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The Thrombin Epitope Recognizing Thrombomodulin Is a Highly Cooperative Hot Spot in Exosite I*

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The functional epitope of thrombin recognizing thrombomodulin was mapped using Ala-scanning mutagenesis of 54 residues located around the active site, the Na⁺ binding loop, the 186-loop, the autolysis loop, exosite I, and exosite II. The epitope for thrombomodulin binding is shaped as a hot spot in exosite I, centered around the buried ion quartet formed by Arg⁶⁷, Lys⁷⁰, Glu⁷⁷, and Glu⁸⁰, and capped by the hydrophobic residues Tyr⁷⁶ and Ile⁸². The hot spot is a much smaller subset of the structural epitope for thrombomodulin binding recently documented by X-ray crystallography. Interestingly, the contribution of each residue of the epitope to the binding free energy shows no correlation with the change in its accessible surface area upon formation of the thrombin-thrombomodulin complex. Furthermore, residues of the epitope are strongly coupled in the recognition of thrombomodulin, as seen for the interaction of human growth hormone and insulin with their receptors. Finally, the Ala substitution of two negatively charged residues in exosite II, Asp¹⁰⁰ and Asp¹⁷⁸, is found unexpectedly to significantly increase thrombomodulin binding.

When thrombin binds to thrombomodulin, a transmembrane receptor present on endothelial cells (1), it loses the ability to cleave fibrinogen or the platelet receptors and “paradoxically” switches into an anticoagulant enzyme with increased (>1,000-fold) specificity toward protein C (2). Human thrombomodulin contains 557 residues and consists of five distinct domains: a 226-residue N-terminal domain with partial similarity to lectin-like molecules, a 236-residue domain containing six epidermal growth factor-like (EGF¹-like) 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 (3, 4). The EGF-like domains 5 and 6 are essential for thrombomodulin binding to thrombin via exosite I (5, 6). Some thrombomodulin molecules contain a chondroitin sulfate moiety linked to the Ser/Thr-rich domain that precedes the transmembrane domain (7). This moiety makes contact with thrombin at exosite II (8), located on the opposite pole of the molecule relative to exosite I (9).

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†The abbreviations used are: EGF, epidermal growth factor; MES, 4-morpholineethanesulfonic acid; FpA, fibrinopeptide A.

Previous investigations of the thrombin-thrombomodulin interaction have documented a surprisingly small change in heat capacity upon formation of the complex (10), suggesting that the interaction is a simple rigid-body association that elicits no significant conformational changes in either thrombin or thrombomodulin. Unequivocal support to this conclusion has come from the recent structural work on thrombin bound with a fragment of thrombomodulin containing EGF-like domains 4–6 (11). The structure reveals extensive contacts made by thrombomodulin with exosite I of thrombin, in agreement with previous x-ray and NMR studies (12, 13), but shows no significant structural changes in the enzyme.

The structural epitope documented recently needs to be validated energetically by Ala scanning mutagenesis of the residues involved in thrombomodulin recognition. Several biological systems studied previously have shown significant differences between structural and functional epitopes (14). Specifically, structural epitopes tend to be larger in size than functional epitopes, because the binding free energy for an interaction often resides at localized hot spots (15). The correct identification of the boundaries of an epitope must therefore rely on Ala scanning mutagenesis and not structural analysis alone (16), because it is the functional epitope that eventually defines the target for drug development and molecular mimicry (15). Here we report the functional epitope of thrombin recognizing thrombomodulin, as revealed by Ala scanning mutagenesis of over 50 residues of thrombin. The results reveal aspects on the energetics of thrombomodulin recognition that have a bearing on protein-protein interactions in general.

MATERIALS AND METHODS

Site-directed mutagenesis of human α-thrombin was carried out in a HPC4-pNUT expression vector, using the QuikChange site-directed mutagenesis kit from Stratagene. Expression of mutant and wild-type thrombin was carried out in baby hamster kidney cells as described previously (17). The enzyme was activated with the prothrombinase complex for 30 min at 37 °C. Some mutants required further activation with the immobilized snake venom enzyme ecarin. Activated thrombin was purified to homogeneity by fast protein liquid chromatography using Resource Q and S columns with a linear gradient from 0.05 to 0.5 M choline chloride, 5 mM MES, pH 6 at room temperature. Mutants were checked for incomplete activation and/or autolytic digestion by N-terminal amino acid sequencing. Mass spectrometry yielded molecular weights consistent with the mutations introduced and indicated identical glycosylation between wild-type and mutant constructs. The active site concentration was determined by titration with hirudin and was found to be >95% in all cases. Human fibrinogen and rabbit thrombomodulin containing chondroitin sulfate were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Rabbit thrombomodulin is functionally equivalent to the human protein containing chondroitin sulfate (10).

