Evidence for Multiple Conformational Changes in the Active Center of Thrombin Induced by Complex Formation with Thrombomodulin: An Analysis Employing Nitroxide Spin-Labels†

Giovanni Musci,‡,§ Lawrence J. Berliner,‡ and Charles T. Esmon*,‖

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Thrombosis/Hematology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

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ABSTRACT: Thrombomodulin (TM) is an endothelial cell surface protein that binds thrombin to form a reversible complex with altered enzyme specificity. The complex rapidly converts protein C to the anticoagulant enzyme activated protein C and has decreased fibrinogen clotting activity. To investigate whether formation of this complex elicits conformational changes in the active center of thrombin, we employed the following fluorosulfonyl spin-label inhibitors: *N*-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolidinyl)-*m*-(fluorosulfonyl)benzamide (m-V); *O*-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyl) *N*-[*m*-(fluorosulfonyl)phenyl]carbamate (m-VI); *N*-[4-(fluorosulfonyl)phenyl]-2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxamide (p-I); *N*-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolidinyl)-*p*-(fluorosulfonyl)benzamide (p-V). To compare the spectra of the free thrombin with those of the complex, the viscosity of the solution was adjusted with sucrose to give similar tumbling rates (isokylindric spectra) or the macromolecular rotational contribution to the spectra was essentially eliminated with saturated sucrose. Both a buffer-soluble proteolytic derivative of TM and the intact molecule elicited changes in the electron spin resonance signals of many of the labeled thrombins employed. Two of the labels, p-I and p-V, had previously been shown to exhibit decreased mobility when indole derivatives were bound to thrombin. When TM complexes with thrombin, the mobility of the p-I label increases while the mobility of the p-V label decreases. Two of the labels, m-V and m-VI, had previously been shown to be sensitive to conversion of α-thrombin to γ-thrombin. The m-V label in thrombin exhibited decreased mobility upon complex formation with thrombomodulin, which is similar to the decrease in probe mobility in γ-thrombin, while m-VI-labeled thrombin exhibited no detectable spectral change. Although Ca^{2+} is required for protein C activation, Ca^{2+} had no influence on the spectra, and Gd(III) produced no dipolar broadening of the p-I spectra, indicating that the binding site on TM is more than 12 Å from the label. 6-Fluorotryptamine did not alter the mobility of the p-I-labeled thrombin–TM complex, indicating either that this site is blocked in the complex or that the probe was insensitive to the additional conformational changes. These studies demonstrate that complex formation between thrombin and TM alters the conformation in at least two distinct regions of the active center. Although some of the changes monitored by selected probes appeared similar to those induced by indole and those resulting from the conversion from α- to γ-thrombin, the responses of other probes were clearly distinguishable.

Thrombin plays a central role as a regulatory protein of the coagulation cascade. It catalyzes many reactions that lead to clot formation, including fibrin formation, platelet aggregation, and activation of factors V, VIII, XIII, and VII (Jackson & Nemerson, 1980). When thrombin interacts with endothelium, it can modulate fibrinolytic activity (Loskutoff, 1986), stimulate prostacyclin formation (Weksler & Jaffee, 1986), and interact with a cell surface receptor, thrombomodulin (TM),[1] to activate protein C (Esmon & Owen, 1981). Several of these reactions serve to inhibit clot formation.

The molecular mechanisms that control which thrombin-dependent reaction is predominant are only poorly understood. The activation of protein C by the thrombin–thrombomodulin complex provides one model for studying the control of thrombin specificity. When thrombin interacts with thrombomodulin on the endothelial cell surface in the presence of Ca^{2+}, the rate of protein C activation is increased ≈20 000-fold (Esmon & Owen, 1981) and with purified detergent-solubilized TM ≈1000-fold (Esmon et al., 1983; Salem et al., 1984; Jakubowski et al., 1986; Hofsteenge et al., 1986). This rate enhancement is achieved both by decreasing the K_m and increasing the k_{cat} for protein C with little change in the hydrolysis of synthetic substrates (Esmon et al., 1983). Although activation requires Ca^{2+}, complex formation is essentially independent of free Ca^{2+}.

