Energetics of Thrombin−Thrombomodulin Interaction†

Alessandro Vindigni,‡ Christopher E. White,§ Elizabeth A. Komives,§ and Enrico Di Cera*,‡

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, Missouri 63110, and Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093-0601

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ABSTRACT: Temperature and salt dependence studies of thrombin interaction with thrombomodulin, with and without chondroitin sulfate, and two fragments containing the EGF-like domains 4−5 and 4−5−6 reveal the energetic signatures and the mechanism of recognition of this physiologically important cofactor. Binding of thrombomodulin is affected drastically by the particular salt present in solution and is positively linked to Na^+ binding to thrombin and the conversion of the enzyme from the slow to the fast form, but is opposed by Cl^- binding to the fibrinogen recognition site and especially to the heparin binding site. Binding of thrombomodulin has an unusually large salt dependence ($\Gamma_{salt} = -4.8$) contributed mostly by the polyelectrolyte-like nature of the chondroitin sulfate moiety that binds to the heparin binding site and increases the affinity of the cofactor by almost 10-fold. On the other hand, the chondroitin sulfate has no effect on the ΔC_p of binding, which is determined predominantly by contacts made by the EGF-like domains 5 and 6 with the fibrinogen recognition site. The modest heat capacity change (−0.2 kcal mol^{-1} K^{-1}) observed when thrombomodulin binds to the fast form suggests a rigid-body association of the cofactor with the enzyme. In the slow form, however, the heat capacity change is significantly more pronounced (−0.5 kcal mol^{-1} K^{-1}) and signals the presence of a conformational transition of the enzyme linked to binding of the cofactor that mimics the slow→fast conversion. These results demonstrate that recognition of thrombomodulin by thrombin is steered electrostatically by the highly charged regions of the fibrinogen recognition site and the heparin binding site, to which the chondroitin sulfate moiety binds and enhances the affinity of the interaction. The recognition event also involves conformational changes of the enzyme in the slow form mediated by binding of the EGF-like domains 5−6 to the fibrinogen recognition site. Consistent with this model, binding of thrombomodulin to the fast form has only a small effect on the hydrolysis of nine chromogenic substrates carrying substitutions at P1, P2, and P3 aimed at probing the environment of the specificity sites S1, S2, and S3 of the enzyme. Binding to the slow form, on the other hand, enhances the specificity toward all substrates up to 15-fold. For substrates carrying a Gly at P2, binding of thrombomodulin changes the relative specificity of the slow and fast forms and makes the slow form more specific. Interestingly, these effects are not specific of thrombomodulin and depend solely on binding to the fibrinogen recognition site of the enzyme. In fact, they are also observed with the hirudin C-terminal fragment 55−65. The characterization of the mechanism of thrombin−thrombomodulin interaction and the effects of the cofactor on the hydrolysis of chromogenic substrates probing the interior of the catalytic pocket bear on the thrombomodulin-induced enhancement of protein C cleavage by thrombin. We propose that this enhancement is due predominantly to an effect of thrombomodulin on the bound protein C in the ternary complex. Therefore, thrombomodulin would carry out its physiological function by making protein C a better substrate for thrombin, rather than making thrombin a better enzyme for protein C.

Thrombomodulin is a cofactor present on the surface of endothelial cells that markedly increases the ability of thrombin to activate protein C while inhibiting in a competitive manner fibrinogen binding (Esmon, 1989). Human thrombomodulin contains 557 residues and consists of 5 distinct domains: a 226-residue N-terminal domain with partial similarity to lectin-like molecules (Petersen, 1988), a 236-residue domain containing 6 epidermal growth factor-like (EGF-like)[1] domains, a glycosylated Ser/Thr-rich domain of 34 amino acids, a transmembrane domain of 23 amino acids, and a C-terminal cytosolic tail of 38 residues (Jackman

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‡ Washington University School of Medicine.

§ University of California at San Diego.

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[1] Abbreviations: rTM, rabbit thrombomodulin; hTM^+, human thrombomodulin with chondroitin sulfate; hTM^-, human thrombomodulin without chondroitin sulfate; EGF, epidermal growth factor; DPR, H-L-Asp-Pro-Arg-*p*-nitroanilide; FGK, H-D-Phe-Gly-Lys-*p*-nitroanilide; FGR, H-D-Phe-Gly-Arg-*p*-nitroanilide; FPK, H-D-Phe-Pro-Lys-*p*-nitroanilide; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; hir^{55-65}, fragment 55−65 H-DEFFIPEEYLQ-OH of hirudin; FpA, fibrinopeptide A; Ch, choline; HPLC, high-performance liquid chromatography; PEG, poly(ethylene glycol); PPACK, H-D-Phe-Pro-Arg-chloromethyl ketone; TM^{4-5}, fragment of human thrombomodulin with EGF-like domains 4 and 5; TM^{4-5-6}, fragment of human thrombomodulin with EGF-like domains 4, 5, and 6; Tris, tris(hydroxymethyl)aminomethane; VGK, H-D-Val-Gly-Lys-*p*-nitroanilide; VGR, H-D-Val-Gly-Arg-*p*-nitroanilide; VPK, H-D-Val-Pro-Lys-*p*-nitroanilide; VPR, H-D-Val-Pro-Arg-*p*-nitroanilide.

