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Thrombomodulin Changes the Molecular Surface of Interaction and the Rate of Complex Formation between Thrombin and Protein C*

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The interaction of thrombin with protein C triggers a key down-regulatory process of the coagulation cascade. Using a panel of 77 Ala mutants, we have mapped the epitope of thrombin recognizing protein C in the absence or presence of the cofactor thrombomodulin. Residues around the Na^+ site (Thr-172, Lys-224, Tyr-225, and Gly-226), the aryl binding site (Tyr-60a), the primary specificity pocket (Asp-189), and the oxyanion hole (Gly-193) hold most of the favorable contributions to protein C recognition by thrombin, whereas a patch of residues in the 30-loop (Arg-35 and Pro-37) and 60-loop (Phe-60h) regions produces unfavorable contributions to binding. The shape of the epitope changes drastically in the presence of thrombomodulin. The unfavorable contributions to binding disappear and the number of residues promoting the thrombin-protein C interaction is reduced to Tyr-60a and Asp-189. Kinetic studies of protein C activation as a function of temperature reveal that thrombomodulin increases >1,000-fold the rate of diffusion of protein C into the thrombin active site and lowers the activation barrier for this process by 4 kcal/mol. We propose that the mechanism of thrombomodulin action is to kinetically facilitate the productive encounter of thrombin and protein C and to allosterically change the conformation of the activation peptide of protein C for optimal presentation to the thrombin active site.

Thrombin is the only enzyme in the blood capable of activating protein C (1). Activated protein C is a natural anticoagulant that inactivates factors Va and VIIIa with the assistance of protein S, thereby promoting the down-regulation of the coagulation cascade (2). In addition to its anticoagulant role, protein C has recently emerged as a regulator of inflammatory response and as an anti-apoptotic agent (3). This has in turn expanded the roles of thrombin in blood coagulation and established an intriguing link between thrombosis and inflammation via the protein C pathway (2, 3).

The interaction of thrombin with protein C has been studied in considerable detail. Under physiologic concentrations of Ca^{2+}, thrombin has only marginal affinity for protein C in the absence of thrombomodulin (4–8). The presence of thrombomodulin increases the k_{cat}/K_m of thrombin for protein C >1,000-fold (4–8). In the absence of Ca^{2+}, thrombomodulin has only a modest effect on protein C activation by thrombin and the reaction proceeds with a k_{cat}/K_m comparable to that observed under physiologic conditions of Ca^{2+} and thrombomodulin (4–8). Because Ca^{2+} has no effect on thrombin structure and function (7) but drastically changes the conformation of the activation peptide of protein C (6), it is reasonable to assume that an important component of thrombomodulin action is to allosterically affect protein C to relieve the inhibitory effect of Ca^{2+} binding on the conformation of the domain containing the scissile bond. However, it is unlikely that such an allosteric effect would explain a >1,000-fold change in k_{cat}/K_m.

Several hypotheses on the action of thrombomodulin have been presented in the literature. Some groups favor the idea that thrombomodulin changes the conformation of thrombin to enable efficient protein C activation. Support to this proposal comes from the observation that binding of thrombomodulin to thrombin alters the spectral properties of probes tethered to the active site of the enzyme (9). Furthermore, several mutations of thrombin have been found to enhance protein C activation in the absence but not in the presence of thrombomodulin (10, 11). These findings suggest that thrombomodulin changes thrombin structure in a way that can be mimicked by ad hoc amino acid substitutions. However, changes in spectral probes are qualitative in nature and may bear little on the mechanism of protein C activation by thrombin. As to the thrombin mutations that increase protein C activation in the absence of thrombomodulin, the effects are small and nowhere close to the large (>1,000-fold) enhancement induced by thrombomodulin. Furthermore, these effects are reproduced entirely by mutations of protein C affecting domains interacting with thrombin (12, 13), thereby raising the possibility that thrombomodulin affects the structure of protein C rather than thrombin. In either case, no evidence has so far been presented that the large enhancement of protein C activation by thrombin induced by thrombomodulin can be reproduced entirely by single or multiple mutations of thrombin or protein C.

In contrast to previous claims of a significant effect of thrombomodulin on the active site of thrombin (9), analysis of the hydrolysis of several chromogenic substrates by thrombin shows little dependence on thrombomodulin binding (14). More importantly, the effect is reproduced both qualitatively and quantitatively by hirugen binding to exosite I, the major determinant of thrombomodulin recognition by thrombin (15), even though hirugen has no effect on the activation of protein C. The crystal structure of thrombin bound to a fragment of thrombomodulin required for protein C activation reveals no changes in the conformation of thrombin (16). Although the structure contains an inhibitor bound to the active site, which may obscure changes within this domain (17), it shows no evidence that thrombomodulin affects the conformation of thrombin away from the active site region.

