Hirudin Binding Reveals Key Determinants of Thrombin Allostery*

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Thrombin exists in two allosteric forms, slow (S) and fast (F), that recognize natural substrates and inhibitors with significantly different affinities. Because under physiologic conditions the two forms are almost equally populated, investigation of thrombin function must address the contribution from the S and F forms and the molecular origin of their differential recognition of ligands. Using a panel of 79 Ala mutants, we have mapped for the first time the epitopes of thrombin recognizing a macromolecular ligand, hirudin, in the S and F forms. Hirudin binding is a relevant model for the interaction of thrombin with fibrinogen and PAR1 and is likewise influenced by the allosteric S→F transition. The epitopes are nearly identical and encompass two hot spots, one in exosite I and the other in the Na⁺ site at the opposite end of the protein. The higher affinity of the F form is due to the preferential interaction of hirudin with Lys-36, Leu-65, Thr-74, and Arg-75 in exosite I; Gly-193 in the oxyanion hole; and Asp-221 and Asp-222 in the Na⁺ site. Remarkably, no correlation is found between the energetic and structural involvements of thrombin residues in hirudin recognition, which invites caution in the analysis of protein-protein interactions in general.

The serine protease thrombin is a member of the large class of enzymes activated by monovalent cations (1) and requires Na⁺ for optimal catalytic activity. The effect of Na⁺ is allosteric (2) and shifts the conformation of the enzyme from a low activity slow (S) form to a high activity fast (F) form. The partitioning of thrombin between these two forms is of physiologic importance because the Na⁺-bound F form accounts for the procoagulant (cleavage of fibrinogen) and prothrombotic/signaling (cleavage of PAR1 and platelet activation) roles of the enzyme, whereas the Na⁺-free S form is responsible for the anticoagulant (cleavage of protein C) role (3, 4). Ancillary procoagulant roles of thrombin, like activation of factor VIII, also require the enzyme to be in the F form (5). The Na⁺-dependent allosteric regulation of catalytic activity is shared by other clotting proteases, such as activated protein C (6, 7) and factor Xα (8), that possess a Na⁺ binding site structurally similar (7, 9) to that found in thrombin (10).

Na⁺ binding to thrombin increases both the rate of substrate diffusion into the active site and the rate of substrate acylation (2, 11). The result is that the F form interacts with chromogenic substrates, fibrinogen, and PAR1 with significantly higher $k_{cat}/K_m$ compared with the S form (4). Recent crystal structures of the S and F forms have helped rationalize this difference (12). Na⁺ binding causes rearrangements of residues close to the Na⁺ site and located up to 15 Å away such as the catalytic Ser-195. The long-range communication of the Na⁺ effect is ensured by a network of water molecules that embed the Na⁺ site, the primary specificity pocket, and the active site. Asp-189 at the bottom of the primary specificity pocket is optimally oriented for substrate binding in the F form, but not in the S form. Likewise, Ser-195 is within H-bonding distance of its catalytic partner His-57 in the F form, but not in the S form, which ensures optimal substrate acylation. Whether such changes around Asp-189 and Ser-195 are also responsible for the higher specificity of the F form toward fibrinogen and/or PAR1 remains to be demonstrated.

One of the most peculiar features of thrombin and related clotting enzymes is that the strategy of macromolecular substrate recognition involves extended regions of the enzyme, in addition to the active site moiety, where small chromogenic substrates bind (4, 13). The observation that the F form cleaves fibrinogen and PAR1 with higher $k_{cat}/K_m$ compared with the S form raises the possibility that these other regions contribute to the difference. Ideally, one would like to address this problem using a molecular probe that makes extensive contacts with the surface of thrombin, including regions involved in fibrinogen and PAR1 recognition, and returns information on the conformational changes that accompany the S→F transition. The potent natural inhibitor hirudin binds to thrombin with a $K_i$ in the fm range (14) and covers >20% of the water-accessible surface area of the enzyme (15). The high affinity of the interaction makes hirudin a particularly suitable probe for site-directed mutagenesis studies because it can retain significant specificity even in the presence of highly disruptive mutations. The interaction is driven by both electrostatic (16–18) and hydrophobic (19) forces. Hirudin contacts residues of exosite I of thrombin through its extended C-tail via acidic (20) and nonpolar (21) residues. The compact N-terminal domain of the inhibitor occludes the active site of the enzyme and contacts the 60-loop, the aryl binding site, and the Na⁺ site (22, 23). These are the same regions probed by fibrinogen and PAR1 upon binding to thrombin (24, 25). Last, but not least, hirudin binding to thrombin is positively linked to Na⁺ binding (19, 26). Hence, hirudin mimics important physiologic substrates in their interaction with thrombin, and, at the same time, it is sensitive to structural changes between the F and S forms that are at the basis of the procoagulant, prothrombotic, and signaling functions of the enzyme.