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et al., 1986; Wen et al., 1987; Suzuki et al., 1987). The EGF-like domains 5 and 6 are essential for thrombomodulin binding to thrombin, while the EGF-like domain 4 is required for cofactor activity (Ye et al., 1992; Hayashi et al., 1990; Tsiang et al., 1992; Lougheed et al., 1995; Meininger et al., 1995). Crystallographic and NMR studies indicate that the EGF-like domains 5 and 6 bind thrombin at the fibrinogen recognition site (Srinivasan et al., 1994; Mathews et al., 1994). Some thrombomodulin molecules contain a chondroitin sulfate moiety linked to the Ser/Thr-rich domain that precedes the transmembrane domain (Bourin et al., 1986; Parkinson et al., 1992). The chondroitin sulfate is present in rabbit thrombomodulin (Bourin et al., 1986), but the fraction of human thrombomodulin that contains the glycosaminoglycan moieties is unknown (Sadler et al., 1993). The chondroitin sulfate moiety does not contribute to the cofactor activity, but it is important for the direct anticoagulant activity of thrombomodulin. Rabbit thrombomodulin devoid of chondroitin sulfate retains the ability to enhance protein C activation, but has a reduced capacity of inhibiting fibrinogen clotting and activation of factor V (Bourin et al., 1990). In addition to promoting direct anticoagulant activity, the chondroitin sulfate moiety modestly accelerates the rate of inhibition of thrombin by antithrombin III, while in the presence of heparin it has the opposite effect, suggesting a possible competition with heparin binding at the highly basic heparin binding site of thrombin (Bourin, 1989). Other studies support this possibility (Ye et al., 1993, 1994; Weisel et al., 1996).

Previous studies have been invaluable to identify the major determinants of thrombomodulin function, but have not provided a mechanistic framework for the interaction of thrombomodulin with thrombin. It is still not clear how regions of thrombin like the fibrinogen recognition site and the heparin binding site contribute to recognition of the cofactor and how this contribution manifests itself energetically. Furthermore, the detailed effects of thrombomodulin on thrombin specificity remain to be elucidated. All of these aspects of thrombin−thrombomodulin interaction demand a thorough elucidation of the mechanism of recognition. To this effect, we have followed a strategy recently introduced for the study of thrombin−hirudin (Ayala et al., 1995) and thrombin−fibrinogen (Vindigni & Di Cera, 1996) interactions. Here we report a systematic investigation of the effects of temperature and salts on thrombomodulin binding to thrombin using rabbit thrombomodulin (rTM), human thrombomodulin with and without the chondroitin sulfate moiety (hTM^+ and hTM^-), and two fragments containing the EGF-like domains 4−5 and 4−5−6 of human thrombomodulin (TM^{4-5} and TM^{4-5-6}). Comparison of the results obtained with these different molecules shows unequivocally the role of the chondroitin sulfate moiety and the EGF-like domains in the recognition process, along with the hydrophobic and electrostatic components. The magnitude of the heat capacity changes helps identify the extent of the structural changes linked to recognition of the cofactor by thrombin. In addition, we examine the effect of thrombomodulin on thrombin specificity using a library of chromogenic substrates carrying substitutions at P1, P2, and P3 that report on the energetics of the S1, S2, and S3 sites of thrombin in the slow and fast forms.

MATERIALS AND METHODS

Human fibrinogen and rabbit thrombomodulin were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The synthetic hirudin fragment hir^{55-65} was synthesized by the solid-phase Fmoc method, purified to homogeneity by HPLC, and analyzed by mass spectrometry. Recombinant human thrombomodulin with and without chondroitin sulfate was obtained from Eli-Lilly (Indianapolis, IN). The thrombomodulin fragments TM^{4-5} and TM^{4-5-6} were expressed in *Pichia pastoris* yeast and purified to homogeneity using a combination of ion-exchange chromatography and reverse-phase HPLC (White et al., 1995). All chromogenic substrates were synthesized by solid phase, purified to homogeneity by HPLC, and analyzed by mass spectrometry.

Binding to the slow and the fast forms of thrombin was studied under experimental conditions of 5 mM Tris, 0.1% PEG, and pH 8.0, over the temperature range from 5 to 45 °C as detailed elsewhere (Ayala et al., 1995). At each temperature, the properties of the fast form were derived as the extrapolation at $[Na^+]\rightarrow\infty$ from the values at 200 mM ChCl and NaCl using the values of Na^+ binding affinity reported elsewhere (Guinto & Di Cera, 1996). The K_d for the binding of thrombomodulin and its fragments, as well as for hir^{55-65}, was measured from the competitive inhibition of the release of fibrinopeptide A using a strategy that minimizes the amount of materials needed for the determinations. This was particularly important for precious reagents like hTM^-, TM^{4-6}, and TM^{4-5-6} in view of their low affinity. The strategy optimizes a previous analysis of thrombomodulin binding based on the inhibition of fibrinogen binding to thrombin (Hofsteenge et al., 1986). First, the progress curve of FpA release was measured under the desired solution conditions in the absence of competitive inhibitor to determine the value of the specificity constant k_{cat}/K_m (Vindigni & Di Cera, 1996). The point along the progress curve most sensitive to competitive inhibition is at time $t_c = K_m e_T k_{cat}$, as it can be demonstrated easily. Then, the amount of FpA released at time t_c was determined as a function of the concentration of competitive inhibitor to derive the value for the K_d of binding using the equation:

$$[\mathrm{FpA}] = [\mathrm{FpA}]_\infty\left\{1 - \exp\left[-k_{cat}e_T t_c/K_m\left(1 + \frac{[\mathrm{L}]}{K_d}\right)\right]\right\} \quad (1)$$

where L stands for thrombomodulin, its fragments, or hir^{55-65}, e_T is the active thrombin concentration, k_{cat}/K_m is the specificity constant for FpA release, and $t_c = K_m/k_{cat}e_T$. All progress curves were measured by HPLC as detailed (Vindigni & Di Cera, 1996). A typical data set is shown in Figure 1. Competitive inhibition of FpA release was observed under all conditions examined for thrombomodulin and its fragments and hir^{55-65}.