The foregoing analysis of the literature reveals that no consensus has been reached so far on how thrombomodulin increases the specificity of thrombin toward protein C. Practi-

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cally all of the current views of this unsolved problem are either in favor or against thrombomodulin-induced conformational changes of thrombin or protein C. The interesting possibility that thrombomodulin may act as a scaffold or a template for the productive encounter of thrombin and protein C has received little attention (16, 18). That possibility cannot be discounted in view of the role that facilitated diffusion plays in many reactions of blood coagulation (19). In this study, we took a direct approach to the problem along two lines of investigation. We dissected the epitopes of thrombin recognizing protein C in the absence and presence of thrombomodulin using a panel of 77 Ala mutants. Ala-scanning mutagenesis is the methodology of choice to identify functional epitopes (20–22) and has been successfully applied to thrombin (15, 23–29). Previous studies have identified thrombin residues potentially important for protein C recognition but have been limited to exosite regions and explored only marginally the 60-loop region and the Na^+ binding site comprising loops 220 and 186 (24). The preeminent role of these regions in substrate recognition by thrombin has become apparent in recent years (28, 29), and their important role in protein C activation was recognized as early as the Na^+ site was characterized structurally (30). A more serious limitation of previous studies is that they have provided no information on the epitope of thrombin recognizing protein C in the presence of thrombomodulin, which is obviously crucial to assess whether the binding of cofactor translates in structural changes at the thrombin-protein C interface. In addition to the mutagenesis studies, we present measurements of the rate of diffusion of protein C into the active site of thrombin in the absence or presence of thrombomodulin to assess directly for the first time the contribution of the cofactor to the kinetic step of enzyme-substrate complex formation. The results offer new insights into how thrombomodulin affects the thrombin-protein C interaction.

MATERIALS AND METHODS

Site-directed mutants of human thrombin were expressed, purified, and tested for activity as described (15, 28). The numbering used in this study refers to chymotrypsin(ogen). For each mutant, the equilibrium dissociation constant for thrombomodulin binding was determined by inhibition of fibrinopeptide A release (14, 15). The activation of protein C (Enzyme Research Laboratories) was determined using progress curves of H-D-Asp-Arg-Arg-*p*-nitroanilide (MidWest Biotech) hydrolysis to extract the value of $s = k_{cat}/K_m$ (7, 31). This chromogenic substrate is highly selective for activated protein C (32), and its hydrolysis monitors quantitatively the thrombin-mediated conversion of protein C into the active enzyme. Control experiments of H-D-Asp-Arg-Arg-*p*-nitroanilide hydrolysis by thrombin were carried out for wild type and all of the mutants and confirmed the absence of any significant activity under all of the conditions tested. Experimental conditions were as follows: 5 mM Tris; 0.1% PEG[1]; 145 mM NaCl; 5 mM $CaCl_2$; pH 7.4 at 37 °C. Thrombin wild type and mutants were used in the concentration range from 0.2 to 10 nM depending on their activity. The concentration of protein C was fixed at 300 nM, well below the estimated values of K_m (33), to ensure accurate determinations of k_{cat}/K_m by progress curve analysis (7, 31). Experiments of protein C activation were run in the absence or presence of 10 nM rabbit thrombomodulin (Enzyme Research Laboratories). The value of s under saturating conditions of thrombomodulin was derived from the linkage expression (34) shown in Equation 1,

$$s = \frac{s_0 + s_1\frac{[TM]}{K_d}}{1 + \frac{[TM]}{K_d}} \qquad \text{(Eq. 1)}$$

where s_0 and s_1 are the values of k_{cat}/K_m in the absence and under saturating conditions of thrombomodulin (TM), respectively. The value of s_1 was inferred from the value of s_0, s, and K_d measured independently by rearrangement of Equation 1 as shown in Equation 2,