In this study we define the boundaries of the epitopes of thrombin recognizing hirudin in the F and S forms using a panel of 79 Ala mutants of residues located in exosite I, exosite II, the S1–S4 sites, the 60-loop, and the Na⁺ binding site. The results reveal unexpected determinants of recognition and
identify residues responsible for the higher affinity of the F form.

MATERIALS AND METHODS

A total of 79 single Ala mutants of human thrombin were expressed, purified, and tested for activity as described previously (24, 27). The numbering used in this work refers to chymotrypsinogen. The new mutants, L99A and I174A, were prepared in this study to target the residues flanking Trp-215 in the aryl binding site and add to the existing panel of 77 mutants used in previous studies (12, 28). Both mutants had normal affinity toward Na+, with Kd values of 13 and 11 mM, respectively, compared with 15 mM for wild-type (12). Highly pure hirudin (HV variant CGP39393, unphosphorylated) was received as a generous gift from Ciba Geigy and Novartis. The chromogenic substrate H-0-Phe-Pro-Arg-p-nitroanilide was obtained from Midwest Biotech (Fishers, IN). For each mutant, the equilibrium dissociation constant Kd for hirudin binding was determined from analysis of progress curves of substrate hydrolysis as a function of inhibitor concentration started by addition of thrombin (14, 26). Binding of hirudin to thrombin was measured under conditions of 5 mM Tris, 0.1% polyethylene glycol 8000, pH 8.0, at 25 °C. The value of Kd depends on [Na+] according to the linkage expression (26, 29)

\[ K_d = K_d^{[Na^+]}/K_d^{[Na^+]_{0}} \]

where \( K_d^{[Na^+]}/K_d^{[Na^+]_{0}} \) and \( K_d^{[Na^+]}/K_d^{[Na^+]_{0}} \) are the values of \( K_d \) for hirudin binding to the S and F forms, respectively. The value of \( K_d^{[Na^+]_{0}} \) was derived in the presence of 200 mM choline chloride. The value of \( K_d^{[Na^+]_{0}} \) was derived in the presence of 200 mM NaCl using a rearrangement of Eq. 1, i.e.

\[ \frac{1}{K_d^{[Na^+]_{0}}} = \frac{1}{K_d^{[Na^+]_{0}}} + \frac{1}{K_d^{[Na^+]}} \]

where \( K_d \) refers to 200 mM NaCl, \( K_d \) is the equilibrium dissociation constant for Na+ binding measured for all mutants as reported previously (12), and [Na+] = 200 mM.

Six progress curves of substrate hydrolysis by thrombin were measured in the presence of different concentrations of hirudin, typically in the range of 0–200 mM, depending on the affinity. The concentration of enzyme was typically 100 pm, and the concentration of substrate was 60 μM. The expression used in the analysis of product (P) formation as a function of time (t) was as follows (30, 31).

\[ P = v_0 + \frac{(v_o - v_{ss})(1 - \gamma)}{\lambda \gamma} \ln \left( \frac{1}{1 - \gamma \exp(-\lambda t)} \right) \]

The terms \( v_0 \) and \( v_{ss} \) are the initial and the steady-state velocities of product formation given by

\[ v_0 = E_{tot}[S] \]

\[ v_{ss} = k_{cat}[S] + [S] \]

where \( k_{cat} \) is the Michaelis-Menten parameter pertaining to the substrate (S) hydrolysis by thrombin, \( K_s \) is the equilibrium dissociation constant of hirudin, and \( E_{tot} \) and \( I_{tot} \) are the total thrombin and hirudin concentrations. The values of \( E_{tot} \) and \( I_{tot} \) were determined from direct titrations (32) and fixed in the data analysis. When these values were floated by allowing for stochiometric factors (14, 30, 31), they changed minimally from the determined values, and the estimated \( K_d \) was within the 5–10% error range of the fit. For each mutant, the values of \( K_d \) and \( k_{cat} \) for the hydrolysis of H-0-Phe-Pro-Arg-p-nitroanilide were determined independently under the same solution conditions used for the hirudin binding assays, using progress curves analyzed by direct integration and correction for product inhibition (33). The values of \( K_d \) ranged from 0.3 to 10 μM. The parameters \( \gamma \) and \( \lambda \) in Eq. 3 are given by the expressions