The values of K_d measured as a function of temperature were analyzed according to the equation (Ayala et al., 1995; Guinto & Di Cera, 1996):

$$\Delta G = RT\ln K_d = \Delta H - T\Delta S = \Delta C_p(T - T_H) - T\Delta C_p \ln\frac{T}{T_S} \quad (2)$$

where T_H and T_S are the temperatures where the binding

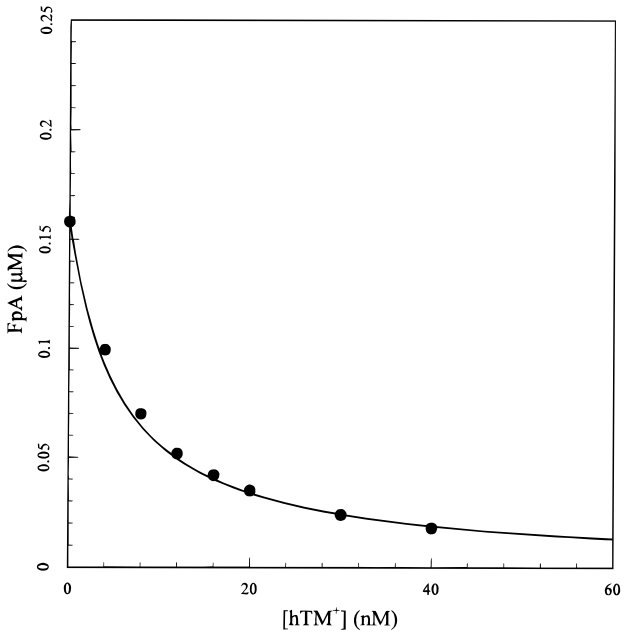

FIGURE 1: Amount of Fpa released time $t_c = K_m/e_T k_{cat}$ as a function of hTM^+ concentration. Experimental conditions are: 5 mM Tris, 200 mM NaCl, 0.1% PEG, pH 8.0 at 25 °C. The continuous line was drawn according to eq 1 in the text, with the best-fit parameters: $K_d = 3.8 \pm 0.1$ nM, $k_{cat}/K_m = 13.7 \pm 0.2$ μM^{-1} s^{-1}, $e_T = 0.2$ nM, $[FpA]_\infty = 0.257 \pm 0.003$ μM.

enthalpy and entropy are zero and ΔC_p is the heat capacity change associated with the reaction.

The salt dependence of the K_d was studeid in the salt concentration range from 100 to 200 mM when using NaCl and ChCl and from 200 to 500 mM when using NaF. The large salt dependence of thrombomodulin binding (see Results) precluded a study over a wider salt concentration range. The results were analyzed according to the equation (Guinto & Di Cera, 1996; Vindigni & Di Cera, 1996):

$$-\ln K_d = A_0 + \Gamma_{salt} \ln [salt] \quad (3)$$

where A_0 is the value of $-\ln K_d$ where [salt] = 1 M and Γ_{salt} is the phenomenological coefficient that quantifies the change in $\ln K_d$ due to a change in ln [salt].

The environment of the specificity sites S1, S2, and S3 of thrombin (Bode et al., 1992) was probed with a library of substrates carrying substitutions at position P1, P2, or P3 meant to perturb the interaction with the corresponding specificity site and to return information on its energetic properties. Details of the theoretical basis for this approach are given elsewhere (Di Cera, 1995; Di Cera et al., 1997). The set of substrates was generated from FPR that mimics the irreversible inhibitor PPACK for which a high-resolution crystal structure of the complex with thrombin is available (Bode et al., 1992). Starting from FPR, seven substitutions were made to generate the three single substitutions (FPK, FGR, and VPR), and three double substitutions (FGK, VPK, and VGR), and the triple substitution (VGK). In addition, H-D-Phe was replaced with H-L-Asp in DPR to mimic the sequence cut by thrombin in the natural substrate protein C. The specificity constant $s = k_{cat}/K_m$ for substrate hydrolysis was measured in all cases from analysis of progress curves. Measurements were carried out under experimental conditions of 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. The properties of the slow form of thrombin were studied in the presence of 200 mM ChCl. Those of the fast form were obtained from measurements carried out at 200 mM [NaCl] and extrapolating at $[Na^+] \rightarrow \infty$ using the known Na^+ binding affinity (Guinto & Di Cera, 1996). The effect of thrombomodulin and hir^{55-65} on the hydrolysis of these substrates was studied in a similar manner using saturating concentrations (>10-fold the value of K_d) of effector.

RESULTS

The thermodynamic parameters for the thrombin−thrombomodulin interaction are summarized in Table 1. Thrombomodulin binds to the fast form of thrombin with higher affinity, consistent with previous findings (Dang et al., 1995), and so do rTM and the two fragments TM^{4-5} and TM^{4-5-6}. The increased affinity observed in the presence of the chondroitin sulfate moiety explains the lower anticoagulant activity of hTM^- compared to hTM^+ (Bourin et al., 1986, 1990). The fragment TM^{4-5-6} and rTM bind with an affinity similar to hTM^+, whereas TM^{4-5} lacking the EGF-like domain 6 has an affinity 100-fold lower. This confirms the importance of the sixth EGF-like domain in thrombin recognition reported by others (Hayashi et al., 1990; Tsiang et al., 1992). The hirudin fragment hir^{55-65} binds to the fibrinogen recognition site (Vijayalakshmi et al., 1994) and provides a control for the specificity of the effects observed with thrombomodulin and its fragments. Hir^{55-65} interacts preferentially with the fast form, as shown previously (Ayala & Di Cera, 1994), but has an affinity about 6-fold lower than that of TM^{4-5} and 600-fold lower than that of TM^{4-5-6}, consistent with its binding to thrombin in a manner similar to portions of the fifth EGF-like domain (Mathews et al., 1994).

