fast Transition†

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ABSTRACT: A recently developed strategy capable of measuring the equilibrium dissociation constant for thrombin–fibrinogen interaction has been used to explore the pH dependence of the interaction and the effects of thrombin conformational transitions. The dependence of fibrinogen binding to thrombin in the pH range 6–10 is bell-shaped and remarkably similar to that obtained in the case of the small synthetic amide substrate tosyl-Gly-Pro-Arg-*p*-nitroanilide-AcOH. Since the synthetic substrate contains no groups that can ionize in the pH range 6–10, the bell-shaped curve must reflect ionization reactions of two groups of the enzyme with $pK_1 = 7.53 \pm 0.09$ and $pK_2 = 8.80 \pm 0.09$. These groups can be identified as the catalytic histidine, His57, and the amino terminus of the B chain, Ile16, respectively. Deprotonation of His57 in the acidic region is important for optimal binding, while protonation of Ile16 in the alkaline region is critical for the formation of a salt bridge with Asp194, which guarantees the conformational stability of the enzyme. The loss of binding free energy at low (<7.0) and high (>9.0) pH values is linked to protonation of His57 and deprotonation of Ile16, respectively. The first 51 residues of the Aα chain of fibrinogen are known to be necessary and sufficient for optimal recognition by thrombin, but none of them contributes to the pH dependence of fibrinogen binding in the pH range examined. Hence, the two possible ionizable groups of the Aα chain, i.e., the amino terminus Ala1 and His24, make no contacts with the thrombin surface. This result is consistent with crystallographic analysis of thrombin bound to fibrinopeptide A and supports a recently proposed structural model for the interaction of the first 51 residues of the Aα chain with thrombin. The slow (Na^+-free) and fast (Na^+-bound) forms of thrombin, that differ in their catalytic competence toward small synthetic amide substrates, have also widely different properties in the recognition of fibrinogen. The slow form binds fibrinogen with an affinity nearly 20 times smaller than that of the fast form. It follows from linkage principles that fibrinogen binding to thrombin must stabilize the fast form and that the slow→fast transition plays a key role in molecular recognition by thrombin. Analysis of the kinetic mechanism of hydrolysis of fibrinogen to yield fibrin I monomer shows that deacylation becomes rate-limiting in the slow form, as seen for small synthetic amide substrates. Clotting of fibrinogen is also drastically affected, with the slow form showing reduced clotting activity. Under conditions of physiological relevance (pH 7.4 and 37 °C), no clotting activity is observed in the presence of the slow form over a time scale during which clotting in the presence of the fast form is complete.

Fibrinogen recognition by thrombin is dependent upon binding to a structural domain of the enzyme distinct from the catalytic pocket (CP).[1] Concerted binding, or bridge binding (Fenton, 1981; Berliner et al., 1985; Fenton et al., 1988), to the fibrinogen recognition site (FRS) and the CP allows thrombin to effectively bind and cleave the natural substrate and to trigger the formation of fibrin monomers, the building blocks of an intricate three-dimensional web of insoluble polymers constituting the fibrin clot. In a series of elegant studies, Scheraga and Blombäck have shown that the first 51 residues of the Aα chain of fibrinogen are necessary for recognition and optimal catalysis (Hageman & Scheraga, 1974; Hogg & Blombäck, 1978). Recent structural studies (Stubbs et al., 1992; Martin et al., 1992) have revealed the spatial arrangements of the portion 7–16 of fibrinopeptide A bound to thrombin and provided a starting point for modeling the interaction of the entire portion of the Aα chain binding to S′ subsites and residues of the FRS. Clearly, the availability of quantitative measurements of thrombin–fibrinogen interaction in solution is now critical for assessing the validity of conclusions arising from recent structural studies. A strategy recently developed in our laboratory has made it possible to measure the equilibrium dissociation constant for thrombin–fibrinogen interaction for the first time (Hopfner & Di Cera, 1992) and explore the fundamental differences in the energetics of fibrinogen and hirudin binding to thrombin (Hopfner et al., 1993). Our strategy has opened the way to a systematic approach to the interaction of fibrinogen with thrombin under conditions where these components are unperturbed by chemical modifications or synthetic inhibitors aimed at interfering with catalytic or polymerization processes. Measurements of the $^F K_m$ for thrombin–fibrinogen interaction as a function of relative viscosity yield the equilibrium dissociation constant and can also identify the rate-limiting step in the conversion of fibrinogen to fibrin I. Neither one of these basic aspects of the interaction can be elucidated by standard experimental methods based on the analysis of clotting curves