\[ \gamma = \frac{E_{tot} + I_{tot} + K_d[1 + [S]/K_s] - 4E_{tot}I_{tot}}{2E_{tot}} \]

\[ \lambda = K_d \left( 1 + \frac{[S]}{K_s} \right) \]

where \( k_1 \) is the second-order rate constant for hirudin binding to thrombin. Eq. 3 contains two independent parameters, \( k_1 \) and \( K_d \). The value of \( K_d \) could be resolved for all mutants, whereas measurements of \( k_1 \) were not possible for mutants that lacked a transient evolution of the progress curve from the initial value of the velocity \( v_0 \) to the steady-state value \( v_{ss} \). The parameter \( K_d \) was necessary and sufficient to map the functional epitopes of thrombin recognizing hirudin in the S and F forms.

The epitopes of hirudin binding to the S and F forms of thrombin were mapped using the strategy recently applied to the study of thrombin interaction with Na+ (12), thrombomodulin (27), and protein C (28). The strategy exploits the linkage between binding and site-directed mutation to identify residues that are energetically involved in the recognition process (34, 35). The Ala substitution of a given residue provides an “adiabatic” perturbation that shaves the side chain down to the Cβ atom while retaining the chirality (with the exception of Gly) (36). Under this assumption, the functional epitope revealed by Ala scans should overlap with the structural epitope determined by crystallographic investigation. In the case of thrombin, mapping of relevant epitopes has so far returned boundaries that are consistent with those defined from x-ray studies (27, 28). Furthermore, recent structures of Ala mutants of thrombin have revealed perturbations that are confined to the site of mutation (37, 38), thereby ruling out undesired long-range effects that are to be expected with more disruptive site-directed mutants. The epitopes for hirudin binding to the S and F forms of thrombin were mapped from measurements of \( K_d \) and \( k_{cat} \) for a panel of 79 single Ala mutants. Comparison of these parameters with the values obtained for wild-type returned a map of the residues of thrombin that affect hirudin recognition in the two allosteric forms of the enzyme.

Mutations that affected predominantly the S or F form were identified from their perturbation of the value of \( c \) (26, 39). When \( c \) increases relative to the value seen in the wild-type, the mutation affects a residue that is more important for hirudin recognition by the S form. On the other hand, when \( c \) decreases relative to the value seen in the wild-type, the mutation affects a residue that is more important for hirudin recognition by the F form.

RESULTS

Seventy-nine residues of thrombin located in exosite I, exosite II, the 60-loop, the specificity sites S1–S4, and the Na+ binding site were targeted by mutagenesis. For each mutant, the value of \( K_d \) for hirudin binding was determined in the S and F forms (Table 1 and Fig. 1 using Eq. 2 and the values of \( K_d^{[Na^+]_{0}} \) for Na+ binding reported elsewhere (12). A structural rendition of the epitopes for hirudin binding is given in Fig. 2. Previous mutagenesis work on hirudin indicated that the inhibitor makes energetically significant contacts with exosite I and the entrance to the active site of the enzyme (20–23). No well-defined hot spot for hirudin recognition emerged from these studies, but that could have been precluded by the relatively small number of hirudin residues targeted by mutagenesis. Our mutagenesis study expands the pioneering work by Stone and collaborators (20–23) by examining the energetic balance of site-directed mutations of thrombin.

Several regions of thrombin participate in hirudin recognition and, with notable few exceptions, the contributions are energetically similar in the S and F forms of the enzyme. This observation is a further testimony to the basic similarities of the two allosteric forms of thrombin (12). Exosite I makes large contributions to binding with the buried ion-quartet Arg-67/Lys-70/Glu-77/Glu-80 standing out in the profile as a hot spot (Fig. 1). The quartet stabilizes the 70-loop (40) and plays a crucial role in the interaction of thrombin with thrombomodulin (27). Given the buried location of the quartet, no appreciable contacts with hirudin are seen in the crystal structure, therefore suggesting that the Ala mutation of any residue in the
quartet produces indirect effects by changing the orientation of other residues on the surface of exosite I. In the case of thrombomodulin, mutation of the quartet produced effects similar to mutation of Tyr-76 or Ile-82 that sit right on top of the 70-loop (27). In the case of hirudin, however, mutations in the quartet are significantly more deleterious than those of Tyr-76 or Ile-82, suggesting that destabilization of the 70-loop extends beyond those residues and that hirudin probed exosite I differently than thrombomodulin. Of the residues in exosite I that make contacts with hirudin, Arg-73, Thr-74, Arg-75, Tyr-76, and Arg-77a together account for 61 of the 70 total contacts < 4 Å (15). Yet, with the exception of Tyr-76, Ala mutation of any of those residues produces a <100-fold drop in affinity for hirudin, which is significantly smaller than the 10,000-fold drop caused by mutation of Arg-67 and Glu-80 that make no direct contacts with the inhibitor. That is consistent with mutagenesis studies of hirudin that reported only modest (<10-fold) drops in affinity upon mutations of negatively charged residues of the C-tail (16). Hence, the ionic interactions of the hirudin C-tail with exosite I of thrombin contribute modestly to recognition (Fig. 1) because Ala mutation of these residues produces a <100-fold drop in affinity. Among all thrombin residues, Trp-60d contributes the largest number of contacts with hirudin (23 total), but Ala replacement of Trp-60d produces a change in affinity of only 20-fold. Although the change is obviously significant, it is well below the level expected from the crystal structure for such large number of contacts and is comparable to that seen upon mutation of residues such as Ser-171 or Arg-187 that make no contacts at all with the inhibitor. Overall, the contribution of the 30- and 60-loops to hirudin recognition does not meet the expectations from the crystal structure and is energetically modest.