The temperature dependence of thrombomodulin binding to the slow and fast forms of thrombin is shown in Figure 2. The nonlinearity of the plots signals the presence of a significant ΔC_p (Table 1). Binding of hTM^+ to thrombin is characterized by a modest ΔC_p in the fast form and a more significant change in the slow form. These effects are also seen for rTM and are not altered significantly by deletion of the chondroitin sulfate moiety. Hence, the heat capacity change must originate from binding of the EGF-like domains to the fibrinogen recognition site. The ΔC_p values measured for thrombin interaction with the fragments TM^{4-5} and TM^{4-5-6} are larger than those pertaining to hTM^+ or hTM^- and confirm the dominant role played by the EGF-like domains in determining the heat capacity change for recognition by thrombin. In all cases, binding to the slow form causes a significantly larger heat capacity change by 0.3−0.6 kcal mol^{-1} K^{-1}, signaling the presence of distinct mechanisms of recognition in the two forms of thrombin. This difference is peculiar to thrombomodulin and its fragments, because it is not observed with hir^{55-65}.

Further information on the energetics of the thrombin−thrombomodulin interaction is derived from the salt dependence studies (Figures 3 and 4). The value of K_d at 200 mM salt is remarkably different in NaCl, ChCl, and NaF (Table 2). For example, in the case of hTM^+ and rTM, the affinity changes by 2 orders of magnitude between ChCl and NaF at the same ionic strength. This difference cannot depend on electrostatic screening that is due only to the charge of the salt species present in solution. Rather, it signals the presence of specific binding interactions of Na^+

Table 1: Thermodynamic Parameters for the Interaction of Thrombomodulin, Its Fragments, and Hir^{55-65} with Thrombin in the Slow and Fast Forms[a]

	K_d (nM)	ΔG (kcal/mol)	ΔC_p (kcal mol^{-1} K^{-1})	T_H (K)	T_S (K)
			Fast Form		
hTM^+	3.4 ± 0.1	−11.55 ± 0.02	−0.2 ± 0.1	282 ± 2	356 ± 7
hTM^-	26 ± 2	−10.35 ± 0.05	−0.27 ± 0.07	297 ± 1	338 ± 11
rTM	1.8 ± 0.1	−11.93 ± 0.03	−0.4 ± 0.1	281 ± 8	309 ± 6
TM^{4-5}	330 ± 20	−8.84 ± 0.04	−0.7 ± 0.2	285 ± 3	298 ± 2
TM^{4-5-6}	3.4 ± 0.1	−11.55 ± 0.02	−0.6 ± 0.1	291 ± 2	310 ± 3
hir^{55-65}	2300 ± 300	−7.69 ± 0.08	−0.7 ± 0.2	286 ± 2	297 ± 2
			Slow Form		
hTM^+	18.8 ± 0.8	−10.53 ± 0.02	−0.5 ± 0.2	286 ± 4	305 ± 3
hTM^-	180 ± 20	−9.20 ± 0.07	−0.8 ± 0.1	290 ± 1	302 ± 1
rTM	16.8 ± 0.9	−10.61 ± 0.03	−0.6 ± 0.2	278 ± 6	295 ± 2
TM^{4-5}	3200 ± 200	−7.50 ± 0.04	−0.99 ± 0.03	285.1 ± 0.2	292.7 ± 0.1
TM^{4-5-6}	22 ± 2	−10.45 ± 0.05	−1.3 ± 0.3	289 ± 2	297 ± 1
hir^{55-65}	14700 ± 800	−6.59 ± 0.03	−0.7 ± 0.2	288 ± 3	297 ± 2

[a] The values of K_d and ΔG are at 25 °C.

and Cl^- that are selectively suppressed in ChCl and NaF because of the inert nature of Ch^+ and F^- (Ayala & Di Cera, 1994; Vindigni & Di Cera, 1996). No evidence of bound Ch^+ is found in crystals of thrombin grown in the presence of ChCl (Zhang, Guinto, Tulinsky, and Di Cera, in preparation), and the properties of thrombin in the presence of F^- are identical to those in the presence of bulky monovalent anions like acetate and cacodylate (Ayala & Di Cera, 1994). Na^+ binding favors the formation of the thrombin−thrombomodulin complex, whereas Cl^- binding opposes it. The difference in the K_d values observed between NaCl and ChCl is almost the same for all ligands and is due to the slow→fast transition of thrombin. On the other hand, the difference between NaCl and NaF depends strongly on the presence of the chondroitin sulfate moiety. Specific Cl^- binding to the fibrinogen recognition site is supported by both experimental (Ayala & Di Cera, 1994; Vindigni & Di Cera, 1996) and computational (Sharp, 1996) studies and explains the difference seen between NaCl and NaF for hTM^-, TM^{4-5}, TM^{4-5-6}, and hir^{55-65}. The larger difference seen for hTM^+ and rTM suggests that additional Cl^- binding sites must be located in the heparin binding site of thrombin where the chondroitin sulfate moiety binds.

This scenario is supported by the more pronounced salt dependence of hTM^+ and rTM compared to hTM^- and the fragments. The value of Γ_{salt} (Table 3) is -4.8 ± 0.6 for hTM^+ and rTM, but drops to -2.2 ± 0.4 for hTM^- and the two fragments TM^{4-5} and TM^{4-5-6}. The values for rTM and hTM^+ under physiological [NaCl] (150 mM) are in agreement with those reported previously (Hofsteenge et al., 1986; Sadler et al., 1993). The chondroitin sulfate moiety contributes more than half the value of Γ_{salt}. The independence of Γ_{salt} on the particular salt used, as recently seen for the thrombin−fibrinogen interaction (Vindigni & Di Cera, 1996), shows that this parameter reflects predominantly the electrostatic screening of ions on the surface of the macromolecules. However, Γ_{salt} for hTM^+ far exceeds the values reported for fibrinogen or hirudin binding (Stone et al., 1989), and signal a much larger electrostatic contribution to the binding of thrombomodulin to thrombin. The value of Γ_{salt} for thrombomodulin binding is unusually high for protein−protein interactions and suggests the involvement of a polyelectrolyte-like domain in the formation of the complex. The only domain capable of such behavior is the chondroitin sulfate moiety in view of its high content in spatially ordered negative charges. Previous studies on the interaction of heparin with thrombin have reported a value of Γ_{salt} of −4.8 (Olson et al., 1991), consistent with the polyelectrolyte-like nature of heparin. The results reported here for hTM^+ and rTM, in conjunction with those obtained with heparin, strongly support an interaction of the chondroitin sulfate with the highly basic heparin binding site of thrombin, consistent also with recent studies (Ye et al., 1993, 1994).