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[1] Abbreviations: Ac, acetyl; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Chromozym-TH, tosyl-Gly-Pro-Arg-*p*-nitroanilide-AcOH; CP, catalytic pocket; DAMPA, N^{α}-dansyl-L-Arg-4-methylpiperidine amide; FRS, fibrinogen recognition site; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-2238, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide; Tris, tris-(hydroxymethyl)aminomethane.

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or the release of fibrinopeptides (Martinelli & Scheraga, 1980; Higgins et al., 1983; Mihalyi, 1988; Naski & Shafer, 1990; Mihalyi et al., 1991). In this study, we apply our recently developed experimental strategy to explore the pH dependence of fibrinogen binding to thrombin. The aim of our studies is to identify the groups that control the pH dependence of binding and compare the results obtained for fibrinogen with those recently reported for synthetic substrates (Di Cera et al., 1991; Stone et al., 1991) and hirudin (Betz et al., 1992). Our measurements also provide a much needed functional analysis of important aspects of the interaction that have emerged from recent crystallographic investigation (Stubbs et al., 1992; Martin et al., 1992). In addition, we analyze the effects of thrombin conformational transitions on fibrinogen binding, and specifically we explore the linkage between fibrinogen binding and the Na^+-induced slow→fast transition of the enzyme (Wells & Di Cera, 1992). We prove that fibrinogen binding to thrombin stabilizes the fast (Na^+-bound) conformation of the enzyme and that the slow→fast transition is a key step of molecular recognition by thrombin.

MATERIALS AND METHODS

Human α-thrombin and human fibrinogen were purified and tested for purity and activity as described in detail elsewhere (Di Cera et al., 1991; Hopfner & Di Cera, 1992). Steady-state measurements of amidase activity were made using the synthetic chromogenic peptides S-2238 (KabiVitrum, Stockholm, Sweden) and Chromozym-TH (Sigma, St. Louis, MO). Thrombin–fibrinogen interaction was studied in terms of the competition with synthetic substrate hydrolysis by thrombin and as a function of relative viscosity (Hopfner & Di Cera, 1992). Assays were performed under solution conditions of 5 mM Tris, 5 mM BisTris, 10 mM CHES, 0.2 M NaCl, 0.1% PEG 8000, and 25 °C, in the pH range 6–10. Fibrinogen was used at concentrations from 3 to 10 μM, depending upon pH. The triple buffer guarantees buffering over the entire pH range and constant ionic strength (Ellis & Morrison, 1982). The relatively high concentration of NaCl was used to stabilize the fast form of thrombin (Wells & Di Cera, 1992). Other assays were carried out under solution conditions of 5 mM Tris, 0.1% PEG 8000, pH 8.0 at 25 °C, and 0.2 M NaCl or choline chloride, in order to study the effect of the slow→fast transition on fibrinogen binding. The inert monovalent cation tetramethylammonium was also used in alternative to choline for analysis of the slow form. Studies carried out in the presence of choline or tetramethylammonium yielded identical results. Fibrinogen was used at concentrations of 2–3 μM in 0.2 M NaCl and 8–10 μM in 0.2 M choline or tetramethylammonium chloride. The viscosity of the solution was changed by addition of sucrose to the buffer and measured under all conditions of interest as described previously (Hopfner & Di Cera, 1992; Wells & Di Cera, 1992). Clotting curves were measured and analyzed as detailed elsewhere (De Cristofaro & Di Cera, 1991). Reproducibility of the results was checked with different thrombin and fibrinogen preparations.