All assays were carried out under experimental conditions of 5 mM Tris, 0.1% polyethylene glycol, 145 mM NaCl, pH 7.4 at 37 °C. The interaction of thrombin with thrombomodulin was studied directly from the inhibition of fibrinopeptide A (FpA) release upon fibrinogen cleavage (10). First, the progress curve of FpA release was measured in the

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In the absence of competitive inhibitor to determine the value of the specificity constant $k_{cat}/K_m$ (18). The point along the progress curve most sensitive to competitive inhibition is at time $t_c = K_m/\epsilon_F$. Then, the amount of FpA released at time $t_c$ was determined as a function of the concentration of thrombomodulin to derive the value for the $K_d$ of binding using the equation,

$$ [\text{FpA}] = [\text{FpA}]_0 \left[1 - \exp \left(\frac{t_c - \epsilon_F}{k_{cat}/K_m} \left(1 + \frac{[\text{L}]}{K_d}\right)\right) \right] \quad \text{(Eq. 1)} $$

where $L$ stands for thrombomodulin, $\epsilon_F$ is the active thrombin concentration, $k_{cat}/K_m$ is the specificity constant for FpA release. Equation 1 implies that thrombomodulin competitively inhibits fibrinogen binding. As in previous studies (10) we observed no evidence of the contrary and the boundaries of the epitope for thrombomodulin binding in exosite I identified in the present study overlap completely with those of fibrinogen recognition (see “Results”).

RESULTS

Previous site-directed mutagenesis studies on thrombin-thrombomodulin interaction have identified thrombin residues involved in thrombomodulin binding by comparing the effect of mutations on protein C activation in the presence and absence of thrombomodulin (19, 20). When the effects of a thrombin mutation that influences thrombomodulin binding are assessed from the reduction of the thrombomodulin-dependent protein C activation relative to wild-type thrombin, the results depend entirely on the thrombomodulin concentration used. A more obvious limitation of this approach is that it does not provide an energetic quantification of the effect of a thrombin mutation on thrombomodulin binding. In this study, we have chosen a direct method of determination of $K_d$ for thrombomodulin binding to wild-type and 54 mutant thrombins, and the results are shown in Fig. 1.

Of the thrombin residues mutated, only a few produce significant ($>3RT$) changes in the binding free energy $\Delta G = RT \ln K_d$ of the mutant relative to wild-type. These residues are Arg$^{67}$, Lys$^{70}$, Tyr$^{76}$, Glu$^{77}$, Glu$^{80}$, and Ile$^{82}$ and cluster in a hot spot of exosite I (Fig. 2). Arg$^{67}$, Lys$^{70}$, Glu$^{77}$, and Glu$^{80}$ form a buried ion quartet under the surface of the 70-loop, on top of which sit Tyr$^{76}$ and Ile$^{82}$. Interestingly, most of the quartet and Tyr$^{76}$ are excised from exosite I upon autocatalytic cleavage at Arg$^{77a}$ and Arg$^{67}$ that produces the derivatives $\beta$- and $\gamma$-thrombins severely compromised in fibrinogen clotting and thrombomodulin binding (21).

Among the regions that seem to be involved significantly in thrombomodulin binding, the domain bridging the active site and exosite I around the 30-loop contains residues of particular importance. Phe$^{34}$ and Lys$^{36}$ are noteworthy, as their mutation drops the affinity for thrombomodulin more than 10-fold. Neighbor residues Pro$^{37}$ and Gln$^{38}$ also make significant contribution to binding, unlike other residues in the region like Arg$^{67}$, Ser$^{68}$, and Glu$^{77}$. Residues defining the entrance to the thrombin active site, like Trp$^{60}$ and Leu$^{69}$, or the autolysis loop and the 186-loop show little involvement in thrombomodulin binding. In the 220-loop, containing residues involved in Na$^+$ binding, mutation of Tyr$^{225}$ affects thrombomodulin binding 10-fold presumably by altering the conformation of thrombin from the fast to the slow form.

In exosite II, there are a few residues that participate in thrombomodulin binding by facilitating the docking of the negatively charged moiety of the chondroitin sulfate. Exosite II contains no hydrophobic patches on its surface, unlike exosite I, and features two ion clusters of partially buried residues organized in the quartet Glu$^{97a}$-Asp$^{100}$-Arg$^{101}$-Arg$^{175}$ and the triplet Arg$^{165}$-Asp$^{178}$-Arg$^{235}$. In the first cluster, mutation of Arg$^{101}$ affects thrombomodulin binding 10-fold, and Arg$^{175}$ makes only a small contribution to binding. The importance of the cluster and Arg$^{101}$ is demonstrated by the fact that mutation of the negatively charged residues Asp$^{178}$ and Glu$^{97a}$ actually results in improved thrombomodulin binding. These residues partially neutralize Arg$^{101}$ and reduce the electrostatic coupling of this residue with the chondroitin sulfate moiety of thrombomodulin. This effect is even more evident in the second cluster, where mutation of Asp$^{178}$ increases thrombomodulin binding almost 10-fold. This residue neutralizes Arg$^{165}$ and
Arg233, which could otherwise interact very favorably with thrombomodulin. The neutralization effect is significant because mutation of either Arg 165 or Arg 233 impairs thrombomodulin binding only slightly.

