The interaction of fibrinogen Aα1–50–β-galactosidase fusion protein with the slow and fast forms of thrombin was studied and compared to thrombin-fibrinogen interaction under identical solution conditions. At equilibrium, the affinity of the fusion protein for the slow form of thrombin is 3 times higher than its affinity for the fast form. The fusion protein and fibrinogen have the same affinity for the fast form. On the other hand, the affinity of the fusion protein for the slow form of thrombin is 40 times tighter than that of fibrinogen. In the transition state, binding of the fusion protein has the same properties as fibrinogen, with the fast form showing higher specificity. The N-terminal fragment of the fibrinogen Aα chain thus contains residues that are responsible for the preferential binding of the fusion protein to the slow form at equilibrium and to the fast form in the transition state. If this fragment binds to thrombin in a similar way for fibrinogen and the fusion protein, then the N-terminal domains of the Bβ and γ chains of fibrinogen, that are not present in the fusion protein, must play a key role in the binding of fibrinogen to thrombin at equilibrium. These chains may destabilize binding to the slow form by nearly 2.4 kcal/mol, thereby favoring binding of fibrinogen to the fast form. We propose that the three chains of fibrinogen play different roles in the thrombin-fibrinogen interaction, with the Aα chain containing residues for preferential binding to the fast form in the transition state and the Bβ and γ chains containing residues that destabilize binding to the slow form at equilibrium.

Thrombin is an allosteric serine protease involved in blood coagulation. The enzyme exists in two forms, slow and fast (1), that have been targeted toward anticoagulant and procoagulant activities (2). The fast form preferentially cleaves fibrinogen, while the slow form cleaves protein C with higher specificity. The molecular basis of preferential binding of fibrinogen to the fast form (3) has been discussed in connection with the structural origin of preferential binding of fibrinogen to the fast form of thrombin remains to be established.

A useful model substrate for thrombin is the tripartite protein consisting of residues 1–50 of the fibrinogen Aα chain linked by a 59-residue segment of collagen to Escherichia coli β-galactosidase (10). The central domain of fibrinogen, represented by the CNBr fragment containing Aα chain residues 1–51, Bβ chain residues 1–118, and γ residues 1–78, makes contact with both the catalytic pocket and the fibrinogen binding loop in thrombin (11). Because the tripartite fusion protein lacks the N-terminal domains of the Bβ and γ chains of fibrinogen, it represents a simplified model for studying molecular recognition events involved in the thrombin-fibrinogen interaction. Specifically, comparative studies of the fusion protein and fibrinogen may shed light on the relative contribution of the three chains of fibrinogen. Functional studies indicate that the FpAα consisting of residues 1–16 of the Aα chain is cleaved from the fusion protein by thrombin at a rate comparable to that of fibrinogen (12). This result suggests that the Bβ and γ chains may have little influence on the interaction of the Aα chain with thrombin. However, perturbation of the N-terminal portion of the Bβ chain of fibrinogen often results in impaired clotting activity (13–15), and interaction of all three chains with the fibrinogen binding loop seems to be crucial for the correct hydrolysis of fibrinogen by thrombin (11, 16). In an attempt to dissect the contribution of Aα1–50 from the N-terminal portion of the three fibrinogen chains, we have decided to explore the interaction of the fusion protein with the slow and fast forms of thrombin at equilibrium and in the transition state. Comparison of the results with those obtained with fibrinogen under identical solution conditions indicates that the Bβ and γ chains play an important role in the preferential binding of the fusion protein to the slow form of thrombin.
ential binding of fibrinogen to the fast form by destabilizing binding to the slow form. The fibrinogen Aα chain, on the other hand, contains all the structural epitopes for preferential deac-

ylation by the fast form. Therefore, the N-terminal portions of the three chains of fibrinogen seem to have been targeted toward different roles in molecular recognition of this substrate by the slow and fast forms of thrombin.