$$s_1 = s + (s - s_0)\frac{K_d}{[TM]} \qquad \text{(Eq. 2)}$$

where s refers to [TM] = 10 nM. The value of s_1 cannot be approximated from the value of s determined from a single [TM] unless [TM] $\gg K_d$ (see Equation 2). That is the reason why the value of K_d must be measured independently for all mutants. Previous studies (24) have used measurements of protein C activation in the presence and absence of thrombomodulin to distinguish between the effects of mutations on protein C recognition and thrombomodulin binding. Mutations that decrease protein C activation in the absence of thrombomodulin can be used to identify unambiguously residues that are involved in protein C recognition by thrombin. On the other hand, mutations that decrease protein C activation in the presence of a fixed concentration of thrombomodulin cannot be used to identify unambiguously thrombin residues involved in thrombomodulin recognition. That is because the value of s in Equation 1 depends not only on K_d but also on s_1. A mutation that reduces s_1 but has no effect on K_d decreases protein C activation in the presence of thrombomodulin, even though it has nothing to do with thrombomodulin binding. In this study, the value of K_d was measured independently for all mutants under identical solution conditions to establish the effect of mutations on s_1 and to map unambiguously the epitope of thrombin recognizing protein C in the presence of thrombomodulin.

Among the individual rate constants defining the mechanism of substrate hydrolysis by serine proteases, the value of k_1 defining the productive encounter between substrate and enzyme is of utmost mechanistic importance. This value can be derived directly from measurements of s as a function of temperature (35–37). The value of s for protein C activation by thrombin in the absence or presence of 100 nM thrombomodulin was measured in the temperature range from 5 to 45 °C under experimental conditions of 5 mM Tris, 0.1% PEG, 100 mM NaCl, and 5 mM $CaCl_2$, pH 8.0. The pH was precisely adjusted at room temperature to obtain the value of 8.0 at the desired temperature. Tris buffer has a $pK_a = 8.06$ at 25 °C and a temperature coefficient of $\Delta pK_a/\Delta T = -0.027$ (38). These properties ensured buffering over the entire temperature range examined. Under these conditions, the K_d for thrombomodulin binding is around 1 nM and changes little with temperature (14). Measurements of s in the presence of 100 nM thrombomodulin are therefore excellent estimates of s_1 (see Equation 2). The explicit expression for s as a function of temperature (35–37) is shown in Equation 3,

$$s = k_1 \exp\left\{-\frac{E_1}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right\}\frac{\alpha\exp\left\{\frac{E_\alpha}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right\}}{1 + \alpha\exp\left\{\frac{E_\alpha}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right\}} \qquad \text{(Eq. 3)}$$

where R is the gas constant, T the absolute temperature, and $\alpha = k_2/k_{-1}$ defines the "stickiness" of substrate as the ratio between k_2 (the rate of acylation) and k_{-1} (the rate of dissociation of the enzyme-substrate complex into the parent species). The Arrhenius terms E_1 and $E_\alpha = E_{-1} - E_2$ define, respectively, the activation energy for substrate binding to the active site of the enzyme and the difference between the activation energies for substrate dissociation and acylation. The values of k_1 and α refer to the reference temperature $T_0 = 298.15$ K. The measurements of s as a function of temperature resolve k_1, α, E_1, and E_α in Equation 3 provided that the plot shows curvature. The curvature is indicative of a change in the rate-limiting step for substrate hydrolysis due to the shift from $k_2 \gg k_{-1}$ at low temperatures to $k_{-1} \gg k_2$ at high temperatures. The shift is caused by the drastic difference in activation energies for substrate acylation and dissociation when $E_{-1} \gg E_2$ or $E_\alpha \gg 0$.

RESULTS

Seventy-seven residues of thrombin located in exosite I, exosite II, the 60-loop region, the specificity sites S1-S4, and the Na^+ binding site were targeted by mutagenesis. For each mutant, the value of K_d for thrombomodulin binding was determined as well as the values of s_0 and s_1 (see Equation 1) for protein C activation (Fig. 1). These are the three independent parameters defining the linkage expression in Equation 1. The measurements of the K_d for thrombomodulin binding are crit-

[1] The abbreviations used are: PEG, polyethylene glycol; TM, thrombomodulin.