Several lines of evidence suggest that thrombomodulin functions by altering the macromolecular specificity of thrombin allosterically: (1) The activation of protein C by the complex requires Ca^{2+} (Esmon et al., 1983). In contrast,

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* Author to whom correspondence should be addressed.

‡ The Ohio State University.

§ Permanent address: University of Rome, "La Sapienza", Department of Biochemical Sciences, Piazzale Aldo Moro 5, Rome, Italy.

‖ Oklahoma Medical Research Foundation.

[1] Abbreviations: TM, thrombomodulin; ε-TM, active fragment of thrombomodulin derived from limited proteolysis with elastase; ESR, electron spin resonance; m-IV, *N*-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyl)-*m*-(fluorosulfonyl)benzamide; m-V, *N*-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolidinyl)-*m*-(fluorosulfonyl)benzamide; m-VI, *O*-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyl) *N*-[*m*-(fluorosulfonyl)phenyl]-carbamate; m-VII, *N*-[*m*-(fluorosulfonyl)phenyl]-*N'*-(2,2,6,6-tetramethyl-1-oxy-4-piperidinyl)urea; p-I, *N*-[4-(fluorosulfonyl)phenyl]-2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxamide; p-V, *N*-(2,2,5,5-tetramethyl-1-oxy-3-pyrrolidinyl)-*p*-(fluorosulfonyl)benzamide; SDS, sodium dodecyl sulfate.

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Ca^{2+} inhibits activation of protein C by free thrombin (Amphlett et al., 1981). Both processes correlate well with conformational changes in protein C, suggesting that the Ca^{2+}-stabilized conformation is a good substrate for the complex and a poor substrate for the free enzyme (Johnson et al., 1983). (2) Complex formation inhibits fibrinogen clotting (Semon et al., 1982; Maruyama et al., 1985; Jakubowski et al., 1986), factor V activation (Esmon et al., 1982), and platelet activation (Esmon et al., 1983a). (3) When thrombin, modified at the active site with dansyl fluoride, complexes with TM, a 47% decrease in dansyl fluorescence emission is observed (Johnson et al., 1984). All of these processes are consistent with a conformational change in thrombin upon complex formation with thrombomodulin.

To gain further insight into whether conformational changes are occurring near the active site, we chose to employ nitroxide spin-labels. These were chosen because previous studies had demonstrated that these probes were sensitive to conformational changes in different regions of the active site (Berliner et al., 1981).

Specifically, ligand-induced conformational changes in thrombin have been detected previously with nitroxide spin-labels (Berliner & Shen, 1977a). Nitroxide spin-labels are stable paramagnetic probes that report information about their structural environment when bound to a macromolecule. The ESR spectrum reflects the tumbling motion of the nitroxide moiety which is sensitive to noncovalent interactions with, e.g., the local protein structure, and to conformational changes that are elicited at the labeling site (Berliner, 1977). These are frequently evident by changes in the broad-line extrema (ca. $2T_{\parallel}$ = 48–70 G), which are related to the tumbling motion of the nitroxide moiety (Shimshick & McConnell, 1974). Previous work with sulfonyl fluoride active site directed irreversible spin-label inhibitors demonstrated the unique sensitivity of the ESR method in distinguishing subtle structural features in the human thrombins. For example, one group of structurally sensitive sulfonyl fluoride spin-labels was sensitive to differences in conformation between highly coagulant human α-thrombin and the estro/amidolytically active, noncoagulant γ-thrombin derivative (Berliner et al., 1981); another group of labels detected conformational changes elicited at the active site by the binding of apolar ligands (indole and tryptamine derivatives). These apolar ligands enhance various thrombin activities (Berliner & Shen, 1977; Conery & Berliner, 1983). Thus, these spin-labels are well suited to monitor potential conformational changes induced in the active site of thrombin by thrombomodulin.