Residues Leu-99, Ile-174, and Trp-215 make up the aromatic pocket together for 61 of the 70 total contacts < 4 Å (15). Yet, with the exception of Tyr-76, Ala mutation of any of those residues produces a <100-fold drop in affinity for hirudin, which is significantly smaller than the 10,000-fold drop caused by mutation of Arg-67 and Glu-80 that make no direct contacts with the inhibitor. That is consistent with mutagenesis studies of hirudin that reported only modest (<10-fold) drops in affinity upon mutations of negatively charged residues of the C-tail (16). Hence, the ionic interactions of the hirudin C-tail with exosite I of thrombin contribute modestly to recognition, whereas hydrophobic interactions involving Tyr-76 correctly stabilized by the ion-quartet buried beneath the surface of the 70-loop provide a more significant portion of the binding free energy.

The 60-loop (Trp-60d and Lys-60f) and the adjacent 30-loop (Phe-34, Lys-36, and Glu-39) make 39 contacts < 4 Å with hirudin (15). However, the contacts contribute modestly to recognition (Fig. 1) because Ala mutation of these residues produces a <100-fold drop in affinity. Among all thrombin residues, Trp-60d contributes the largest number of contacts with hirudin (23 total), but Ala replacement of Trp-60d produces a change in affinity of only 20-fold. Although the change is obviously significant, it is well below the level expected from the crystal structure for such large number of contacts and is comparable to that seen upon mutation of residues such as Ser-171 or Arg-187 that make no contacts at all with the inhibitor. Overall, the contribution of the 30- and 60-loops to hirudin recognition does not meet the expectations from the crystal structure and is energetically modest.

A key observation from our mutagenesis study points to the Na’ binding environment as the dominant determinant of hirudin recognition. The 220-loop harbors a second hot spot (Fig. 1) that was not predicted from previous investigations of...
The epitope of thrombin recognizing hirudin in the expressed as log $c_{\text{mut}}/c_{\text{wt}}$ (mut, mutant, wt, wild-type). The value of $c_{\text{mut}}$ is 62 ± 3 and reflects the ratio of $K_i$ values between the two forms (see Eq. 1). Negative values in the plot indicate residues (e.g. Asp-221 and Asp-222) that stabilize binding of hirudin to the F form. Positive values, on the other hand, indicate residues (e.g. Glu-192 and Ser-214) that stabilize binding of hirudin to the S form. Bottom panel, Ala scanning mutagenesis mapping of the epitope of thrombin recognizing hirudin in the S (black bars) and F (white bars) forms. Plotted is the change in binding affinity due to mutation, expressed as log $K_{\text{mut}}/K_{\text{wt}}$, under experimental conditions of 5 mM Tris, 0.1% polyethylene glycol 8000, pH 8.0, at 25 °C (see also Table I). Values for the S form were obtained in the presence of 200 mM choline chloride. Values for the F form were obtained from those in the presence of 200 mM NaCl using Eq. 2. The values of $K_{\text{mut}}$ are 2.6 ± 0.1 μM and 42 ± 2 μM for the S and F forms, respectively.