The results reported here compare well with and extend those reported in previous studies. The extensive Ala-scanning mutagenesis study of the Leung’s group uncovered residues Lys36, Gln38, Arg67, Lys60, Arg73, Thr74, Tyr76, Arg77a, Lys81, and Lys109 as part of the epitope for thrombomodulin binding (20). Our study confirms the important role of Arg67, Lys70, and Tyr76, but assigns a lesser role to Lys36, Gln38, and Arg77a, and practically no role to Arg73, Thr74, Lys81, and Lys109. Our study also rules out the importance of residue Arg75 to thrombomodulin binding suggested previously from charge reversal substitution (22), but confirms the important role of the hydrophobic residues Phe34, Tyr76, and Ile82 supported by recent hydrogen/deuterium exchange experiments (23). Finally, Ala mutants of Arg93, Arg97, and Arg101 have shown that Arg93 is important for heparin binding, whereas Arg97 and Arg101 play a dominant role in thrombomodulin binding (8, 24). We confirm the important role of Arg101 in view of its coupling with Asp100 and Glu97a, but find no contribution of Arg97 to thrombomodulin recognition.

Our study enables a direct comparison of the functional epitope for thrombomodulin binding to thrombin (red and yellow residues in Fig. 2, bottom left) with the structural epitope (red and yellow residues in Fig. 2, bottom right) recently determined by x-ray crystallography of thrombin bound to the thrombomodulin fragment containing the EGF-like domains 4–6 (11). We find that the functional epitope is less extended than the structural epitope (Fig. 2), with most of the energy for thrombomodulin binding being concentrated in a hot spot defined by the residues of the buried ion-paired quartet Arg67–Lys70–Glu77–Glu80, topped by the hydrophobic residues Tyr76 and Ile82. The crystal structure shows that the thrombin-thrombomodulin interface in exosite I is dominated by hydrophobic contacts between Phe34, Ile82, and Tyr76 of thrombin, and Ile414, Leu415, Ile424, Phe432, and Thr422 of thrombomodulin (11). The side chains of residues Ser266, Gln38, Thr74, and Arg77a make polar contacts with thrombomodulin, and so do the backbones.

FIG. 2. Molecular mapping of the thrombin epitope-recognizing thrombomodulin. Thrombin is displayed as its solvent-accessible surface based on the coordinates 1PPB. The orientation is centered around the active site, exosite II, or exosite I. The residues subject to mutagenesis are color-coded based on the extent of change in the Kd for thrombomodulin binding (see Fig. 1, a–c) calculated as ΔGmut = RTln(Kd,w/Kd,mut) in RT units according to the scale (top panels and bottom left: red (5–7 RT units), yellow (3–5 RT units), green (1–3 RT units), cyan (−1–1 RT units), blue (−3–1 RT units)). Residue Lys81 is buried under Tyr76 in exosite I and is not visible. Note the hot spot (red residues) under the surface of exosite I contributing most of the binding energy for thrombomodulin recognition. Also notable, in exosite II, is the effect of mutating Asp100 and Asp178, which translates in enhanced thrombomodulin binding. The structural epitope for thrombomodulin binding is shown at the bottom right panel using the coordinates 1DX5 (11). The epitope is color-coded according to the interatomic distance of the thrombin-thrombomodulin contacts: red (<3 Å), yellow (<4 Å), green (<5 Å), cyan (<6 Å), blue (<10 Å). The functional epitope (residues in red and yellow, bottom left) is only a small portion of the structural epitope (residues in red and yellow, bottom right). An electronic version of the thrombin epitope for thrombomodulin ready for viewing, rotating, and zooming can be downloaded from www.biochem.wustl.edu/~enrico.
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The results reported in this study define the regions of thrombin interacting with the receptor thrombomodulin. We find that thrombomodulin binding is controlled by a hot spot buried under the surface of the 70-loop in exosite I and supporting Tyr^{76} and Ile^{82}. Other notable findings are the contribution of Phe^{34} located in close proximity to this hot spot and the unanticipated role of Asp^{100} and Asp^{178} in exosite II in enhancing thrombomodulin binding when mutated to Ala. No other region of thrombin appears to be significantly involved in thrombomodulin binding. This allows the active site, the 186-loop, and the autolysis loop to remain available for binding protein C in the ternary complex formed with thrombomodulin and thrombin.