Materials and Methods

Proteins. Electrophoretically pure human α-thrombin was purified from prothrombin concentrate (a generous gift from Dr. J. W. Fenton, II, New York State Department of Health, Albany). On SDS gels, the thrombin appeared homogeneous with and without disulfide bond reduction. Rabbit thrombomodulin was purified as described by Galvin et al. (1987) and converted to its buffer-soluble form by limited proteolysis with porcine elastase (Cooper Biomedicals) and separated from inactive cleavage products by chromatography on a Mono-Q column as described (Kurosawa et al., 1987a). This proteolytic derivative is referred to as ϵ-TM.

Chemicals. The (fluorosulfonyl)phenyl spin-labels were synthesized as described previously (Wong et al., 1974). Spin-labeled human α-thrombins were prepared as described by Berliner and Shen (1977a). Tryptamine, 6-fluorotryptamine, and 5-fluorotryptamine were from Sigma Chemicals, Inc. Octyl glucoside was purchased from Behring Diagnostics.

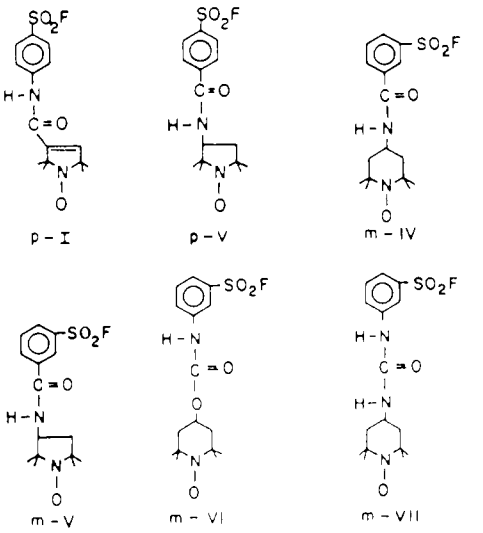

FIGURE 1: Structures of sulfonyl spin-labels examined in this study.

Methods. Protein concentrations were estimated spectrometrically using $\epsilon_{280}^{1\%}$ = 18.3 for thrombin (in 0.1 M NaOH) (Fenton et al., 1977), 6.0 for ϵ-TM, and 8.8 for TM, respectively (Kurosawa et al., 1987a). Molecular weights used were 50 000 for ϵ-TM, 74 000 for TM, and 36 000 for α-thrombin. ESR spectra were measured in quartz capillaries (Berliner, 1978) at room temperature on a Varian E-4 spectrometer equipped with a E-935 data system. For ESR experiments with intact TM, the protein was solubilized with 20 mM octyl glucoside.

Results

Figure 1 shows structures of the spin-labels employed in these studies, which were chosen on the basis of their previously reported specific sensitivity to thrombin structure and conformational changes (Berliner et al., 1981).

Approximately equimolar complexes of spin-labeled α-thrombin with ϵ-TM complexes were compared with spin-labeled α-thrombin alone in 24% (w/v) sucrose containing buffers. Parts A and B of Figure 2 depict X-band ESR spectra of human α-thrombin derivatized with the apolar site sensitive spin-label p-I and complexed with ϵ-TM, respectively. The ESR spectrum of a covalently bound nitroxide spin-label is comprised of two motional contributions: the rotational tumbling motion of the nitroxide label with respect to the macromolecule and the overall tumbling rate of the macromolecule complex. When comparing the ESR spectrum of spin-labeled α-thrombin with, e.g., that of the ϵ-TM–thrombin complex, one must account for the change in macromolecular rotation as the overall molecular weight shifts from 36 600 to 86 600 for the protein–enzyme complex. Therefore, one must measure ESR spectra for those spin-labeled thrombins alone under viscosity conditions where their overall rotational tumbling rate is equivalent to that for a 86 600-dalton molecule (i.e., *isokytindric*). In order to mimic those conditions, sucrose containing buffers have been employed in the past. In this viscosity range, no apparent perturbation of the protein structure is observed (Shimshick & McConnell, 1972; Berliner & Wong, 1974; Berliner et al., 1981). Thus, the viscosity of ca. 2.4 P was chosen by assuming that the molecular weight of a spherical globular protein is directly related to its (radius)^3 as the partial specific volumes of most proteins are similar. Alternatively, Berliner and Wong (1974) demonstrated that the most sensitive method to distinguish nitroxide motional changes (i.e., protein conformation changes) was measuring the ESR spectra in saturated sucrose, where overall macro-