The high affinity of hirudin enabled a thorough investigation of the thrombin-hirudin interaction and could not have been anticipated from the crystal structure. Here, the massive contribution of Lys-224 dwarfs that seen for any other residue of thrombin. Mutation of Lys-224 to Ala produces a staggering drop in hirudin affinity of 7 orders of magnitude, a change that is at least 1000-fold larger than that observed upon mutation of the second most important residue for hirudin recognition, Arg-221a. Lys-224 and Arg-221a make, respectively, 3 and 13 contacts < 4 Å with hirudin. Lys-224 forms a H-bond with the backbone O atom of Ser-19 of hirudin, a residue never mutated in previous studies. It is very hard to rationalize how such a tenuous crystal contact could store >10 kcal/mol of binding free energy between thrombin and hirudin. Arg-221a makes an ion-pair with Asp-5 of hirudin, and mutation of Asp-5 to Ala reduces hirudin binding 10-fold (22). In this case, at least, the ion-pair provides a structural basis for the energetic contribution. Other residues in the hot spot make significant contributions to hirudin binding. Glu-217 and Thr-172 both affect hirudin binding >100-fold, although only Glu-217 makes extensive contacts with the inhibitor. Residues important for Na⁺ binding such as Tyr-184a, Asp-189, and Tyr-225 affect hirudin binding >100-fold. Finally, residue Arg-173 makes an ion-pair with Glu-17 of hirudin (15), but its mutation to Ala is energetically inconsequential on hirudin binding.

The high affinity of hirudin enabled a thorough investigation of the properties of the S form of thrombin in addition to those of the F form. For the first time, the interaction of thrombin with a macromolecular ligand could be studied in the two allosteric forms by extensive site-directed mutagenesis. The epitopes of hirudin recognition in the S and F forms differ little (Figs. 1 and 2). In general, residues important for recognition in the F form are also important in the S form. That is particularly evident for residues of the two hot spots in the Na⁺ site (Arg-221a and Lys-224) and in exosite I (Arg-67, Lys-70, Tyr-76, Glu-77, and Glu-80). The residues of thrombin responsible for the functional differences between the S and F forms differ from those that make dominant contributions to binding. There are seven residues whose mutation affects hirudin binding >10-fold and decreases the value of $c_i$ >5-fold (Fig. 1). These are residues that promote hirudin binding to the F form. The most significant effect on the value of $c_i$ is seen upon mutation of Asp-221 and Asp-222 in the Na⁺ binding loop that significantly reduces the differences between the S and F forms. Interestingly, Asp-222 is required for optimal Na⁺ binding, and Asp-221 controls the allosteric transduction of Na⁺ binding into enhanced catalytic activity (12). Neither residue contacts hirudin directly (Fig. 3), which suggests that the effects observed are due to stabilization of the S form caused by compromised Na⁺ binding (D222A mutant) or allosteric transduction (D221A mutant). Among the other five residues that affect hirudin binding predominantly in the F form, four of them (Lys-36, Leu-65, Thr-74, and Arg-75) are located in or near exosite I and contact hirudin directly (Fig. 3). These residues do not affect Na⁺ binding (12) and presumably orient their side chains differently in the S and F forms, thereby explaining the differential contribution to hirudin binding. Indeed, inspection of the crystal structures of the S and F forms shows movements around Lys-36 and Arg-75 upon Na⁺ binding (12). The fifth residue, Gly-193, is in the oxyanion hole and does not make contacts with the inhibitor but affects Na⁺ binding slightly...
Thrombin-Hirudin Interaction

As for Asp-221 and Asp-222, the effect of Gly-193 may be indirect and mediated by stabilization of the S form. Another interesting observation is the presence of residues of thrombin, like Glu-192 and Ser-214, that promote binding of hirudin to the S form (Fig. 1). Mutation of these residues increases the value of c and the difference in affinity between the F and S forms. The observation is consistent with previous mutagenesis and structural studies. Ser-214 also affects substrate hydrolysis predominantly in the S form (11, 12), whereas Glu-192 is oriented differently in the S form to enable better protein C activation (12). Both residues make contacts with hirudin and specifically with the N-terminal portion of the inhibitor around residues Ile-1, Thr-2, and Thr-4.

Fig. 3 depicts the surface of recognition for the thrombin-hirudin interaction with the residues controlling preferential binding to the F (red) or S (green) forms. Residues that cause the F form to bind hirudin with higher affinity are distributed over a large surface area that spans the hirudin epitope in its entirety. Residues around exosite I (Lys-36, Leu-65, Thr-74, and Arg-75) promote hirudin binding to the F form through direct interactions with the inhibitor, whereas residues in the oxyanion hole (Gly-193) and the Na\(^+\) site (Asp-221 and Asp-222) influence the binding indirectly through their effect on Na\(^+\) binding. It should be pointed out that mutations of Asp-189, Tyr-225, and Glu-217, which affect Na\(^+\) binding significantly, do not affect the value of c significantly, perhaps as a result of direct interactions with the inhibitor. That is certainly the case for Glu-217, which makes a H-bonding interaction with the N backbone atom of Val-21 and other contacts of < 4 Å with Tyr-3, Leu-15, Ser-19, and Asn-20 of hirudin (15). Finally, Ser-14 and Glu-192 promote binding to the S form, and their contribution counters that provided by the residues stabilizing binding to the F form. The energetic balance of hirudin recognition in the two forms of thrombin is therefore the result of numerous interactions that make opposite contributions to binding. These interactions tend to be weaker than those observed for residues of the hot spots in exosite I and the Na\(^+\) environment but nonetheless are at the basis of the allosteric control of macromolecular recognition.