Competition experiments of synthetic substrate hydrolysis in the presence of fibrinogen can be analyzed in terms of a kinetic scheme where the free enzyme, E, interacts with either the synthetic substrate, S, or fibrinogen, F, as follows

$$E + S \underset{{}^{S}k_{-1}}{\overset{{}^{S}k_1^*}{\rightleftarrows}} ES \xrightarrow{{}^{S}k_2} ES' \xrightarrow{{}^{S}k_3} E + P \quad (1a)$$

$$E + F \underset{{}^{F}k_{-1}}{\overset{{}^{F}k_1^*}{\rightleftarrows}} EF \xrightarrow{{}^{F}k_2} EF' \xrightarrow{{}^{F}k_3} E + P' \quad (1b)$$

where P and P′ are the products of the two reactions and k_2 and k_3 are the acylation and deacylation rates, respectively, while k_1^* and k_{-1} are the rate constants for binding and dissociation, respectively. In the case of thrombin–fibrinogen interaction, the product P′ refers to fibrinopeptide A and fibrin I monomer. In fact, the release of fibrinopeptide A precedes that of fibrinopeptide B (Higgins et al., 1983; Mihalyi, 1988), and the latter reaction is negligible over the time scale (15–30 s) of our initial velocity measurements. The steady-state velocity of product formation in the first reaction, $v = d[P]/dt$, is given by

$$v = e_T \frac{{}^{S}k_{cat}[S]}{{}^{S}K_m\{1 + [F]/{}^{F}K_m\} + [S]} \quad (2)$$

where e_T is thrombin concentration, ${}^{S}k_{cat} = {}^{S}k_3{}^{S}k_2/({}^{S}k_3 + {}^{S}k_2)$, ${}^{S}K_m = {}^{S}k_3({}^{S}k_{-1} + {}^{S}k_2)/[{}^{S}k_1^*({}^{S}k_3 + {}^{S}k_2)]$, and ${}^{F}K_m = {}^{F}k_3({}^{F}k_{-1} + {}^{F}k_2)/[{}^{F}k_1^*({}^{F}k_3 + {}^{F}k_2)]$. The Michaelis–Menten parameters can be obtained from analysis of measurements of v collected in a matrix of [S] and [F] values. The equilibrium dissociation constant, ${}^{F}K_d$, for thrombin–fibrinogen interaction can be derived from the values of ${}^{F}K_m$ obtained as a function of the relative viscosity, η_{rel}, as detailed elsewhere (Hopfner & Di Cera, 1992). The relevant expression is

$${}^{F}K_m = {}^{F}K_d \frac{1 + \alpha\eta_{rel}}{1 + \beta\eta_{rel}} \quad (3)$$

where ${}^{F}K_d = {}^{F}k_{-1}/{}^{F}k_1^*$, $\alpha = {}^{F}k_2/{}^{F}k_{-1}$, and $\beta = {}^{F}k_2/{}^{F}k_3$. Measurements of ${}^{F}K_m$ as a function of η_{rel} yield the value of ${}^{F}K_d$ as the extrapolation of ${}^{F}K_m$ for $\eta_{rel} \to 0$. These measurements also yield information on the parameters α and β in eq 3 and hence the rate-limiting steps in the catalytic conversion of fibrinogen.

RESULTS

The pH dependence of ${}^{F}K_d$ is shown in Figure 1. A bell-shaped curve peaked around pH 8 is observed, with two branches showing slightly different but constant slope. This indicates that binding of fibrinogen is controlled by two ionizable groups, with the value of ${}^{F}K_d$ given by

$${}^{F}K_d = {}^{F}K_d^{opt}(1 + [H]/K_1 + K_2/[H]) \quad (4)$$

where ${}^{F}K_d^{opt}$ is the optimized (pH independent) value of ${}^{F}K_d$, while K_1 and K_2 are the acid dissociation constants of the two groups. Equation 4 is typically used when dealing with bell-shaped profiles such as the one in Figure 1, that arise when ionizable groups change their pK_a values by more than 1 unit upon binding (Cleland, 1977). In the case of thrombin–fibrinogen interaction, it indicates that the complex is formed only when one of the groups is protonated and the other is deprotonated. The values of $pK_1 = 7.53 \pm 0.09$ and $pK_2 = 8.80 \pm 0.09$ are very similar to the values of $pK_1 = 7.56 \pm 0.06$ and $pK_2 = 8.96 \pm 0.08$ obtained for the binding of DAMPA (Stone et al., 1991), a synthetic inhibitor of thrombin which only binds to the CP (Bode et al., 1990). The pH dependence of Chromozym-TH binding to thrombin is also given in Figure 1. Again, a bell-shaped curve is observed with