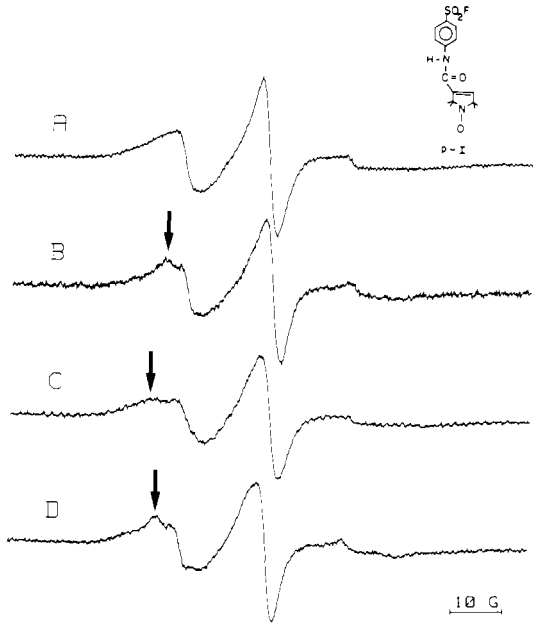

FIGURE 2: X-band ESR spectra of p-I spin-labeled α-thrombin–TM complexes: (A) p-I-labeled thrombin in buffer; (B) p-I spin-labeled α-thrombin complexed with ϵ-TM; (C) an *isokylindric* control of thrombin spin-labeled with p-I in 24% (w/v) sucrose; (D) p-I-labeled thrombin complexed with intact TM. Experimental conditions: 98 μM labeled thrombin (A), 32 μM labeled thrombin and 35 μM ϵ-TM (B); 150 μM labeled thrombin (C); 60 μM labeled thrombin and 98 μM TM (D). Scan range 100 G; field set 3395 G; modulation amplitude 2 G; microwave power 20 mW; scan time 2 min (4 scans were averaged for each spectrum).

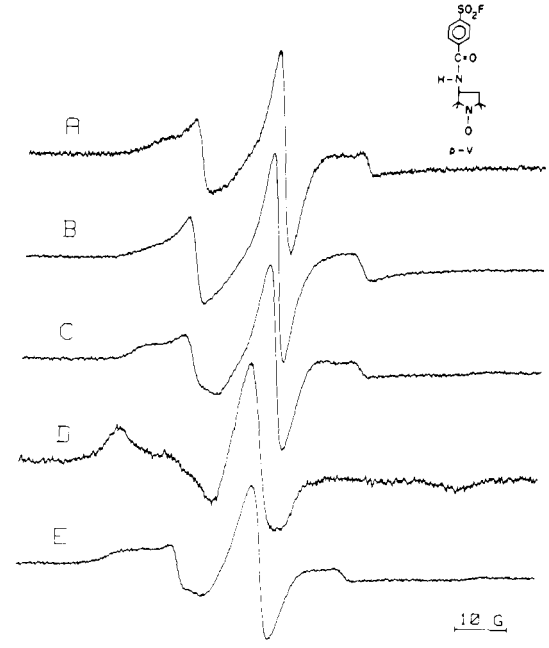

FIGURE 3: X-band ESR spectra of p-V spin-labeled α-thrombin–TM complexes: (A) p-V spin-labeled α-thrombin–ϵ-TM; (B) *isokylindric* control of p-V-labeled thrombin in 24% (w/v) sucrose; (C) p-V-labeled thrombin–TM; (D) as in (C) but in saturated sucrose; (E) p-V-labeled thrombin in saturated sucrose. Experimental conditions: 0.16 M NaCl; 33 μM labeled thrombin and 35 μM ϵ-TM (A); 200 μM labeled thrombin (B); 66 μM labeled thrombin and 98 μM TM (C,D); 66 μM labeled thrombin (E). All other conditions as in Figure 2.