**DISCUSSION**

Hirudin binding to thrombin shares important similarities with fibrinogen and PAR1 binding. Structural and modeling information on the complexes shows that these macromolecular ligands bind to exosite I and the active site of the enzyme, making numerous contacts with the 60-loop and the 220-loop (15, 24, 25). Furthermore, hirudin binding to thrombin is linked to the S→F transition in a significant (60-fold change in K\(_i\)) manner, as found in the case of fibrinogen and PAR1 (24, 41). Energetic mapping of the epitopes of thrombin recognizing hirudin in the S and F forms is therefore relevant to understand how the enzyme fulfills its procoagulant, prothrombotic, and signaling roles and to identify the residues of thrombin responsible for the higher affinity of the F form.

The results reported in this study reveal that hirudin binding to thrombin is controlled mainly by residues in exosite I and the Na\(^+\) site, as found in the case of fibrinogen and PAR1. The shape of the epitope changes little upon Na\(^+\) binding, and the difference in affinity between the F and S forms can be assigned to the preferential contribution in the F form of seven residues: Asp-221 and Asp-222 in the Na\(^+\) site, Gly-193 in the oxyanion hole, and Lys-36, Leu-65, Thr-74, and Arg-75 in exosite I. In addition, Ser-214 and Glu-192 are found to stabilize binding to the S form. Asp-221 and Asp-222 are key players in thrombin allostery. Asp-222 forms an ion-pair with Arg-187, and the integrity of this interaction is required for Na\(^+\) binding (12). On the other hand, Asp-221 is required for transduction of Na\(^+\) binding into enhanced catalytic activity (12). Notably, both Asp-221 and Asp-222 make no direct contacts with hirudin according to the crystal structure (15), and mutation of either residue produces a hirudin affinity similar to that of the S form of wild-type. Consistent with this observation, the thrombin double mutant D221A/D222K does not bind Na\(^+\) (37) and binds hirudin with an affinity similar to that of the S form of wild-type (10). In addition to Asp-221 and Asp-222, there are five other residues that promote hirudin binding to the F form. Their individual contribution is small, but together they provide a significant source of difference in affinity between the two forms of thrombin. Residues Lys-36 and Leu-65 reside strategically at the boundary between the 30-loop and exosite I and make numerous contacts with the hirudin C-tail. Their role is assisted by Thr-74 and Arg-75 in exosite I that engage the hirudin acidic C-tail in several polar interactions. Finally, within the active site moieties, Gly-193 in the oxyanion hole makes a significantly larger contribution to hirudin binding in
the F form by indirectly affecting Na\textsuperscript{+} binding. The action of these residues is counterbalanced by the role of Glu-192 and Ser-214, which promote binding of hirudin to the S form. In summary, the allosteric control of hirudin recognition depends on the occupancy of the Na\textsuperscript{+} site (Asp-221 and Asp-222), residues in the 30-loop (Lys-36) and exosite I (Leu-65, Thr-74, and Arg-75), as well as residues within the active site (Gly-193 and Ser-214) and its lower rim (Glu-192).