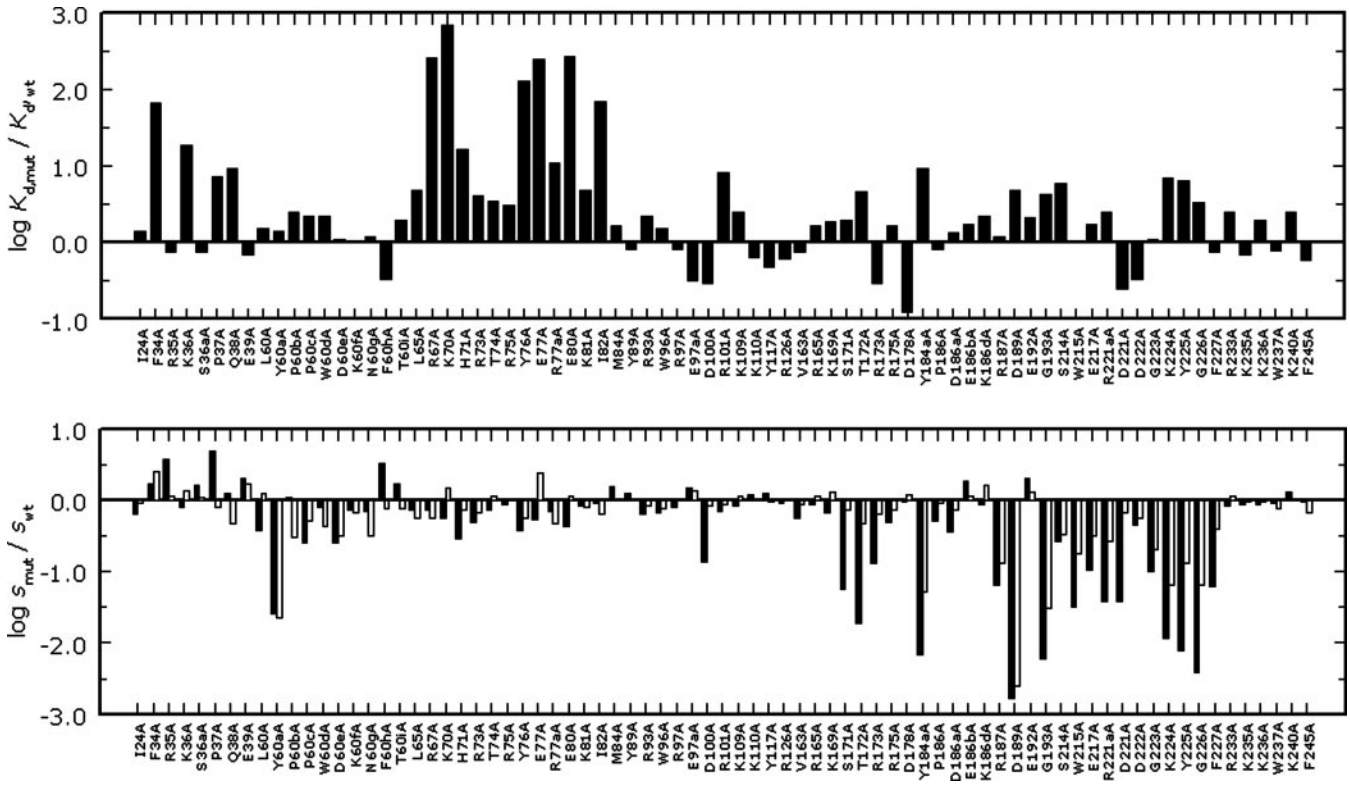

FIG. 1. *Top panel*, Ala-scanning mutagenesis mapping of the epitope of thrombin recognizing thrombomodulin. Shown is the change in binding affinity due to mutation, expressed as log $K_{d,mut}/K_{d,wt}$, under experimental conditions of 5 mM Tris, 0.1% PEG, 145 mM NaCl, and 5 mM $CaCl_2$, pH 7.4 at 37 °C. The value of $K_{d,wt}$ is 1.2 ± 0.1 nM. The profile extends and completes the results of previous mutagenesis studies (15). *Bottom panel*, Ala-scanning mutagenesis mapping of the epitopes of thrombin recognizing protein C in the absence (*black bars*) or presence (*white bars*) of thrombomodulin. Shown is the change in the specificity constant due to mutation, expressed as log s_{mut}/s_{wt}, under experimental conditions of 5 mM Tris, 0.1% PEG, 145 mM NaCl, and 5 mM $CaCl_2$, pH 7.4 at 37 °C. *White bars* refer to saturating conditions of thrombomodulin derived from measurements in the presence of 10 nM thrombomodulin and extrapolated using Equation 2. The values of s_0 and s_1 for wild type are 59 ± 3 M^{-1} s^{-1} and 0.25 ± 0.02 μM^{-1} s^{-1}.

ical to the definition of the boundaries of the epitope-recognizing protein C in the presence of cofactor. The results presented in Fig. 1 extend our recent mapping of the thrombin epitope recognizing thrombomodulin (15) by adding information on the role of the 60-loop region and the 220 and 186 loops defining the Na^+ site. These domains make only small (<10-fold changes in K_d) contributions to thrombomodulin binding and maintain exosite I as the dominant determinant for thrombomodulin recognition.