The results reported in this study address important points on protein-protein interactions and their value extend beyond the realm of thrombin. The observation that binding epitopes identified by Ala scanning mutagenesis are often small subsets of structural epitopes is not new and was elegantly documented in the case of the human growth hormone binding to its receptor (14, 15) and the barnase-barstar interaction (25). The incomplete overlap between structural and functional epitopes makes Ala scans absolutely necessary to correctly define residues energetically involved in protein-ligand interactions (16).

Another issue of interest in protein-protein interactions is whether residues of an epitope participate in ligand binding in an independent or cooperative manner (16). It has been proposed that such regions can be uncovered by studying hydrogen/deuterium exchange properties of specific residues, because residues that become buried upon formation of a complex are likely to contribute most of the binding free energy (23). Fig. 4 shows the change in binding free energy due to the Ala substitution versus the change in accessible surface area for residues defining the structural epitope of thrombomodulin in exosite I. The correlation in the plot is $r = -0.01$. As the identification of molecular contacts in a crystal structure cannot reveal the energetics of the interaction, the change in water accessibility of a residue upon formation of the complex cannot reveal the extent to which that residue participates energetically to binding.

DISCUSSION

Fig. 4. Energetic perturbation of thrombomodulin binding in exosite I plotted as a function of the change in exposed surface area upon formation of the complex, calculated from the coordinates 1DX5 (11). Shown are 17 residues of exosite I subject to Ala substitution in this study and that change their exposed surface area upon binding of thrombomodulin, according to the crystal structure (11). The values of $\Delta G_{\text{mut}} = R\ln(K_{\text{mut}}/K_{\text{wild}})$ in kcal/mol and the change in exposed surface area in $\text{Å}^2$ per each residue are: Phe^{34} (2.6, −16); Lys^{50} (1.8, −4); Ser^{36} (−0.2, −18); Pro^{77} (1.2, −53); Gln^{38} (1.4, −118); Glu^{39} (−0.2, −7); Leu^{52} (1.0, −22); Arg^{67} (3.4, −14); Thr^{74} (0.8, −43); Arg^{75} (0.7, −32); Tyr^{76} (3.0, −91); Arg^{77} (1.5, −80); Glu^{80} (3.4, −3); Lys^{110} (1.0, −45); Ile^{82} (2.6, −72); Met^{84} (0.3, −53); Lys^{178} (−0.3, −52). The correlation between the perturbation of thrombomodulin binding and the change in exposed surface area upon binding is $r = −0.01$. The change in water accessibility of a given residue is not a reliable predictor of the energetic involvement of that residue in ligand recognition.

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replacement of 24 residues in exosite I is $-32.3 \text{ kcal/mol}$, whereas the binding free energy for wild-type thrombin interacting with thrombomodulin is $-12.7 \text{ kcal/mol}$. The excess free energy of $19.6 \text{ kcal/mol}$ calculated from summing the individual perturbations from the Ala scans underscores the cooperative nature of the binding epitope (16, 26). The excess of $19.6 \text{ kcal/mol}$ documented for the thrombin-thrombomodulin interaction compares well with the values of $13.6 \text{ kcal/mol}$ (17) with their receptors. Interestingly, a good portion of the excess binding free energy due to residue-residue coupling in the thrombomodulin epitope can be assigned structurally to the buried quartet in exosite I. Residues Arg$_{67}$, Lys$_{70}$, Glu$_{77}$, and Glu$_{80}$ define a highly coupled ion quartet where mutation of any residue produces the same loss in thrombomodulin binding free energy of $-3.5 \text{ kcal/mol}$ (see Fig. 1a). The structural origin of this energetic perturbation is disruption of the architecture of the 70-loop with loss of optimal orientation of Tyr$_{76}$ and Ile$_{82}$ and is achieved by replacing any one of the four members of the quartet. In fact, the Ala replacement of either Tyr$_{76}$ or Ile$_{82}$ produces a binding defect practically identical to that caused by the Ala replacement of any member of the quartet. The structural coupling among Arg$_{67}$, Lys$_{70}$, Glu$_{77}$, and Glu$_{80}$ produces an excess in the predicted binding free energy for thrombomodulin of $-3.5 \times 4 = 14 \text{ kcal/mol}$ when the energetic perturbations of the single Ala mutations are added up, which accounts for 70% of the total excess (19.6 kcal/mol) found experimentally. The thrombin-thrombomodulin interaction can therefore be used as an instructive model system for illustrating the role of hot spots in protein-protein interactions and to dissect the structural origin of residue-residue coupling in epitopes involved in protein recognition.

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