The value of Γ_{salt} correlates with the net total charge of the ligand in the case of hir^{55-65} and the two thrombomodulin fragments (net charge of −4 and −11, respectively) and scales as −0.25 per unit of total charge (in absolute value) of the ligand. Hence, a singly charged ligand binding to thrombin should have a value of $\Gamma_{salt} = -0.25$. This is in fact the value measured recently for the interaction of Na^+ with thrombin (Guinto & Di Cera, 1996). Therefore, in the case of four ligands with unrelated structure, the value of Γ_{salt} can be predicted by multiplying the absolute total charge of the ligand by −0.25. This allows an estimation of the net total charge of hTM^+ as −19, with the chondroitin sulfate moiety carrying a charge of approximately −12.

Important clues on the effect of thrombomodulin on thrombin specificity come from the results on the hydrolysis of chromogenic substrates summarized in Table 4. The substrate FPR was synthesized to optimize the interactions with the specificity sites of thrombin. Arg at P1 ion-pairs to D189 at S1; Pro at P2 interacts with the apolar moiety of S2 lined up by P60b, P60c, and W60d, whereas Phe at P3 interacts with the aromatic ring of W215 at S3. Substitutions were then made at each P1−P3 position to study the response of the enzyme to single- and multiple-site replacements. H-D-Phe in the "optimal" substrate FPR was replaced with H-D-Val in VPR, VPK, VGR, and VGK, because the two residues are almost isosteric and the sequence VPR is identical to that cleaved by thrombin in the natural substrate factor XIII. Pro was replaced with Gly in FGR, FGK, VGR, and VGK, because S2 can accommodate only small apolar side chains. Arg was replaced with Lys in FPK, FGK, VPK, and VGK, to preserve the positive charge at P1 needed to contact D189 at S1. The library of substrates constructed in this way also enables the identification of coupling effects among the specificity sites of thrombin (Di Cera et al., 1997).

All substrates interact more specifically with the fast form, although they span a range of k_{cat}/K_m values of nearly 5 orders of magnitude. In FPR, replacement of Arg at P1 with Lys

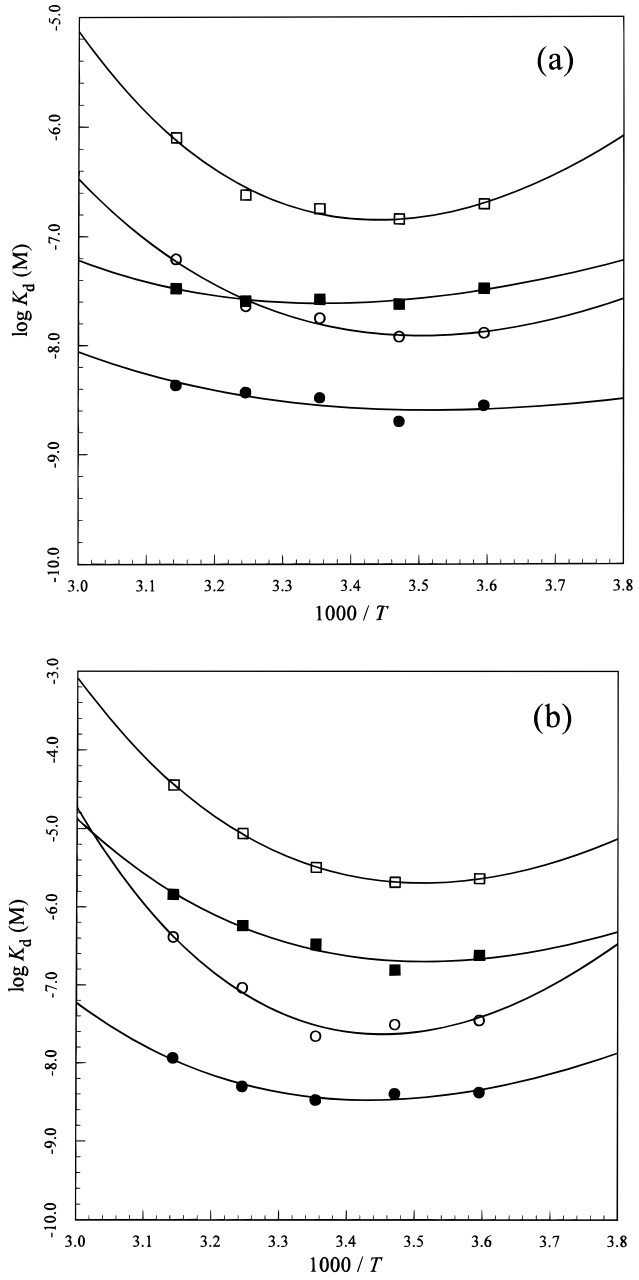

FIGURE 2: van't Hoff plots in the temperature range from 5 to 45 °C for the binding of thrombomodulin and its fragments to the slow (○, □) and fast (●, ■) forms of thrombin. (a) hTM^+ (●, ○) and hTM^- (■, □); (b) TM^{4-5} (●, ○) and TM^{4-5-6} (■, □). Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0, at a constant ionic strength of 200 mM. Continuous lines were drawn according to eq 2 with best-fit parameter values listed in Table 1.