The functional epitopes for protein C recognition in the absence or presence of thrombomodulin are depicted on the surface of thrombin in Fig. 2. There are significant differences in the structure of the epitopes that underscore molecular changes in the thrombin-protein C interface due to cofactor binding. In the absence of thrombomodulin, there are several residues of thrombin critically involved in protein C recognition. These residues cluster around the Na^+ site (Thr-172, Tyr-184a, Lys-224, Tyr-225, and Gly-226) and define the primary specificity pocket (Asp-189), the oxyanion hole (Gly-193), and portions of the aryl binding site in the 60-loop region (Tyr-60a). With the exception of Lys-224, none of these residues was targeted by previous Ala-scanning mutagenesis studies (24). Contrary to previous claims (24), mutations of Trp-60d, Glu-217, and Arg-221a were found to have a small effect on protein C recognition and certainly not comparable to that of the critical residues identified in this study and are labeled in *yellow* and *red* in Fig. 2. A striking property of the epitope is that it contains residues that once mutated to Ala produce a small but significant enhancement of protein C activation. These residues are Arg-35 and Pro-37 in the 30-loop region and Phe-60h at the distal end of the 60-loop region. Interestingly, these residues lay close to each other (*blue* in Fig. 2) and define a patch on the surface of thrombin next to the active site where docking of protein C is unfavorable in the wild type. The role of Arg-35 has been pointed out in previous studies (11, 24), together with the unfavorable contribution of other residues in the 30-loop region like Ser-36a, Gln-38, and Glu-39 (24). We did not see any significant effect on protein C activation upon mutation of these residues, although our mutation of Pro-37 may have non-local effects involving neighbor residues like Ser-36a and Gln-38 combined. The spatial contiguity of Phe-60h, Arg-35, and Pro-37 is striking and suggests that a domain of protein C experiences unfavorable contacts with this region upon binding to thrombin. Indeed, Grinnell *et al.* (13) have identified a cluster of positively charged residues in the primed sites of protein C (Lys-174, Arg-177, and Arg-178) that once mutated to Glu produces a mutant that is activated 16-fold faster by thrombin in the absence but not in the presence of thrombomodulin. They suggested that the cluster experiences electrostatic repulsion with the positively charged region of exosite I of thrombin. Given that exosite I makes no contribution to protein C recognition (Fig. 2), it is likely that some of these positively charged residues of protein C clash with Arg-35 and the 30-loop region of thrombin.

The scenario changes significantly when thrombomodulin binds to thrombin (Figs. 1 and 2). Accurate measurements of the K_d for thrombomodulin binding enabled estimation of s_1 in Equation 1 from which a detailed picture of the epitope could be obtained unambiguously for the first time. Recognition of protein C by thrombin involves a much smaller set of residues compared with the absence of cofactor. Practically only Asp-189 in the primary specificity pocket and Tyr-60a in the aryl bind-