molecular tumbling was essentially zero on the spin-label time scale. Spectrum 2C is thus the p-I–α-thrombin derivative in 24% (w/v) sucrose. Note the apparently *decreased* immobilization in the ϵ-TM–thrombin complex (Figure 2B) vs the *isokylindric* control in 24% (w/v) sucrose (Figure 2C), as reflected by the position of the low-field shoulder (arrows). It is worth noting that the considerably larger intact thrombomodulin in complex with p-I-labeled thrombin also resulted in a similar *decreased* immobilization (Figure 2D). The system appears saturated with respect to thrombomodulin since increasing the ratio of thrombomodulin to thrombin from 1:1 to 1.5:1 had no discernible influence on the spectra (data not shown). As an interesting contrast, the other apolar site spin-label, p-V, yields the spectral results shown in Figure 3. Here the p-V-labeled thrombin–ϵ-TM (Figure 3A) complex shows an increased immobilization vs the control in 24% (w/v) sucrose (Figure 3B). The conformational changes upon TM binding to p-V spin-labeled α-thrombin are even more evident when the complex with intact TM (Figure 3D) is compared to the control (Figure 3E) in saturated sucrose.

Since it was previously shown by Conery and Berliner (1983) that tryptamine analogues effect nitroxide mobility in thrombin derivatives spin-labeled with apolar site sensitive probes, we examined the effect of 2 mM 6-fluorotryptamine on the line shape of ESR spectra of the thrombin–TM complexes with spin-labels p-I or p-V. No change in mobility of the label was found.

Figure 4 depicts ESR spectra for ϵ-TM complexes with the label m-V, which have been shown previously to be sensitive to differences in the active-center regions of human α- and γ-thrombins, respectively (Berliner et al., 1981). Here again, as in Figure 3, the nitroxide in the thrombin–ϵ-TM complex (Figure 4A) is more immobilized than the isokylindric control in 24% (w/v) sucrose (Figure 4B). Again, as in Figure 3C,D, the comparisons in saturated sucrose with intact TM show

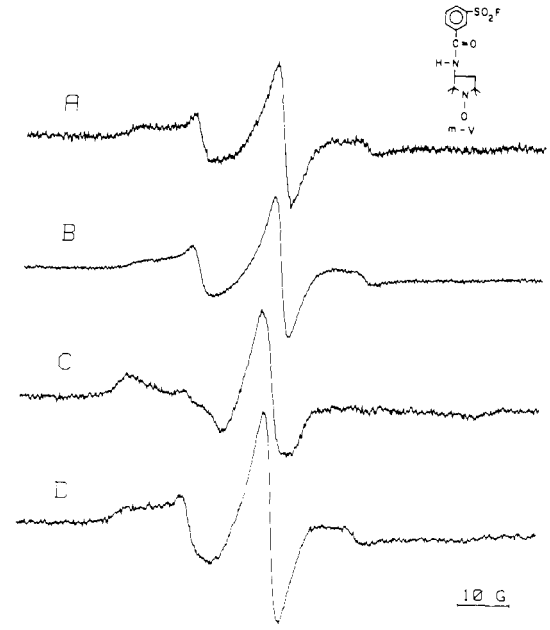

FIGURE 4: X-band ESR spectra of m-V spin-labeled α-thrombin–TM complexes. (A) m-V-labeled thrombin–ϵ-TM; (B) *isokylindric* control in 24% (w/v) sucrose; (C) m-V-labeled thrombin–TM complex in saturated sucrose; (D) m-V-labeled thrombin in saturated sucrose. Experimental conditions: 0.12 M NaCl; 28 μM labeled thrombin and 32 μM ϵ-TM (A); 140 μM labeled thrombin (B); 48 μM labeled thrombin and 98 μM TM (C); 48 μM labeled thrombin (D). All other conditions as in Figure 2.

distinct conformational differences (Figure 4C,D). Similar differences were found with m-IV-labeled thrombin. On the other hand, the two para-substituted piperidinoxy spin-labeled thrombins (m-VI and m-VII) were much less sensitive to conformational changes with ϵ-TM or TM. The similar behavior of these two labels was not surprising since their molecular structures are extremely similar.