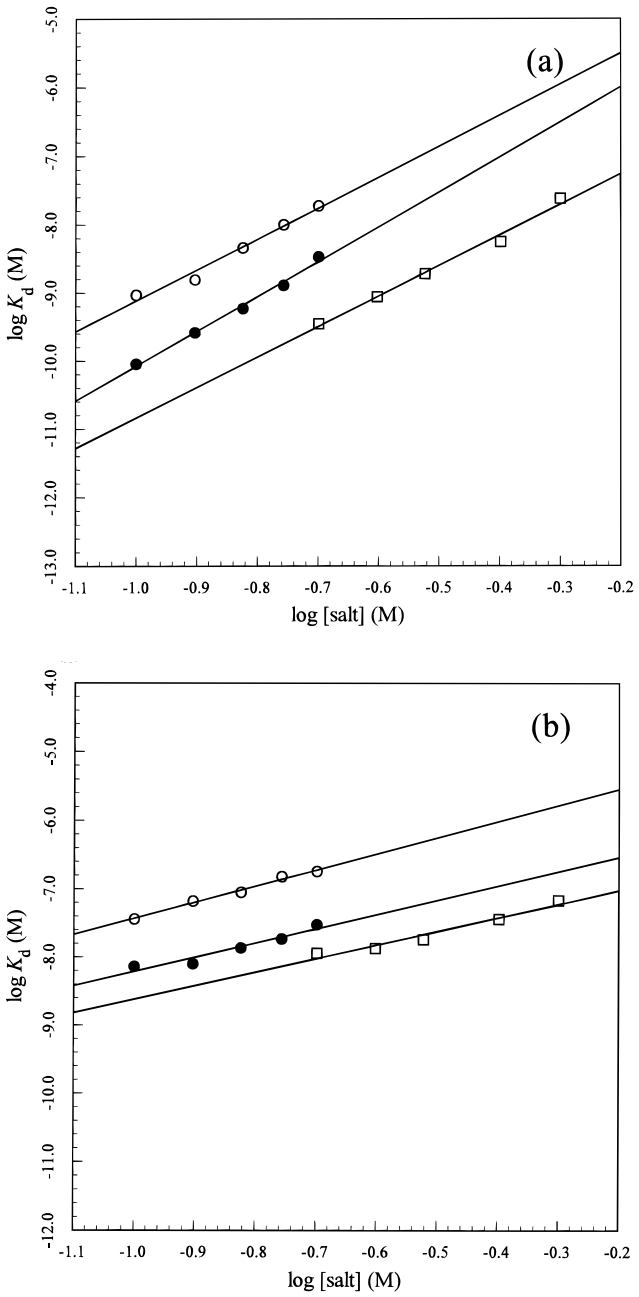

FIGURE 3: Salt dependence of the binding of hTM^+ (a) and hTM^- (b) to thrombin. Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C, in the presence of NaCl (●), ChCl (○), or NaF (□). Continuous lines were drawn according to eq 3, with the best-fit parameter values for Γ_{salt} listed in Table 3.

produces a loss of specificity of about 10-fold, whereas replacement of Pro with the more flexible Gly at P2 reduces specificity by more than 30-fold. Replacement of Phe with Val at P3 actually enhances specificity by 1.1-fold. The change in specificity due to perturbation at a given site also depends on substitutions made at other sites, indicating the presence of substantial cooperative effects. For example, the loss of specificity caused by the triple replacement in VGK far exceeds the product of the individual changes.

When thrombomodulin binds to the fast form, there is at most a 2-fold enhancement of specificity for all synthetic substrates, including DPR which mimics the sequence cut by thrombin in protein C. On the other hand, binding of thrombomodulin to the slow form produces a consistently higher increase in specificity by as much as 15-fold. Interestingly, the effects are similar for all substrates and cause a uniform reduction of the ΔG_c for allosteric switching. ΔG_c is the coupling free energy in the thermodynamic cycle that links the slow↔fast equilibrium to the binding of the substrate in the transition state and is a useful quantity to map the structural domains of the enzyme that are energetically linked to the allosteric equilibrium (Ayala & Di Cera, 1994; Guinto et al., 1995). A reduced value of ΔG_c observed upon thrombomodulin binding demonstrates that this cofactor tends to abolish the differences between the slow and fast forms. The energetic contribution of this effect is about 1 kcal/mol for all substrates. This causes the slow form to become more specific in the case of substrates like FGR and VGR when thrombomodulin binds. The effect of thrombo-

Table 2: Specific Salt Dependence of K_d (nM) for the Binding of Thrombomodulin, Its Fragments, and Hir^{55-65} to Thrombin[a]

	ChCl	NaCl	NaF	r_{Na^+}	r_{Cl^-}
hTM^+	18.8 ± 0.8	3.8 ± 0.1	0.35 ± 0.02	4.9 ± 0.3	0.092 ± 0.005
hTM^-	180 ± 20	30 ± 2	11.4 ± 0.4	6.0 ± 0.6	0.38 ± 0.03
rTM	16.8 ± 0.9	2.1 ± 0.1	0.14 ± 0.02	8.0 ± 0.5	0.067 ± 0.004
TM^{4-5}	3200 ± 200	380 ± 20	150 ± 5	8.4 ± 0.6	0.39 ± 0.04
TM^{4-5-6}	22 ± 2	3.8 ± 0.1	1.42 ± 0.04	5.8 ± 0.6	0.37 ± 0.02
hir^{55-65}	14700 ± 300	2600 ± 300	1700 ± 200	5.7 ± 0.6	0.65 ± 0.07

[a] Salts are at a concentration of 200 mM. Also shown are the apparent relative effects of Na^+, r_{Na^+} (affinity in NaCl versus ChCl), and Cl^-, r_{Cl^-} (affinity of NaCl versus NaF).