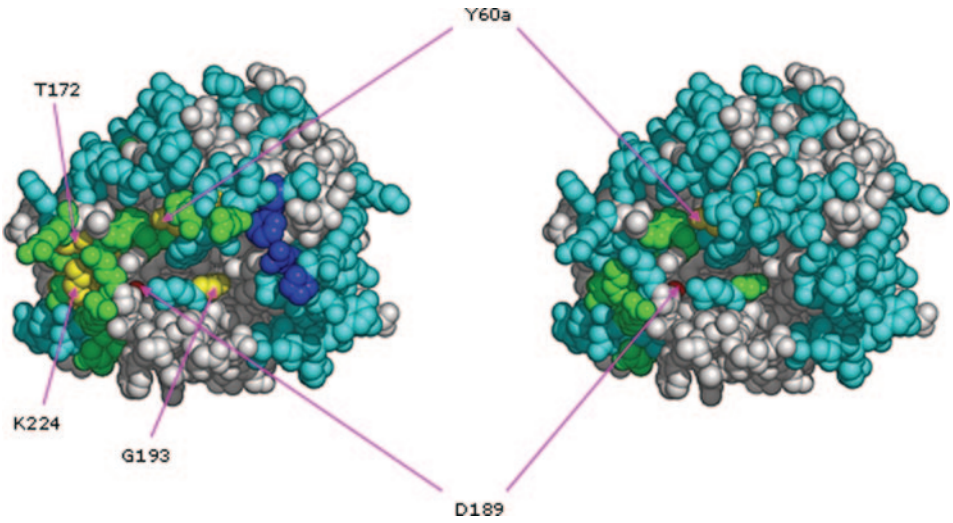

FIG. 2. **Space-filling model of thrombin in the Bode orientation (active site, *center*; exosite I, *right*; exosite II, *top left*; Na^+ site, *bottom left*; 60-loop, *top*) depicting the structural organization of the epitopes recognizing protein C in the absence (*left*) or presence (*right*) of thrombomodulin.** Residues affected by Ala replacement are color-coded based on the log change in the value of *s* for protein C activation (see Fig. 1). *Blue*, −1.5/−0.5 units (1/30- to 1/3-fold change in *s*); *cyan*, −0.5/0.5 units (1/3- to 3-fold change in *s*); *green*, 0.5/1.5 units (3- to 30-fold change in *s*); *yellow*, 1.5/2.5 units (30- to 300-fold change in *s*); *red*, >2.5 units (>300-fold change in *s*). Residues not subject to Ala-scanning mutagenesis are in *gray*. Crucial residues are labeled. The epitope in the absence of thrombomodulin is split into one domain providing favorable interactions (residues in *yellow* and *red*: visible are Tyr-60a, Thr-172, Asp-189, Gly-193, and Lys-224) and a second domain providing steric/electrostatic hindrance (residues in *blue*: from top to bottom, Phe-60h, Arg-35 and Pro-37). In the presence of thrombomodulin, the shape of the epitope changes drastically and only Tyr-60a and Asp-189 make significant contribution to protein C recognition. The region of unfavorable contributions to recognition (residues in *blue* at *left*) disappears.

ing site make a dominant (>30-fold change in s_1 relative to wild type) contribution to binding. The critical role played by the oxyanion hole (Gly-193) and the Na^+ site (Thr-172, Tyr-184a, Lys-224, Tyr-225, and Gly-226) is diminished when thrombomodulin binds. The unfavorable contribution from residues in the 30-loop region and the distal portion of the 60-loop region disappears in the presence of thrombomodulin. Because the binding of thrombomodulin depends little on residues located in the 30 loop, the 60 loop, the oxyanion hole, and the Na^+ site (Fig. 1), the change in the epitope is not due to hijacking of these domains by the cofactor. The drastic difference in the epitopes is due to the mode of interaction of protein C with thrombin and proves that the thrombin-protein C interface changes when thrombomodulin is bound. The interface is reduced to the primary specificity pocket and the aryl binding site around Tyr-60a in the presence of cofactor.

A crucial component of any recognition process is the formation of the enzyme-substrate complex. The rate constant, k_1, defining this step (see Equation 3) is limited by diffusion (39) or conformational rearrangements that facilitate docking. Its energetic balance is determined by the Arrhenius energy of activation E_1 (see Equation 1). Typically, diffusion-limited enzyme-substrate encounters feature k_1 values in excess of $10^7\ M^{-1}\ s^{-1}$ linked to small activation energies ($E_1 < 10$ kcal/mol) (39). Values of E_1 that exceed 20 kcal/mol are conducive to structural rearrangements of the enzyme-substrate complex that facilitate productive complex formation and are usually linked to slower ($<10^7\ M^{-1}\ s^{-1}$) values of k_1. Therefore, information on k_1 and E_1 is quite valuable in establishing mechanisms of enzyme-substrate interaction. In the case of thrombin interacting with protein C, these values can be used to establish the effect of thrombomodulin on how protein C docks on the thrombin active site to enable productive binding and activation. Fig. 3 presents the Arrhenius plots of log *s versus* 1/*T* from which the values of k_1 at 25 °C and E_1 can be extracted using Equation 3. The specificity constant *s* increases three orders of magnitude in the presence of cofactor. The shape of the Arrhenius plot changes little in the presence of thrombomodulin (see *discontinuous line*). The value of E_1 decreases by 4 kcal/mol, and k_1

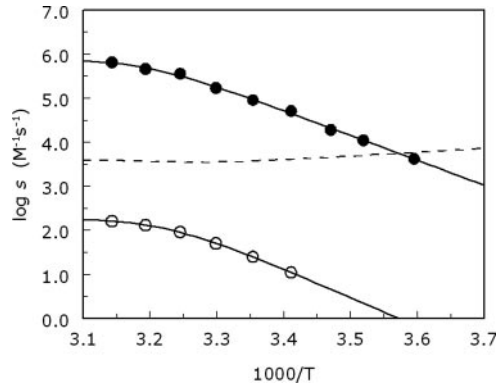

FIG. 3. **Arrhenius plots of the specificity constant $s = k_{cat}/K_m$ for the cleavage of protein C by thrombin in the absence (○) or presence (●) of 100 nM thrombomodulin over the temperature range from 5 to 45 °C.** Values in the absence of cofactor could only be measured accurately at temperatures >20 °C. Note the use of the decimal logarithm in the ordinate. Experimental conditions are as follows: 5 mM Tris; 0.1% PEG; 100 mM NaCl; 5 mM $CaCl_2$; pH 8.0. *Continuous lines* were drawn according to Equation 3 with best-fit parameter values listed in Table I. The *discontinuous line* depicts the difference in the temperature dependence of log *s* between the two sets of data and gives a direct representation of the effect of thrombomodulin on the hydrolysis of protein C by thrombin. This difference is almost temperature independent and underscores the dominant role of thrombomodulin as facilitating the diffusion of protein C into the thrombin active site.

