Crystallographic Structures of Thrombin Complexed with Thrombin Receptor Peptides: Existence of Expected and Novel Binding Modes†,‡

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ABSTRACT: Many of the vital actions of thrombin on platelets and other cells appear to be mediated by the recently cloned seven-transmembrane-domain thrombin receptor. Thrombin activates this receptor by a novel proteolytic mechanism. The amino-terminal exodomain of the receptor contains the sequence LDPRSFLLRNPNDKYEPF. Structure–activity studies with mutant receptors and receptor peptides suggest that this sequence binds to thrombin at two sites: LDPR with the active center of thrombin and KYEPF with the fibrinogen recognition exosite of thrombin. Thrombin then cleaves the Arg41–Ser42 bond to unmask a new amino terminus, which functions as a tethered peptide ligand binding to as yet undefined sites within the body of the receptor to effect receptor activation. We have determined eight crystal structures of thrombin complexed with receptor-based peptides. Each of the two components of the bidentate docking model was captured in individual cocrystals. In one crystal type, the LDPR sequence docked in the active center of thrombin in a manner analogous to *d*-PheProArg chloromethyl ketone. In other crystals, the KYEPF sequence bound in the fibrinogen anion binding exosite of thrombin in a manner analogous to the DFEEI sequence of the carboxylate-terminal peptide of hirudin. Strikingly, however, generation of a single crystal that includes both components of the anticipated bidentate binding mode was not achieved, apparently because the peptides have a dominant solution S-like conformation that does not bind in a productive way at the active center. This peptide structure apparently favored a novel alternative mode of receptor peptide–thrombin interaction in which the receptor peptides formed an intermolecular bridge between neighboring thrombin molecules, resulting in an infinite peptide thrombin chain in crystals. In this structure, the KYEPF sequence docked in the expected manner at the exosite of one thrombin molecule, but the LDPR sequence docked in an unusual nonproductive mode with the active center of a neighboring molecule. Mutations that removed important determinants of the S-like receptor peptide structure underlying the bridging mode in the receptor itself did not significantly alter thrombin signaling. Additionally, a comparison of receptor density to the responsiveness of a cell did not support a role for receptor oligomerization in signaling. The physiological role for this unexpected intermolecular binding mode, if any, remains to be identified. Docking of the KYEPF sequence of receptor peptides was associated with conformational changes in the active center and the autolysis loop of thrombin; the former may account for changes in substrate specificity reported to be induced by exosite binding and may be potentially important for receptor activation.

Thrombin, a multifunctional serine protease generated at sites of vascular injury, is a potent stimulus for a variety of cellular events. It initiates platelet aggregation (Davey & Luscher, 1968; Charo et al., 1977), activation of monocytes and neutrophils, and proliferation of mesenchymal cells [reviewed in Coughlin (1994)]. The diverse actions of thrombin may be viewed as mediating hemostatic, inflammatory, and proliferative responses to wounding. The recently cloned thrombin receptor (Vu et al., 1991a) appears to be capable of mediating many of these responses and has provided a framework for understanding how the thrombin protease signals cell activation.

The thrombin receptor is a member of the seven-transmembrane-domain family of receptors, but is activated by a novel mechanism. Thrombin cleaves the long extracellular amino-terminal domain of the receptor at Arg41 to unmask

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‡ The coordinates of the complexes have been deposited in the Brookhaven Protein Data Bank under the following filenames: NRS, 1NRN; NRP, 1NRO; NR′S, 1NRP; *d*-FPR′S, 1NRQ; (NRP+H), 1NRS; (PPACK+XA), 1NRR.

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Table 1: Amino Acid Sequences of the Thrombin Receptor Peptides Studied[a]

abbreviations	sequences
	38 40 45 50 55 60 64
NRS (native)	L D P R S F L L R N P N D K Y E P F W E D E E
NRP	L D P R P F L L R N P N D K Y E P F W E D E E K N E S
NR'S	L D P R R' S F L L R N P N D K Y E P F W E D E E
d-FPR'S	d-F P R' S F L L R N P N D K Y E P F W E D E E
(PPACK+XA)	Ac-F L L R N P N D K Y E P F W E D E E K-amide
(NRP+H)	L D P R P F L L R N P N D K Y E P F W E D E E K N E S
hirugen (H)[b]	N G D F E E I P E E Y* L
XA	Ac-F L L R N P N D K Y E P F W E D E E K-amide
(nR'S+XB)	L D P R' S F L L R
	N P N D K Y E P F W E D E E

[a] N, native receptor peptide from 38–60 (also used for 38–64 peptide); n, from 38–46 only; two letters trailing N are residues at the scissile bond (41–42); all in receptor peptide numbering; X, fibrinogen binding exosite fragment of receptor A (from 43–61) or B (from 47–60); R', β-homoarginine (Argh); *d* indicates *d*-enantiomer; Y*, sulfated tyrosine; plus sign used to designate ternary complexes. [b] Hirugen sequence (H) aligned with receptor peptides in receptor peptide numbering.

a new amino terminal beginning with the sequence SFLLR. This new amino terminal then functions as a tethered peptide ligand, binding to an as yet undefined domain in the body of the receptor to effect activation (Vu et al., 1991a,b). Indeed, the synthetic peptide SFLLR is a full agonist for receptor activation.