**MATERIALS AND METHODS**

Human α-thrombin was purified and tested for activity as described (1, 10, 17). The chromogenic substrate S2238 was purchased from Chromogenix (Malmö, Sweden). The fusion protein was expressed, purified, and tested for activity as described (10, 12). The release of FpA from the fusion protein was quantified by reverse-phase HPLC (12) using a Vydac C18 column. Elution was carried out at a flow rate of 1 ml/min, with a gradient containing 25 mM Na₂HPO₄/NaH₂PO₄ buffer at pH 6.0 (solvent A) and 50% acetonitrile in solvent A (solvent B). Opti-

mal separation was obtained using a 30-min linear gradient to 40% of solvent B. The effluent was monitored at 206 nm. A molar absorption coefficient for FpA was determined by calibration curves. The slow and fast forms of thrombin were studied under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0, at 25 °C, in the presence of 200 mM ChCl (slow form) or NaCl (fast form). Progress curves for the release of FpA were analyzed using the expression for first-order kinetics shown in Equation 1, where $e_0$ is the active thrombin concentration, $t$ is time, $k_{e0}$ and $K_m$ refer to the hydrolysis of FpA, $[F]$ is the concentration of FpA at time $t$, and $[F]^*$ is the asymptotic concentration of FpA.

$$[F] = [F]^*\left(1 - \exp\left(-\frac{k_{e0}}{K_m}t\right)\right)$$  \hspace{1cm} (Eq. 1)

This value was consistent with the concentration of fusion protein estimated from SDS-polyacrylamide gel electrophoresis analysis of the purified protein.

The equilibrium constant for the binding of the fusion protein to thrombin was measured using the viscosimetric method introduced for the study of fibrinogen binding and described in detail elsewhere (19). In the viscosimetric method, the $K_m$ for the hydrolysis of FpA from fibrinogen is measured as a result of the competition of the hydrolysis of S2238 by thrombin as a function of fibrinogen concentration. Values of $K_m$ determined as a function of the relative viscosity of the solution are analyzed using the expression shown in Equation 2, where $\alpha$ is the ratio between the acylation and dissociation rates, $\beta$ is the ratio between acylation and deacylation, and $\eta_{rel}$ is the relative viscosity of the medium.

$$K_m = \frac{1 + \alpha\eta_{rel}}{1 + \beta\eta_{rel}}$$  \hspace{1cm} (Eq. 2)

The value of the equilibrium dissociation constant $K_d$ is obtained in a plot of $K_m$ versus $\eta_{rel}$ as the extrapolation of $K_m$ for $\eta_{rel}\rightarrow 0$. The viscosimetric method yields information on the equilibrium components of the binding interaction ($K_m$), as well as on the rate-limiting events during the catalytic conversion of the substrate leading to the release of FpA ($\alpha$ and $\beta$). The value of $K_d$ for the release of FpA from the fusion protein was derived from the competitive effect on the hydrolysis of S2238. Measurements of $K_m$ were then carried out as a function of relative viscosity, with thrombin either in slow or fast form. The results were expressed in units of [FpA] to allow a direct comparison between the fusion protein, containing 4 FpA fragments/molecule, and fibrinogen, containing 2 FpA fragments/molecule.

**RESULTS**

The results of the fusion protein binding to the slow and fast forms of thrombin are shown in Fig. 1 as a plot of $K_m$ versus $\eta_{rel}$. The data obey a straight line in the plot, over the range of relative viscosity values examined, suggesting that $\beta \approx 0$ in Equation 2. This implies that deacylation and diffusion of the FpA away from the catalytic pocket of thrombin occur on a time scale much faster than acylation, in either the slow or fast forms. The value of $\alpha$ in either form indicates that the fusion protein behaves as a "sticky" substrate, with the dissociation rate being comparable to acylation. This situation is seen for the cleavage of FpA from fibrinogen by the fast form, but not by the slow form (3). The extrapolation of $K_m$ for $\eta_{rel}\rightarrow 0$ in Fig. 1 gives the value of $K_m$ for the fusion protein binding to the slow