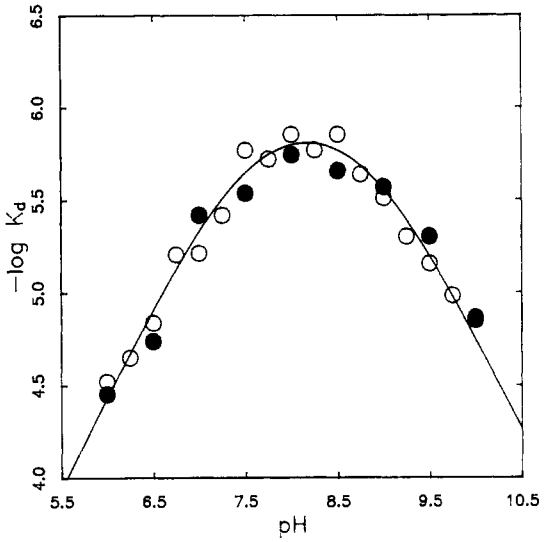

FIGURE 1: pH dependence of the equilibrium dissociation constant, K_d, for thrombin interaction with fibrinogen (○) and Chromozym-TH (●), under conditions of 1 nM human α-thrombin, 5 mM BisTris, 5 mM Tris, 10 mM CHES, 0.1% PEG, and 0.2 M NaCl, 25 °C. The continuous line is the best-fit of the fibrinogen data points according to eq 4 in the text, using the parameter values ${}^{F}K_d^{opt} = 1.1 \pm 0.2$ μM, $pK_1 = 7.53 \pm 0.09$, and $pK_2 = 8.80 \pm 0.09$. The best-fit parameter values for Chromozym-TH are ${}^{S}K_d^{opt} = 1.5 \pm 0.3$ μM, $pK_1 = 7.41 \pm 0.07$, and $pK_2 = 8.98 \pm 0.09$.

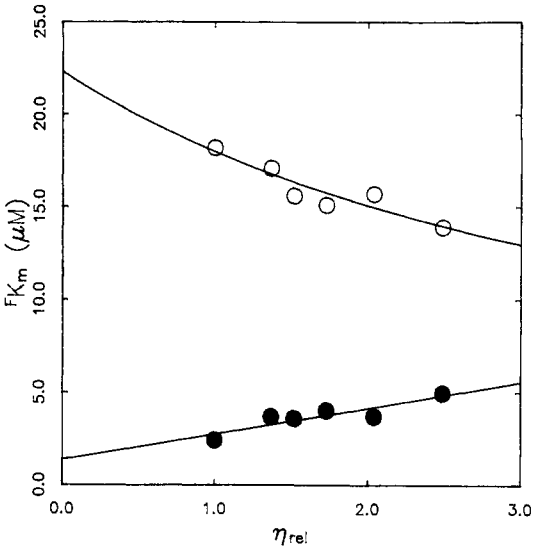

FIGURE 2: Determinations of ${}^{F}K_m$ for the hydrolysis of fibrinogen by thrombin as a function of relative viscosity, under conditions of 1 nM human α-thrombin, 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C, and 0.2 M NaCl (●) or 0.2 M choline chloride (○). Continuous lines were drawn according to eq 3 in the text, using the best-fit parameter values (●) ${}^{F}K_d = 1.3 \pm 0.3$ μM, $\alpha = 1.0 \pm 0.1$, and $\beta = 0$ and (○) ${}^{F}K_d = 22 \pm 3$ μM, $\alpha = 0$, and $\beta = 0.24 \pm 0.03$. In this plot, the value of the equilibrium constant ${}^{F}K_d$ for thrombin–fibrinogen interaction is derived as the extrapolation of ${}^{F}K_m$ for $\eta_{rel} \rightarrow 0$.