The nature of the epitope for hirudin recognition supports the notion that the binding free energy concentrates at two hot spots in exosite I and the Na\textsuperscript{+} site of thrombin. This conclusion complements and expands information from the pioneering mutagenesis studies of hirudin from Stone and collaborators (20–23). An unexpected feature of the thrombin epitope recognizing hirudin is that Lys-224 by itself makes a staggering contribution to binding. Mutation of this residue to Ala results in a drop in binding affinity of 7 orders of magnitude. Studies on other protein-protein interactions characterized in exquisite detail both structurally and by site-directed mutagenesis, like the human growth hormone-receptor interaction (42) and the barnase-barstar interaction (43), have documented the presence of hot spots for recognition, but in no case was mutation of a single residue found to have such a profound effect on ligand binding. Why, then, is Lys-224 so uniquely important to hirudin binding by thrombin? The observation is difficult to rationalize from the crystal structure of the complex (Fig. 3). According to the structure (15), Lys-224 makes an H-bond with the backbone O atom of Ser-19 of hirudin. The length of the H-bond (3.1 Å) and the three contacts <4 Å hardly explain why removal of the positively charged \epsilon-amino group would cause a free energy penalty of 11 kcal/mol for an interaction that is worth ~18 kcal/mol in the F form and ~16 kcal/mol in the S form. The observation is also difficult to rationalize in terms of the contribution of this residue to thrombin interaction with fibrinogen, PAR1, protein C, or anti-thrombin (24, 25, 44), which make direct contacts with the 220-loop. Lys-224 is important for fibrinogen recognition (25), and the naturally occurring mutant thrombin Scranton, in which Lys-224 is replaced by Thr, is associated with a bleeding phenotype (45). However, the K224A mutation causes a 30-fold drop in affinity for fibrinogen (25), which is an effect 6 orders of magnitude smaller than that experienced by hirudin. We propose that replacement of the Lys-224 side chain must destabilize binding of the entire N-terminal domain of hirudin, perhaps by shifting the register of key interfacial contacts between the inhibitor and the 220-loop of thrombin. The crucial role of Lys-224 uncovered in this study calls into question the uniqueness of the current structural information on the thrombin-hirudin complex (15). New structural work is necessary to shed light on how hirudin contacts the 220-loop of thrombin, and future crystal structures should be obtained under conditions that mimic more closely
the biochemical work presented in this study.

The results reported in this study also point to a lack of correlation between the number of thrombin-hirudin contacts <4 Å and the energetic consequences of removing such contacts by Ala scanning mutagenesis. Lys-224 provides a striking example of the lack of such correlation, but it is by no means an exception. The data in Fig. 4 (open circles) portray the energetic cost of Ala replacement at residues that make contacts <4 Å with hirudin according to the crystal structure (15). Plotted are data from 25 thrombin residues that together account for 188 of the 217 total contacts <4 Å reported in the 4HCG structure (15). The expectation is that mutations of residues involved in crystal contacts with hirudin become more deleterious as the number of contacts increases. A strong positive correlation is therefore expected for the data in Fig. 4. Instead, the number of contacts proves to be a very poor predictor of the energetic contribution to binding. Interestingly, the same conclusion is reached with the mutagenesis data on hirudin (Fig. 4, filled circles) previously reported by Stone and collaborators (20–23). It is possible that the number of contacts observed in the crystal structure is not a faithful reporter of the exact mode of interaction between thrombin and hirudin. Furthermore, it is possible that although the listed contacts are all short range, whether van der Waals or polar, their energetic contribution to binding may be different and not necessarily proportional to their number. Finally, it is possible that the number of contacts may correlate better with the enthalpy rather than the free energy of binding, as observed in the case of intestinal fatty acid-binding protein (46). Although these alternative possibilities cannot be discounted, they likely apply to a small fraction of the large number of mutations presented in this study. More importantly, none of the alternative possibilities explains the significant disruption of hirudin binding upon mutation of residues that make hardly any contacts with the inhibitor. We conclude that, in the case of the thrombin-hirudin interaction, the number of short range contacts detected in the crystal structure of the complex does not correlate with the extent of contribution to binding. This comes as a cogent reminder of the potential pitfalls of using structural information to assess the energetics of protein-protein interactions in general (34).