![Figure 1](http://www.jbc.org/Downloaded from http://www.jbc.org/)
Properties of the slow and fast forms of thrombin in their interaction with fibrinogen and the fusion protein

All values are in units of $F_pA$ concentration. The third column reflects the ratio of affinity or specificity between the fast and slow forms. The last column gives the coupling free energy (in kcal/mol), $\Delta G_c = -RT\ln r$, where $R$ is the gas constant and $T$ is the absolute temperature (2).

|                | Slow            | Fast           | $r$ | $\Delta G_c$ |
|----------------|-----------------|----------------|-----|-------------|
| **K_{m} values** |                 |                |     |             |
| Fibrinogen     | $44 \pm 6 \mu M$| $2.6 \pm 0.6 \mu M$ | 17  | $-1.7 \pm 0.1$ |
| Fusion protein  | $1.1 \pm 0.2 \mu M$ | $3.3 \pm 0.4 \mu M$ | 0.33 | $0.7 \pm 0.1$ |
| **$k_{cat}/K_{m}$ values** | | | | |
| $F_pA$ (fibrinogen)* | $1.88 \pm 0.04 \mu M^{-1} s^{-1}$ | $13.4 \pm 0.2 \mu M^{-1} s^{-1}$ | $7.1 \pm 0.2$ | $-1.16 \pm 0.02$ |
| $F_pA$ (fusion protein) | $0.27 \pm 0.06 \mu M^{-1} s^{-1}$ | $2.4 \pm 0.3 \mu M^{-1} s^{-1}$ | $9 \pm 2$ | $-1.3 \pm 0.1$ |

* Data from Ref. 3.

**TABLE II**

Kinetic rate constants for the interaction of fibrinogen and the fusion protein with the slow and fast forms of thrombin

The Michaelis-Menten constants are given by $K_m = \frac{k_3 k_{-1} + k_2}{k_1 k_3 + k_2}$ (1, 19), and $k_{cat} = \frac{k_1 k_3}{k_2 + k_3}$ (19).

|                | $k_1$ | $k_{-1}$ | $k_2$ | $k_3$ |
|----------------|-------|----------|-------|-------|
| **Fast form**  |       |          |       |       |
| Fibrinogen     | $25 \pm 4$ | $64 \pm 10$ | $64 \pm 10$ | $>410$ |
| Fusion protein  | $4 \pm 1$ | $14 \pm 4$ | $19 \pm 3$ | $>120$ |
| **Slow form**  |       |          |       |       |
| Fibrinogen     | $>10$ | $>441$ | $84 \pm 16$ | $350 \pm 65$ |
| Fusion protein  | $0.4 \pm 0.1$ | $0.4 \pm 0.2$ | $0.9 \pm 0.2$ | $>41$ |

DISCUSSION

The fusion protein is the first substrate to be found to bind with higher affinity to the slow form of thrombin. All substrates, effectors, and inhibitors studied previously have been reported to bind to the fast form with higher affinity. Such is the case of fibrinogen (3), hirudin and its C-terminal fragment (4), thrombomodulin (2), and a variety of synthetic substrates and inhibitors (1, 4). Although the fusion protein binds preferentially to the slow form at equilibrium, $F_pA$ is cleaved by the fast form with higher specificity. This result bears directly on thrombin-fibrinogen interaction. Fibrinogen binds preferentially to the fast form (3) and is cleaved by the fast form with higher specificity (2). This observation suggests that the structural components responsible for preferential binding to the fast form at equilibrium are also involved in the preferential stabilization of the transition state in this form. The results for the fusion protein, however, demonstrate that different structural domains of fibrinogen may control molecular recognition.
at equilibrium and at the transition state. In addition, the results reinforce the notion that the N-terminal domains of the Bβ and γ chains of fibrinogen play a key role in molecular recognition of the natural substrate. These chains presumably destabilize binding to the slow form. The binding affinities of the fusion protein and fibrinogen are the same when thrombin is in the fast form, but differ by a factor of 40 when thrombin is in the slow form. If the sequence 1–50 of the Aα chain present in the fusion protein makes contacts with thrombin as the analogous sequence in the fibrinogen molecule, then this sequence must bind to the slow form with higher affinity. Preferential binding to the fast form, as seen in the case of fibrinogen, would result from contacts made by the N-terminal domains of the Bβ and γ chains with thrombin. These contacts would provide 2.4 kcal/mol toward the stabilization of the fast isomer in the fast form, but differ by a factor of 40 when thrombin is destabilized binding to the slow form. The binding affinities of these chains presumably hold the key to unraveling the contribution of the Bβ and γ chains to the destabilization of binding to the slow form. Alternatively, the Bβ and γ chains may constrain the Aα chain of fibrinogen such that the contacts with thrombin are not analogous to those in the fusion protein.