values of $pK_1 = 7.41 \pm 0.07$ and $pK_2 = 8.98 \pm 0.09$. The striking similarity of the pK values obtained for DAMPA, Chromozym-TH, and fibrinogen indicates that groups of the enzyme must control all these reactions. Consistent with previous findings (Di Cera et al., 1991; Stone et al., 1991), we conclude that the catalytic His57 is responsible for the acidic portion of the pH profile, while Ile16 controls the alkaline portion. In the case of DAMPA and Chromozym-TH, assignment of these groups is straightforward, since these molecules do not contain groups that ionize in the pH range 6–10. In the case of fibrinogen, on the other hand, the first 51 residues of the Aα chain should be examined since these are the residues important for recognition by thrombin (Hageman & Scheraga, 1974; Hogg & Blombäck, 1978).

Analysis of the binding of fibrinogen to the slow and fast forms of thrombin (Wells & Di Cera, 1992) has yielded the values of ${}^{F}K_m$ plotted in Figure 2 as a function of relative viscosity. Fibrinogen binds to the fast form of thrombin with a ${}^{F}K_d = 1.3 \pm 0.3$ μM. The linear dependence of ${}^{F}K_m$ on η_{rel} implies that deacylation is much faster than acylation ($\beta \approx 0$ in eq 3), while the dissociation rate is comparable to acylation. Hence, under these conditions, fibrinogen behaves as a sticky substrate for thrombin, as already reported (Hopfner & Di Cera, 1992). In the case of the slow form, however, the affinity drops significantly, and ${}^{F}K_d = 22 \pm 3$ μM. Also, the dependence of ${}^{F}K_m$ on η_{rel} is no longer linear but hyperbolic and indicates that deacylation has become comparable to acylation, while the dissociation rate has become much faster than acylation ($\alpha \approx 0$ in eq 3). The inversion of the rate-limiting step in the catalysis of fibrinogen parallels that observed for the hydrolysis of synthetic amide substrates, for which acylation is rate-limiting in the fast form and deacylation becomes rate-limiting in the slow form (Wells & Di Cera, 1992). The drastic drop in binding affinity linked to the fast→slow transition is a most unexpected result. In the case of synthetic amide substrates, the binding affinity of the slow form shows only a minor change since the slow→fast transition affects significantly, but nearly to the same extent, both the

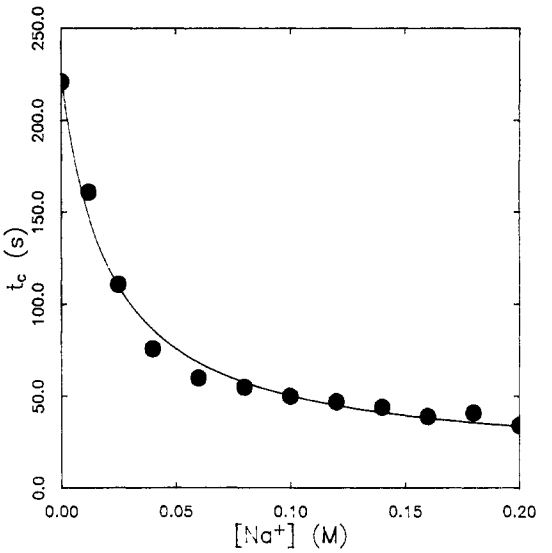

FIGURE 3: Dependence of the clotting time, t_c, on Na^+ concentration, at an ionic strength $I = 0.2$ M kept constant with choline chloride, under conditions of 4 nM human α-thrombin, 0.25 μM human fibrinogen, 5 mM Tris, and 0.1% PEG, pH 8.0 at 25 °C. The clotting time decreases in a seemingly hyperbolic fashion with Na^+ concentration, as depicted by the continuous line.