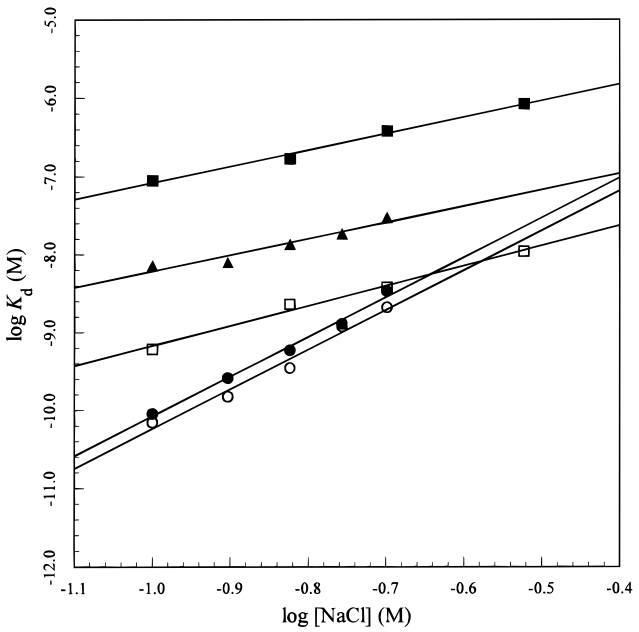

FIGURE 4: Salt dependence of the binding of hTM^+ (●), hTM^- (▲), rTM (○), TM^{4-5} (■), and TM^{4-5-6} (□) to thrombin in the presence of NaCl. Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C. Continuous lines were drawn according to eq 3, with the best-fit parameter values for Γ_{salt} listed in Table 3.

Table 3: Values of Γ_{salt} for the Interaction of Thrombomodulin, Its Fragments, and Hir^{55-65} with Thrombin in the Presence of Different Salts

	NaCl	ChCl	NaF
hTM^+	−5.2 ± 0.3	−4.5 ± 0.4	−4.5 ± 0.3
hTM^-	−2.1 ± 0.3	−2.4 ± 0.1	−2.0 ± 0.3
rTM	−5.1 ± 0.4	−5.4 ± 0.1	−4.2 ± 0.2
TM^{4-5}	−2.1 ± 0.1	−2.3 ± 0.4	−1.9 ± 0.2
TM^{4-5-6}	−2.6 ± 0.1	−2.5 ± 0.2	−2.4 ± 0.2
hir^{55-65}	−1.06 ± 0.02	−1.35 ± 0.09	−1.0 ± 0.1

modulin is energetically uniform on the S1, S2, and S3 specificity sites, and hence on all substrates probing these sites. A surprising result, however, is that these effects are not peculiar to thrombomodulin, because they are also elicited in an almost identical manner by binding of hir^{55-65}.

DISCUSSION

The temperature studies reported here have revealed the magnitude of the heat capacity changes associated with binding of thrombomodulin and its fragments to thrombin and enable the formulation of a possible mechanism for the formation of the complex. The ΔC_p does not depend on the presence of the chondroitin sulfate moiety, but on binding of the EGF-like domains of thrombomodulin to the fibrinogen recognition site of thrombin. In the case of binding to the fast form, the ΔC_p comes entirely from removal of the nonpolar surface area from water, and its value can be predicted from calculations of buried surface areas using hirudin as a model in view of the profound similarities in the binding to the fibrinogen recognition site (Rydel et al., 1991; Mathews et al., 1994; Vijayalakshmi et al., 1994). The calculated amount of surface area removed from water in the thrombin−hir^{55-65} interface yields a predicted ΔC_p of −0.21 kcal mol^{-1} K^{-1} (Ayala et al., 1995), which is identical to that observed for hTM^+ binding to the fast form. The contribution of burial of highly polar surface area at the level of the heparin binding site upon binding of chondroitin sulfate should make only a negligible contribution to the ΔC_p, based on theoretical considerations (Spolar & Record, 1994; Makhatadze & Privalov, 1995), and that is indeed found experimentally. Hence, the interaction of thrombomodulin with the fast form of thrombin is a simple rigid-body association, and the heat capacity change can be predicted entirely from the surface area removed from the solvent upon formation of the complex. This implies that the fast form is the optimal conformation for recognizing thrombomodulin, which must be a very rigid molecule in solution and does not change its conformation upon binding to the enzyme. On the other hand, binding to the slow form brings about additional contributions to the heat capacity change and does not conform to a simple rigid-body association. These contributions cannot come from conformational transitions of the cofactor, because they would be seen also in the interaction with the fast form. Rather, they come from conformational changes of the enzyme.

Thrombomodulin induces a conformational change in thrombin that mimics the slow→fast transition, and the larger ΔC_p observed in the slow form contains the partial contribution of the allosteric switch that is known to have a large and negative ΔC_p (Guinto & Di Cera, 1996). In the case of hir^{55-65}, the ΔC_p is dominated by the ligand, which is disordered in solution (Haruyama & Wütrich, 1989) and folds upon binding. The heat capacity change can be predicted from surface area calculations using the same analysis as for the thrombin−hirudin interaction (Ayala et al., 1995). An interesting aspect of the results reported here is the more pronounced ΔC_p measured for the binding of TM^{4-5} and TM^{4-5-6} compared to the intact cofactor, which stands in contrast to the results observed for hirudin and its fragments (Ayala et al., 1995). This finding can only be explained by invoking folding transitions linked to binding of the EGF-like domains 5 and 6 in TM^{4-5} and TM^{4-5-6}, but not in the intact thrombomodulin. Hence, these domains would have a quite different conformation in solution when isolated from the rest of the thrombomodulin molecule.