The lack of structural information on the thrombin-fibrinogen complex makes assignment of residues involved in recognition very difficult. A structure of thrombin covalently bound to the fragment 1–16 of the fibrinogen A chain has documented the expected contacts with primary recognition sites in the catalytic pocket, along with hydrophobic contacts with the arginine binding site of thrombin and Gly-216 (8). A seemingly important salt bridge between Arg-173 of thrombin and Glu-11 of fibrinogen has also been reported in the crystal structure. This assignment, however, may be questionable since the side chain of Arg-173 is disordered. The E11A replacement in the fusion protein is without effect (11, 12), but the E11G mutation in fibrinogen Mitaka II impairs thrombin binding (20). The mutation R173E of thrombin decreases the release of FpA and FpB from fibrinogen by a factor of 3 and 2, respectively, which is an effect too small to be assigned to the lack of an important salt bridge interaction. The fibrinogen binding loop provides a significant portion of the binding free energy (19), but contributes very little to the value of ΔG (4). This is because residues of the fibrinogen binding loop contribute almost equally to binding in the slow and fast forms. Mutagenesis studies of thrombin have indicated that Lys-60f, located strategically in between the catalytic pocket and the fibrinogen binding loop, may play a significant role in the recognition of fibrinogen since the mutant K60f has a reduced clotting activity (9). This residue is also important in the binding of hirudin (5). The portion of the NSDK of fibrinogen interacting with the region of thrombin surrounding Lys-60f may hold the key to unravel the contribution of the Bβ and γ chains to the destabilization of binding to the slow form.

The significant differences seen at equilibrium between fibrinogen and the fusion protein disappear in the transition state. Preferential binding to the fast form in the transition state must originate from contacts made with residues within the catalytic moiety and the recognition subsite Asp-189 of thrombin. All the molecular components responsible for the preferential interaction are contained in the Aα1–50, with no apparent contribution from the N-terminal domains of the Bβ and γ chains. The three chains in the NSDK of fibrinogen appear to have different roles in the recognition mechanism. The Aα chain contains the structures required for recognition by the fast form in the transition state, while the Bβ and γ chains contain the structures that destabilize binding to the slow form at equilibrium. In this model the Bβ and γ chains act as intramolecular allosteric effectors of the Aα chain. They destabilize binding to the slow form, inducing the slow → fast transition, which in turn facilitates binding of the Aα chain in the transition state and the release of FpA by the fast form.

The observation that the fusion protein binds to the slow form with higher affinity is intriguing and represents a significant step toward our understanding of the molecular basis of the slow → fast transition of thrombin and the structural epitopes important for fibrinogen recognition. An important implication of our results is that synthetic inhibitors tailored after the 1–50 segment of the Aα chain of fibrinogen may work as effective stabilizers of the anticoagulant slow form of thrombin. Such inhibitors would also be effective in enhancing the enzyme specificity toward protein C and may reveal key details involved in the interaction of thrombin with thrombomodulin.

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Binding of Fibrinogen Aα1–50-β-Galactosidase Fusion Protein to Thrombin Stabilizes the Slow Form

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