on and off rates for binding (Wells & Di Cera, 1992). The differences seen in fibrinogen binding to the two forms of thrombin carry over to the clotting process itself. The clotting time is significantly prolonged when the Na^+ concentration decreases, as shown in Figure 3. This effect is observed at constant ionic strength and must specifically involve Na^+ as the responsible agent. Since the clotting time is derived from turbidity measurements, the effect observed can in principle be due to effects not only on the enzyme, but also on fibrinogen, fibrin monomers, and the aggregation process. Both specific and nonspecific ion effects on fibrin assembly have largely been documented by turbidimetric studies since the pioneering work of Ferry (Ferry & Morrison, 1947; Shulman et al., 1953; Latallo et al., 1962; Weisel, 1986; De Cristofaro & Di Cera,

1992). Effects on the clotting curve may also arise from interference with the thrombin-induced, nonenzymatic enhancement of fibrin polymerization (Kaminsky & McDonagh, 1983; Kaminsky et al., 1991). However, in view of the differential binding properties of the slow and fast forms of thrombin, it is reasonable to suggest that a significant portion of the effect on the clotting time is due to the enzyme switching from the fast to the slow form when Na^+ is removed from the solution. The slow form is less catalytically competent toward amide substrates and fibrinogen, as shown by the data in Figure 2, and is expected to yield a clotting curve with a lag phase considerably prolonged. Also, the release of fibrinopetide A is retarded in the presence of the slow form (De Cristofaro and Di Cera, unpublished results).