approaches the diffusion-limited regime (Table I). The data in Fig. 3 proves that thrombomodulin improves diffusion of protein C into the thrombin active site and that this effect is linked to a small but significant conformational change that reduces the energetic barrier for formation of the productive enzyme-substrate complex by nearly 4 kcal/mol. The large value of E_1 in the absence of thrombomodulin indicates substantial structural strains linked to the formation of the thrombin-protein C complex. The strains are partially relieved by the presence of cofactor. The main function of thrombomodulin is to facilitate diffusion of protein C into the thrombin active site. An ancillary

TABLE I
Kinetic rate constants and activation energies for substrate hydrolysis by thrombin

Experimental conditions are as follows: 5 mM Tris; 0.1% PEG; 100 mM NaCl; 5 mM $CaCl_2$, pH 8.0.

	PC	PC + TM	Fibrinogen[a]	FPR[b]
k_1 (μM^{-1} s^{-1})	$27 \pm 1 \times 10^{-6}$	0.10 ± 0.02	19 ± 3	120 ± 15
$\alpha = k_2/k_{-1}$	11 ± 3	10 ± 4	2.6 ± 0.4	2.6 ± 0.3
E_1 (kcal/mol)	30 ± 1	26 ± 1	12 ± 2	19 ± 2
$E\alpha$ (kcal/mol)	33 ± 1	24 ± 2	28 ± 3	36 ± 3

[a] Data from reference (36). The values of k_1 and α refer to 25 °C.
[b] Data from reference (37). Abbreviations: PC, protein C; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide.

function is to induce changes in the molecular surface of recognition between thrombin and protein C.

The parameters α and $E\alpha$ in Equation 3 reveal further aspects of the thrombin-protein C interaction. Protein C behaves as a "sticky" substrate with the rate of acylation at 25 °C exceeding that of substrate dissociation by one order of magnitude. The stickiness of protein C is not altered by the presence of thrombomodulin, suggesting that the cofactor does not influence the propensity of the enzyme-substrate complex to undergo acylation rather than dissociation. On the other hand, the value of $E\alpha$ changes significantly in the presence of thrombomodulin. The large positive nature of this term signals a substantial difference in the energetic cost of dissociating the substrate compared with acylating it. Thrombomodulin reduces this difference, presumably by lowering the barrier for diffusion of protein C back into solution once in complex with thrombin. This effect echoes the energetic facilitation of diffusion of protein C into the thrombin active site and further supports the role of thrombomodulin as a scaffold promoting the formation of the thrombin-protein C complex.

It is of interest to compare the mechanism of thrombin-protein C interaction with that involving fibrinogen or chromogenic substrate recognition (Table I). In the case of fibrinogen, the value of k_1 is very fast and the value of E_1 is very small (36). The interaction is a rigid body association controlled by diffusion. Interaction of thrombin with the chromogenic substrate H-D-Phe-Pro-Arg-*p*-nitroanilide is also extremely fast (37). However, the value of E_1 is significantly higher than in the case of fibrinogen, underscoring a conformational rearrangement of substrate to promote complex formation. The large value of E_1 observed for thrombin-protein C interaction rules out a rigid body association as seen for thrombin-fibrinogen interaction. Also, the large variability of the value of E_1 seen among different substrates indicates that any conformational rearrangement linked to binding involves the substrate rather than the enzyme (Table I). Finally, both fibrinogen and the chromogenic substrate are less sticky than protein C, whereas their $E\alpha$ values are in the same range as those observed for protein C in the absence and presence of cofactor.