We examined the effect of Ca(II) binding to TM on the TM-induced thrombin conformational change for the case of spin-label p-I (Figure 2). However, upon titrating Ca(II) into the sample in Figure 2B,D absolutely no change in the nitroxide mobility was detected. We then substituted up to 1.2 mM Gd(III) for Ca(II) in order to examine if any paramagnetic dipole–dipole interactions existed between the lanthanide (at the calcium site) and the nitroxide moiety at the thrombin active center. No paramagnetic broadening of the nitroxide spectrum was observed, indicating that their intramolecular distance exceeded ca. 12 Å (Dwek, 1973).

Discussion

From the studies presented here, the TM (or ϵ-TM) interaction with thrombin clearly results in several alterations of the thrombin active-site region. These conformational changes may contribute not only to the increased rate of protein C activation (Musci & Berliner, 1987) but also to the decreased rates of procoagulant reactions such as fibrinogen clotting (Esmon et al., 1982; Jakubowski et al., 1986; Hofsteenge et al., 1986). It is interesting that the binding of thrombin to TM appears to be inhibited competitively by fibrinogen (Jakubowski et al., 1986; Hofsteenge et al., 1986). These results have been interpreted to indicate that TM sterically hinders the thrombin binding interaction with fibrinogen.

In the studies performed here we have employed both a proteolytic derivative of TM prepared by treatment with elastase and intact TM. The ϵ-TM derivative was first chosen because it is buffer soluble and therefore circumvents requirements for detergents that might complicate interpretation of the spectra (although control experiments with labeled thrombin in octyl glucoside showed no changes). This derivative serves as a reasonable model since it retains both the ability to accelerate protein C activation and to inhibit fibrinogen clotting activity (Kurosawa et al., 1987a). The only major differences in function relate to a lower affinity for thrombin ($K_d \approx 5$ nM) and an altered dependence on calcium ions for protein C activation. The similarity in the changes in the spectra of p-I-derivatized thrombin bound to ϵ-TM or intact TM further supports the concept that these two forms of thrombomodulin function similarly.

The results with the labels p-V and m-V demonstrate the advantages in utilizing multiple labels. Both clearly showed evidence for a ϵ-TM- or TM-induced conformation change in the active-center region of human α-thrombin. That this change is elicited in several specific regions of the active center is supported by the fact that the labels p-V and m-V reside in different regions of the active center (Berliner et al., 1981).

The comparative ESR spectra for p-V- and m-V-labeled thrombin and their TM complexes with and without exogenous sucrose display several powerful features of the spin-label approach. Upon complexation of, e.g., p-V-labeled thrombin with intact TM (total complex M_r 111 000, probably as the tetramer complex, M_r 440 000), the overall macromolecular motion is essentially "rigid" on the time scale of spin-label motion. Yet one notes that while p-V–thrombin in saturated sucrose (Figure 3E) displays a slightly more mobile nitroxide moiety than that for p-V–thrombin–TM (Figure 3C), the corresponding spectrum of the latter sample also in saturated sucrose (Figure 3D) displays a nitroxide that is even more immobilized. The significance of these results is manifold. While high sucrose produces an exceptionally high macroviscosity that slows macromolecular motion, it may also contribute to the *microviscosity* at the nitroxide moiety if sufficient solvent access exists at the spin-label site. Thus, the increased nitroxide immobilization from Figure 3C to Figure 3D is due to microviscosity at the spin-label site. The *differences* between p-V-labeled thrombin and its TM complex, both in high sucrose (parts D and E of Figure 3), are, of course, still valid diagnostics since the measurements were made under precisely the same conditions.

The spectra in 24% (w/v) sucrose were chosen for the studies with ϵ-TM since the macromolecular tumbling for both the thrombin–ϵ-TM complex and the isokylindric control were comparable. While microviscosity effects in 24% w/v sucrose are relatively minor, the experiments in saturated sucrose both serve as cross-check and allow comparison of both ϵ-TM and intact TM.