DISCUSSION

The pH dependence of thrombin–fibrinogen interaction is remarkably similar to that observed with the synthetic amide substrate Chromozym-TH or the inhibitor DAMPA (Stone et al., 1991). Fibrinogen binding to thrombin is linked to proton release below pH 8.0 and to proton uptake above pH 8.0. From the pK_a values of the groups controlling fibrinogen binding, it can be concluded that only two groups of the enzyme participate in the ionization reactions. The first group is the catalytic His57, which must be unprotonated for optimal binding. Fibrinogen binds to the configuration of thrombin with the unprotonated His57 and therefore induces the release of protons in the pH range where His57 can significantly protonate. The second group is the amino terminus of the B chain, Ile16, which is salt-bridged to Asp194. This salt-bridge is also present in related serine proteases and is meant to stabilize the active conformation of the enzyme (Fersht, 1985). Deprotonation of Ile16 causes the salt-bridge to break and yields an enzyme form with reduced binding and catalytic competence toward synthetic amide substrates (Di Cera et al., 1991; Stone et al., 1991) and reduced binding affinity for hirudin (Betz et al., 1992) and fibrinogen. Hence, fibrinogen binding to thrombin has the effect of stabilizing this salt-bridge and is linked to proton uptake in the pH range where Ile16 is partially deprotonated. For this simple scenario to be realistic, one has to exclude the contribution of other groups of the fibrinogen molecule. Assignment in the case of the synthetic compounds Chromozym-TH and DAMPA is straightforward, since these molecules do not contain groups that ionize in the pH range 6–10. The similarity of the pK_a values obtained for DAMPA, Chromozym-TH, and fibrinogen is striking, which *per se* strongly supports the idea that indeed there is no other contribution arising from groups of the fibrinogen molecule. Additional support comes from the observation that the pK_a values obtained for fibrinogen are similar to those reported for hirudin binding when the amino terminus of this potent inhibitor is acetylated (Betz et al., 1992), or those obtained for synthetic amide substrates with the amino terminus blocked (Stone et al., 1991). This group is in fact responsible for the pH dependence of hirudin and synthetic substrate binding along with His57 and Ile16 of the enzyme. However, it is useful to rationalize our results also from known structural details. It has long been known that the first 51 residues of the Aα chain are important for recognition by thrombin (Hageman & Scheraga, 1974; Hogg & Blombäck, 1978). The sequence of the first 51 residues of the Aα chain is A^1DSGE^5GDFLA^{10}EGGGV^{15}RGPRV^{20}-VERHQ^{25}SACKD^{30}SDWPF^{35}CSDED^{40}WNYKC^{45}-PSGCR^{50}M. Cleavage of the chain occurs at Arg16, a residue conserved in all species (Doolittle et al., 1979; Henschen et al., 1983), consistent with the preference of Arg at P1 in the hydrolysis of amide bonds by thrombin. The released segment Ala1–Arg16 is fibrinopeptide A. Among the 51 residues, there are only 2 possible good candidates for ionization in the pH range 6–10. They are the amino terminus of the Aα chain (Ala1) and His24. The crystal structure of thrombin covalently bound to the decapeptide Ac-Asp7–Arg16-CH_2Cl, which is analogous to the segment 7–16 of the Aα chain, shows Asp7 with its amino terminus blocked, pointing away from the enzyme moiety (Stubbs et al., 1992). It is therefore conceivable that the amino terminus of the Aα chain is located well away from the thrombin surface and its protonation state should not affect fibrinogen binding. If it did, one would observe a pH profile for thrombin–fibrinogen interaction similar to that of thrombin–hirudin interaction (Betz et al., 1992), with a much steeper drop in affinity at pH >8.0. In fact, the amino terminus of Ile1 of hirudin makes contacts with O^γ of the catalytic Ser195 and the carbonyl oxygen of Ser214 (Rydel et al., 1991). In the case of His24, no structural information is available. Whether or not this residue makes contacts with thrombin remains to be established. A structural model for thrombin–fibrinogen interaction (Stubbs et al., 1992) proposes that Pro18 makes numerous contacts with residues of the rigid Trp60D loop of thrombin and that Arg19 may form a salt-bridge with either Glu192 or Glu39 of thrombin. Starting with Val20, the Aα chain would leave the thrombin surface and form a loop peaked around Cys28, which is disulfide-bonded with Cys28 of the second Aα chain. The chain would then fold back to allow the portion 35–43 of the Aα chain to make contacts with the FRS, consistent with the results of some functional studies (Hogg & Blombäck, 1978; Hofsteenge & Stone, 1987; Binne & Lord, 1991). Hence, His24 would be located in the intervening portion that links the primed residues 17–19 to the Cys28–Cys28 disulfide bridge, which should make no contacts with the thrombin surface. It is worth mentioning that this spatial arrangement of His24 has probably no bearing on the position of His51 of hirudin, which forms a salt-bridge with Glu39 of thrombin (Rydel et al., 1991). Interestingly enough, His51 does not contribute to the pH profile of thrombin–hirudin interaction since binding of the mutant H51Q has the same pH dependence as the wild-type hirudin (Betz et al., 1992). Thus, our results seem to provide substantial support for the structural model of Stubbs et al. (1992) and imply that the ionizable groups controlling thrombin–fibrinogen interaction are located only on the enzyme and that Ala1 and His24 of the Aα chain make no contribution.

We have shown that the slow→fast transition of thrombin significantly affects fibrinogen binding and clotting. Linkage thermodynamic principles imply that if the fast form has higher affinity for fibrinogen, then fibrinogen binding must induce the slow→fast transition of thrombin. Hence, the slow→fast transition discovered upon Na^+ binding to the enzyme (Wells & Di Cera, 1992) is a key step of molecular recognition by thrombin, since it is part of the molecular mechanism of fibrinogen binding. We have already reported that Na^+ binding to thrombin is accompanied by a significant increase of the intrinsic fluorescence of Trp residues (Wells & Di Cera, 1992). We predict that similar changes will be found upon fibrinogen binding to thrombin under conditions where both the slow and fast forms are significantly populated. Given the similarity in the bridge-binding strategy between fibrinogen and hirudin, one may wonder if hirudin binding to the slow and fast forms of thrombin is subject to the same regulatory control. Indeed, the fast form binds hirudin with an affinity about 16 times higher than the slow form (Ayala & Di Cera,