REFERENCES

1. Di Cera, E. (2004) C. R. Biol. 327, 1065–1076
2. Wells, C. M., and Di Cera, E. (1992) Biochemistry 31, 11721–11730
3. Dang, Q. D., Vindigni, A., and Di Cera, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5977–5981
4. Di Cera, E. (2003) Chest 124, 118–178
5. Nogami, K., Zhou, Q., Myles, T., Leung, L. L., Wakabayashi, H., and Fay, P. J. (2005) J. Biol. Chem. 280, 18476–18487
6. He, X., and Reznik, A. R. (1999) J. Biol. Chem. 274, 4970–4976
7. Schmidt, A. E., Padmanabhan, K., Underwood, M. C., Bode, W., Mather, T., and Bajaj, S. P. (2002) J. Biol. Chem. 277, 28987–28995
8. Reiher, A. R., and He, X. (2000) Biochemistry 39, 1817–1825
9. Zhang, E., and Tulinsky, A. (1997) Biophys. Chem. 61, 185–200
10. Di Cera, E., Guinto, E. R., Vindigni, A., Dang, Q. D., Ayala, Y. M., Wuyi, M., and Tulinsky, A. (1995) J. Biol. Chem. 270, 22069–22092
11. Krems, M. M., Prasad, S., and Di Cera, E. (2002) J. Biol. Chem. 277, 40260–40264
12. Pineda, A. O., Carrell, C. J., Bush, L. A., Prasad, S., Caccia, S., Chen, Z.-W., Mathews, F. S., and Di Cera, E. (2004) J. Biol. Chem. 279, 31842–31853
13. Krishnaswamy, S. (2005) J. Thromb. Haemost. 3, 54–67
14. Stone, S. R., and Hofsteenge, J. (1986) Biochemistry 25, 4622–4628
15. Rydel, T. J., Tulinsky, A., Bode, W., and Huber, R. (1991) J. Mol. Biol. 221, 631–681
16. Betz, A., Hofsteenge, J., and Stone, S. R. (1991) Biochem. J. 275, Pt 3, 801–803
17. Karshikov, A., Bode, W., Tulinsky, A., and Stone, S. R. (1992) Protein Sci. 1, 727–735
18. Stone, S. R., Dennis, S., and Hofsteenge, J. (1989) Biochemistry 28, 6857–6863
19. Ayala, Y. M., Vindigni, A., Nayal, M., Spolar, R. S., Record, M. T., Jr., and Di Cera, E. (1995) J. Mol. Biol. 253, 787–798
20. Braun, P. J., Dennis, S., Hofsteenge, J., and Stone, S. R. (1988) Biochemistry 27, 6517–6522
21. Betz, A., Hofsteenge, J., and Stone, S. R. (1991) Biochemistry 30, 9848–9853
22. Betz, A., Hofsteenge, J., and Stone, S. R. (1992) Biochemistry 31, 4557–4562
23. Wallace, A., Dennis, S., Hofsteenge, J., and Stone, S. R. (1999) Biochemistry 28, 10079–10084
24. Ayala, Y. M., Cantwell, A. M., Rose, T., Bush, L. A., Arosio, D., and Di Cera, E. (2001) Proteins 45, 107–116
25. Rose, T., and Di Cera, E. (2002) J. Biol. Chem. 277, 18875–18880
26. Ayala, Y., and Di Cera, E. (1994) J. Mol. Biol. 235, 733–746
27. Pineda, A. O., Cantwell, A. M., Bush, L. A., Rose, T., and Di Cera, E. (2002) J. Biol. Chem. 277, 32015–32019
28. Xu, H., Bush, L. A., Pineda, A. O., Caccia, S., and Di Cera, E. (2005) J. Biol. Chem. 280, 7956–7961
29. Di Cera, E. (1995) Thermodynamic Theory of Site-specific Binding Processes in Biological Macromolecules. Cambridge University Press, Cambridge, UK
30. Cha, S. (1975) Biochem. Pharmacol. 24, 2177–2185
31. Cha, S. (1976) Biochem. Pharmacol. 25, 2695–2702
32. Dang, Q. D., and Di Cera, E. (1994) J. Protein. Chem. 13, 367–372
33. Krem, M. M., Prasad, S., and Di Cera, E. (2002) Biophys. Chem. 100, 315–323
34. Greenspan, N. S., and Di Cera, E. (1999) Nat. Biotechnol. 17, 936–937
35. Di Cera, E. (1998) Adv. Protein Chem. 51, 59–119
36. Wells, J. A. (1990) Biochemistry 29, 8509–8517
37. Pineda, A. O., Chen, Z. W., Caccia, S., Cantwell, A. M., Savvides, S. N., Waksman, G., Mathews, F. S., and Di Cera, E. (2004) J. Biol. Chem. 279, 38924–38938
38. Biskofchak, K. M., Pineda, A. O., Mathews, F. S., and Di Cera, E. (2005) J. Biol. Chem. 280, 25644–25650
39. Guinto, E. R., Vindigni, A., Ayala, Y. M., Dang, Q. D., and Di Cera, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11185–11189
40. Bode, W., Turk, D., and Karshikov, A. (1992) Protein Sci. 1, 426–471
41. Vindigni, A., and Di Cera, E. (1996) Biochemistry 35, 4447–4446
42. Clackson, T., and Wells, J. A. (1995) Science 268, 383–386
43. Schreiber, G., and Peshrl, A. R. (1995) J. Mol. Biol. 248, 478–486
44. Dang, Q. D., Guinto, E. R., and Di Cera, E. (1997) Nat. Biotechnol. 15, 146–149
45. Sun, W. Y., Smirnow, D., Jenkins, M. L., and Degen, S. J. (2001) Thromb. Haemostasis 85, 651–654
46. Richieri, G. V., Low, P. J., Ogata, R. T., and Kleinfeld, A. M. (1998) J. Biol. Chem. 273, 7397–7405
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