Most convincing, however, are the results with p-I (Figure 2A) spin-labeled α-thrombin, where the nitroxide mobility increased upon complexation with ϵ-TM as well as with the even larger intact TM! Here one need not consider the appropriateness of the assumptions used in matching macromolecular correlation times or the effects of saturating sucrose since the larger complex showed more rapid nitroxide motion than the spin-labeled thrombin alone. Again, this is clearly evidence for an ϵ-TM (or intact TM) induced conformational change in human α-thrombin. Here the change apparently involved structural changes that reflected either less steric hindrance or reduced binding interactions with the enzyme surface. It is also interesting to note that spin-labels p-I and p-V respond similarly to the binding of apolar ligands, i.e., the unique indole site in human thrombin (Berliner et al., 1981). However, from these results it is possible to discriminate the effects of TM binding to α-thrombin in finer detail than with apolar ligands.

The ESR results found previously when tryptamine analogues bind to spin-labeled thrombin derivatives correlated well with alterations in catalytic activity of the unlabeled enzyme (Conery & Berliner, 1983). Furthermore, the analogue 6-fluorotryptamine was shown to be the most effective enhancer of human α-thrombin catalysis of both fibrinogen hydrolysis (Berliner et al., 1986) and protein C activation (Musci & Berliner, 1987). Since no change was observed for both p-I and p-V spin-labeled α-thrombin–TM, we may surmise either that the tryptamine site was covered by the extremely strong α-thrombin–TM interaction, $K_d \approx 0.5$ nM (Kurosawa et al., 1987a), or that the site is distinct but unable to exert the same conformational change observed for spin-labeled thrombin alone.

One of the properties of the thrombin–TM complex is markedly reduced clotting activity (Esmon et al., 1983). A structural model that is similar to the above is the conversion of highly coagulant α-thrombin to the noncoagulant γ-form. This is accomplished by structural changes near the active center that have been detected by specific sulfonyl fluoride spin-labels (Berliner et al., 1981). In particular, the spin-label m-V reported significantly hindered mobility in the γ- vs α-form. What is interesting to note here are the results with m-V-labeled α-thrombin–ϵ-TM complex, which also resulted in a more immobilized probe. It is enticing to speculate that the ϵ-TM-induced conformational change shares some properties in common with γ-thrombin. Clearly, however, these structural changes cannot be identical since conformational changes affecting other regions of the active center were occurring upon complex formation with thrombomodulin. Thus, some of the transitions may be similar but not precisely identical.

The existence of a Ca^{2+} binding site on TM was first suggested by the unusual Ca^{2+} dependence of protein C activation

with ϵ-TM, which was consistent with a Ca^{2+} bridge between TM and protein C. Further evidence for this hypothesis comes from recent fluorescence studies that show Ca^{2+}-dependent fluorescence quenching of ϵ-TM (Kurosawa et al., 1987b). The metal ion binding experiments presented here, e.g., Ca(II) and Gd(III), while yielding negative results, were nonetheless significant to certain aspects of the thrombin–TM interaction. The calcium binding site may be just a "structural cation" site whose function is only to stabilize the TM conformation. On the other hand, should this site be involved in more subtle aspects of TM function, it is *not* manifested in the TM-induced conformational change of α-thrombin at the active site. Furthermore, we have a lower limit from the spin-labeled complex with Gd(III) of ≥ 12 Å for the distance between the cation and the free electron on the N–O moiety. While 12 Å is admittedly a small distance relative to the molecular sizes of α-thrombin and TM, it confirms other evidence suggesting that the TM α-thrombin contact region is sufficiently *distant* from the thrombin active site.

We have examined two general subgroups of thrombin spin-labels, one group classified as apolar (indole) site sensitive (i.e., p-I and p-V), the other group as sensitive to differences between α- and γ-thrombin (i.e., m-IV, m-V, m-VI, and m-VII). They show complex responses to TM binding represented by increases, decreases, and slight (if any) changes in label mobility. It is clear that the conformational changes induced by TM are complex and influence multiple distinct regions of the active center. These structural changes probably contribute to the changes in substrate specificity induced by TM binding.

In conclusion, this study again shows the applicability of these active site directed spin-label probes for differentiating conformational states in the active center of a common enzyme, i.e., thrombin, induced by the binding not only of different effectors (Berliner et al., 1981; Conery & Berliner, 1983) but of a physiological macromolecular effector.

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