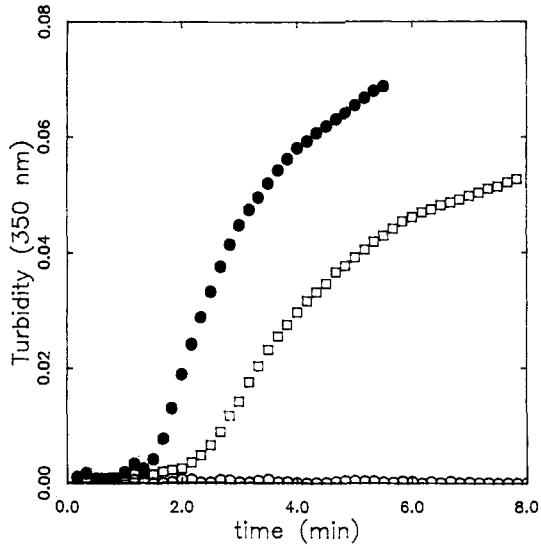

FIGURE 4: Clotting curves as a function of Na^+ concentration, at an ionic strength I = 0.2 M kept constant with choline chloride, under conditions of 4 nM human α-thrombin, 0.25 μM human fibrinogen, 5 mM Tris, and 0.1% PEG, pH 7.4 at 37 °C. In the absence of Na^+ (○), there is no appreciable clotting activity over a time scale during which clotting by the fast form (●) is complete. Turbidity starts to develop in the absence of Na^+ only after 10 min. The curve at physiological concentration of Na^+ (□) is significantly different from that at 0.2 M NaCl (●), thereby indicating that both the slow and fast forms of thrombin are significantly populated under physiological conditions.

1993), just as seen for fibrinogen. Consequently, the binding of hirudin to thrombin must stabilize the fast form and induce an increase in the intrinsic fluorescence of Trp residues. It should be pointed out that the enhancement of fibrinogen binding to thrombin linked to the slow→fast transition has a bearing on the mechanism of bridge-binding. No such enhancement is observed for synthetic substrates that only bind to the catalytic pocket (Wells & Di Cera, 1992). This important observation draws attention to the structural components involved in the slow→fast transition. In the case of small synthetic substrates, that only make contacts with the specificity subsites S1, S2, and S3, the slow→fast transition causes only a small increase in affinity. This may be due to the fact that the specificity pocket is not involved in the transition in a way that significantly affects the energetics of binding of small synthetic substrates. Since fibrinogen makes contacts with the specificity pocket and other regions of the enzyme, it is reasonable to assume that other structural components are responsible for the change in the binding free energy linked to the slow→fast transition. Possible candidates are the Trp60D and Trp148 loops that shape the upper and lower rims of the CP (Bode et al., 1992), and of course the FRS. Clearly, the origin of the reduced affinity of the slow form must be looked for in changes that affect these regions. We have addressed this point by studying the binding energetics to the slow and fast forms in systems involving thrombin and hirudin derivatives. These studies indicate that the mobile Trp148 loop and the region surrounding the access to the CP play a role in controlling the affinity of bridge-binding (Ayala & Di Cera, 1993). A final comment sould be made on the effects seen on the clotting curve. The clotting time is quite sensitive to the amount of Na^+ present in solution and changes in the direction indicated by our steady-state measurements of thrombin–fibrinogen interaction, i.e., it increases with decreasing Na^+ concentration since the value of ^FK_m for the slow form is higher and changes linearly with ^FK_m. The effect of [Na^+] is even more pronounced under conditions of physiological pH and temperature, as shown in Figure 4. The clotting curve measured in the absence of Na^+ shows no development of turbidity over a time scale of several minutes, during which clotting by the fast form is complete. Most of this effect is to be attributed to the existence of two thrombin forms with widely different affinity and catalytic competence toward fibrinogen. The curve obtained at physiological concentration of Na^+ (140 mM) is significantly different from that obtained in 0.2 M Na^+, thereby indicating that a significant fraction of human thrombin must exist in the slow form under physiological conditions. This observation, already anticipated from steady-state measurements of substrate hydrolysis (Wells & Di Cera, 1992), may have important implications for understanding how thrombin accomplishes its multifunctional roles in the pathophysiology of blood coagulation. We have demonstrated that the slow→fast transition is a key step of fibrinogen binding to thrombin. This transition is likely to have a bearing on other functionally important interactions of this and other enzymes involved in the coagulation cascade.

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