DISCUSSION

In this study, we have identified in greater detail the epitope of thrombin recognizing protein C, and for the first time, we have provided evidence that the epitope changes significantly upon thrombomodulin binding. Recognition of protein C involves two main regions of thrombin that contribute favorably and unfavorably to binding in the transition state. The region contributing favorably to protein C recognition encompasses residues of the Na^+ site (Thr-172, Tyr-184a, Lys-224, Tyr-225, and Gly-226), the primary specificity pocket (Asp-189), the oxyanion hole (Gly-193), and portions of the aryl binding site (Tyr-60a). The region providing unfavorable contribution to protein C recognition is shaped as a small patch in the 30-loop region (Arg-35 and Pro-37) and the adjacent distal protion of the 60-loop region (Phe-60h). The role of this region has partially been uncovered in previous studies (11, 24). The spatial contiguity of Phe-60h, Arg-35, and Pro-37 (Fig. 1) suggests that an extended domain of protein C is sterically or electrostatically hindered in its docking onto the thrombin surface. This domain may coincide with the cluster of positively charged residues identified by Grinnell *et al.* (13). The epitope changes drastically when thrombomodulin binds to thrombin, and only Asp-189 and Tyr-60a make a significant contribution to protein C recognition.

The Ala scan unravels the functional epitope for recognition, which often but not always overlap with the structural epitope (22). To first approximation, the Ala substitution of any residue is an "adiabatic" perturbation that shaves the side chain down to the Cβ atom while retaining the chirality (with the exception of Gly) (40). Under this assumption, the functional epitope revealed by Ala scans should overlap substantially with the structural epitope determined by crystallographic investigation. In the case of thrombin, the current information on the possible overlap between functional and structural epitopes favors the conclusion that Ala scans reproduce quite well the boundaries emerged from structural investigation. This is particularly evident in the case of the thrombin-thrombomodulin interaction (15) for which relevant crystallographic information has recently emerged (16). Furthermore, recent structures of Ala mutants of thrombin reveal perturbations that are confined to the site of mutation even when they cause substantial disruption of catalytic activity (17, 41). These findings support the conclusion that our Ala scan (Fig. 2) reveals to a first approximation the topology of the thrombin-protein C interface and that the surface of recognition between the two proteins is drastically reduced in the presence of thrombomodulin. This represents a new insight into the action of the cofactor.

Studies on the temperature dependence of s for protein C activation indicate that the major effect of thrombomodulin is to facilitate diffusion of protein C in the thrombin active site. A small but significant change was observed in the value of E_1 linked to complex formation. The value of activation energy decreases 4 kcal/mol and underscores a conformational change that facilitates binding. This energetic signature complements the changes in the epitope documented by Ala-scanning mutagenesis. Because the epitope changes in the presence of cofactor, the conformation of thrombin and/or protein C must change as necessary. If thrombomodulin only acted to enhance diffusion of protein C into the thrombin active site, the residues recognizing protein C in the absence of thrombomodulin would be the same as those recognizing the substrate in the presence of cofactor. In this case, the Ala scan would have returned nearly identical consequences for Ala replacements of thrombin residues on the activation of protein C in the absence or presence of thrombomodulin.

We conclude that thrombomodulin promotes diffusion of protein C into the thrombin active site, which is indicated eloquently by the drastic change in the value of k_1 when thrombomodulin binds. Additional contributions must come from conformational rearrangements of either thrombin or protein C as evidenced by the small but significant decrease in the value of E_1. Because this parameter changes with different substrates (Table I), it is quite likely that thrombomodulin changes the conformation of protein C rather than thrombin. This possibility was first voiced by Hayashi *et al.* (42) and is consistent with a large body of kinetic, thermodynamic, and structural data (9, 14, 16, 43) as well as with the recent observation that Ca^{2+} and Na^+ binding affect the conformation of the activation peptide of protein C (6). The flexibility of this domain documented by fluorescence spectroscopy suggests that thrombo-

modulin may change the orientation of the scissile bond, which should cause the change in E_1 observed experimentally. As the scissile bond is presented faster and optimally to thrombin by the action of the cofactor, the contacts made by protein C with thrombin are reduced to the primary specificity pocket (Asp-189) and portions of the aryl binding site (Tyr-60a). Because these domains are involved in protein C recognition regardless of the presence of thrombomodulin, it is reasonable to expect no functionally relevant changes in the active site region of thrombin upon thrombomodulin binding as indeed found experimentally (14). The effect of thrombomodulin on the molecular surface of recognition between thrombin and protein C is felt mainly at the Na^+ site region and at the 30-loop region (Fig. 2) to which chromogenic substrates do not have access. Future analysis of the action of thrombomodulin should be aimed at providing information on how the cofactor influences allosterically the conformation of the activation peptide of protein C. We predict that thrombomodulin reverses the negative influence of Ca^{2+} on the conformation of this peptide (6) and that this change results in the reduction or abrogation of contacts with the Na^+ site and the 30-loop region of thrombin. This conformational change, together with the facilitated diffusion provided by the thrombomodulin scaffold, enables protein C to be efficiently activated by thrombin.

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