The conclusion that thrombomodulin binding induces a conformational change of thrombin similar to the slow→fast

Table 4: Effect of Thrombomodulin and Hir$^{55-65}$ on the Specificity Constant, $s = k_{cat}/K_m$ (in μM^{-1} s^{-1}), for the Hydrolysis of Synthetic Substrates by Thrombin[a]

	fast form					slow form					coupling		
	s	s +hTM$^+$	s +hir$^{55-65}$	r_{TM}	r_{hir}	s	s + hTM$^+$	s + hir$^{55-65}$	r_{TM}	r_{hir}	ΔG_c	ΔG_c + hTM$^+$	ΔG_c +hir$^{55-65}$
FPR	90	94	117	1.0	1.3	3.0	20	21	6.7	7.0	−1.8	−0.7	−0.8
FPK	7.9	10	6.3	1.3	0.8	0.35	3.4	1.6	9.7	4.6	−1.8	−0.6	−0.8
FGR	2.0	2.1	1.7	1.0	0.8	0.86	4.4	3.1	5.1	3.6	−0.5	+0.4	+0.4
VPR	100	96	98	1.0	1.0	6.7	27	24	4.0	3.6	−1.6	−0.7	−0.8
FGK	0.021	0.035	0.023	1.7	1.1	0.0026	0.020	0.0084	7.7	3.2	−1.2	−0.3	−0.6
VPK	2.1	3.7	2.0	1.8	1.0	0.11	1.6	0.57	15	5.2	−1.7	−0.5	−0.7
VGR	0.34	0.44	0.32	1.3	0.9	0.17	0.73	0.66	4.3	3.9	−0.4	+0.3	+0.4
VGK	0.0047	0.010	0.0064	2.1	1.4	0.00079	0.0061	0.0027	7.7	3.4	−1.1	−0.3	−0.5
DPR	5.2	6.1	7.1	1.2	1.4	0.70	3.1	2.9	4.4	4.1	−1.2	−0.4	−0.5

[a] Errors of typically ±2%. r_{TM} and r_{hir} denote the change in specificity induced by thrombomodulin and hir$^{55-65}$ relative to the absence of effector. Also listed are the values of coupling free energy for the slow→fast transition in the transition state, $\Delta G_c = RT \ln r$ (in kcal/mol), where r is the ratio between the specificities of the fast and slow forms. Errors are typically ±0.1 kcal/mol.

transition is strongly supported by the results obtained with the library of chromogenic substrates. Thrombomodulin has only a small effect on the fast form, whereas it enhances the specificity of the slow form up to 15-fold. As a result, binding of thrombomodulin drastically reduces the difference between the slow and fast forms. This result is also seen with the natural substrate protein C, which is cleaved by the slow form with significantly higher specificity in the absence, but not in the presence, of thrombomodulin (Dang et al., 1995).

The effects on the synthetic substrates seen with thrombomodulin are also elicited by hir$^{55-65}$, whereas this ligand has no effect on the hydrolysis of protein C. These results have a bearing on the mechanism that leads to the drastic (~1000-fold) enhancement of thrombin specificity toward protein C upon thrombomodulin binding (Esmon, 1989), which is seen in both the slow and fast forms (Dang et al., 1995). The effect of thrombomodulin on the specificity sites S1, S2, and S3 of the enzyme produces a change in specificity that is either small (fast form) or at most 15-fold (slow form). Hence, thrombin must enhance its specificity toward protein C using sites other than those probed by the library of chromogenic substrates. Mutation of E192 to Gln enhances protein C cleavage in the absence of thrombomodulin (Le Bonniec & Esmon, 1991), but this residue makes contacts with the charged amino group of substrates like those used in this study (Nienaber et al., 1996) and therefore cannot be a major factor in the change of thrombin specificity toward protein C. Residues in the Na^+ binding loop affect protein C cleavage both directly and through the slow↔fast equilibrium (Di Cera et al., 1995; Dang et al., 1997), while they make no direct contacts with small chromogenic substrates. These residues may be responsible for the thrombomodulin-induced switch in specificity toward protein C. Furthermore, other mechanisms to enhance protein C cleavage by thrombin, independent of the fast→slow conversion and thrombomodulin binding, have recently been documented (Richardson et al., 1992; Berg et al., 1996).

We propose an alternative and more likely possibility for the thrombomodulin-induced switch in specificity of thrombin toward protein C. Thrombomodulin exerts its physiologically important function by influencing the conformation of the bound protein C in the thrombin−thrombomodulin−protein C ternary complex, thereby enhancing the specificity of the enzyme by turning protein C into a better substrate. A similar hypothesis was originally formulated by Hayashi et al. (1990). It is extremely unlikely that the structural domains responsible for the enhancement in specificity are entirely located in regions of the enzyme other than the critical sites within the catalytic pocket that can be probed with small chromogenic substrates. It is also extremely unlikely that thrombomodulin would induce such a large effect on thrombin without a large conformational transition and that this transition would not be detected experimentally as a large change in heat capacity as seen for the slow→fast transition (Guinto & Di Cera, 1996) and myriad other cases (Spolar & Record, 1994). Thrombomodulin makes extensive contacts with thrombin, bridging regions diametrically disposed on the surface of the enzyme like the fibrinogen recognition site and the heparin binding site (Rydel et al., 1991; Bode et al., 1992). The thrombin−thrombomodulin complex would therefore have the W60d loop and especially the Na^+ binding loop available for contacting protein C to form the ternary complex. It is conceivable that the bound protein C would make contacts with the bound thrombomodulin, perhaps at the level of the external portion of W60d loop of thrombin that is located in between the fibrinogen recognition site and the heparin binding site (Bode et al., 1992). If this were the case, any chromogenic substrate contacting only the interior of the catalytic pocket would not experience the large change in specificity observed for protein C because it would lack the critical direct interaction with the cofactor. This would explain the results obtained in this study and especially the similarity of effects seen on the chromogenic substrates with thrombomodulin and hir$^{55-65}$, but the lack of effect of hir$^{55-65}$ on protein C hydrolysis. If our model is correct, it should be possible to find mutations of thrombomodulin that do not affect binding to thrombin but reduce the ability of thrombin to cleave protein C.

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