In the context of known thrombin structures complexed with other polypeptides (Skrzypczak-Jankun et al., 1991; Qiu et al., 1992, 1993), functional studies with mutant receptors and receptor-based peptides have suggested a model for the thrombin–receptor interaction. The amino-terminal exodomain of the receptor contains the sequence LDPRSFLLRNPNDKYEPFWEDEE. The LDPR sequence is identical to the known thrombin P1–P4 sites in protein C. The DKYEPFWEDEE sequence resembles the carboxylate-terminal peptide of hirudin, which is known to interact with the fibrinogen recognition or anion binding exosite of thrombin (Rydel et al., 1991). Thus, by analogy, the hypothesis was advanced that the amino-terminal exodomain of the receptor might interact with thrombin in a bidentate manner, very much like hirulog3[1] (Qiu et al., 1992), with the LDPR sequence bound in the active center of thrombin and the hirudin-like domain docked in the anion binding exosite of thrombin (Vu et al., 1991b). Whether additional receptor domains participate in the thrombin receptor association has not yet been addressed.

This model of thrombin–receptor interaction has been buttressed by structure–activity studies. Substitution of the enteropeptidase recognition sequence DDDDK for the LDPR sequence of thrombin receptor changed the receptor specificity, creating an enteropeptidase receptor. This observation, along with others, confirms that cleavage of the Arg41–Ser42 peptide bond (Table 1) is necessary and sufficient for receptor activation (Vu et al., 1991b). Deletion of the hirudin-like domain of the receptor markedly shifted the concentration response of the receptor to thrombin, but substitution of the carboxylate terminal of hirudin itself restored receptor function. Alanine scanning of the hirudin-like region of the receptor revealed residues Tyr52, Glu53, and Phe55 to be important for thrombin–receptor interaction, suggesting roles analogous to those of Phe 56, Glu57, and Ile59 of hirudin (Vu et al., 1991b).

The bidentate nature of the interaction of the receptor with thrombin was also supported by studies with receptor-based peptides. Native receptor peptides that included both the LDPR sequence and the hirudin-like domain were good substrates for thrombin, some 300-fold better than peptides lacking the hirudin-like domain (Vu et al., 1991b). Similar peptides that contained a Ser42Pro substitution at the P1′ site and slow cleavage by thrombin inhibited thrombin esterolytic activity. Inhibitory peptides that included both the LDPRP sequence and the hirudin-like domain were 3 orders of magnitude more potent than peptides that included the LDPRP sequence alone. Addition of a peptide mimicking the hirudin-like domain did not boost the inhibitory activity of the lone LDPRP peptide, suggesting that the two domains had to be covalently joined for high-affinity binding of thrombin (Liu et al., 1991).

To test this model of the thrombin–thrombin receptor interaction and to identify the docking associations of thrombin with its receptor, we have determined the crystallographic structures of a number of thrombin complexes with receptor-based peptides. We obtained cocrystals in which the LDPR sequence docked productively in the active center and crystals in which the KYEPF sequence docked in the fibrinogen recognition site of thrombin, which is similar in binding to that of the carboxylate-terminal peptide of hirudin. However, the bidentate binding interaction was not observed in a single crystal. Instead, a novel alternate intermolecular binding mode for the thrombin receptor peptides was revealed, where the LDPR sequence bound at the active site of one thrombin molecule in an unexpected nonproductive binding mode while the KYEPF sequence of the same receptor peptide bound in the usual way at the fibrinogen recognition site, but of another thrombin molecule.

The alternate binding mode is apparently the result of a relatively stable solution S-like conformation of the peptides. When crystallization conditions were changed to conform to those that produced crystals of hirugen, hirulog1, and hirulog3 thrombin complexes (the latter in a bidentate binding mode), bidentate binding still was not observed. The crystals were either the same or cleavage of the receptor peptide occurred and γ-thrombin crystals were produced. In the production of γ-thrombin, it is presumed that the bidentate binding mode existed transiently, but resulted in cleavage of the receptor peptide with eventual autolytic conversion of α- to γ-thrombin.

EXPERIMENTAL PROCEDURES

Crystallization. The receptor peptide sequences that were examined crystallographically are given in Table 1. The

[1] Abbreviations: hirulog3, *d*-FPR′(G)$_5$ hirudin 53–64, where *d* is the *d*-enantiomorphic form, R′ is β-homoarginine (Argh), and Tyr63 is not sulfated; hirugen, hirudin 53–64 with sulfated Tyr63; hirulog1, *d*-FPRP-(G)$_4$ hirudin 53–64 with unsulfated Tyr63; PPACK, *d*-FPR chloromethyl ketone; MDL 28050, SuccYEPI PEEA Cha *d*-E, where Succ is succinyl and Cha is β-cyclohexylalanine; hirullin P18, SDFEEFSLDDIEQ.

Table 2: Summary of Intensity Data Collection and Least-Squares Refinement Statistics/Parameters

	NRS	NRP	NR'S	*d*-FPR'S	(NRP+H)	(PPACK+XA)	XA	(nR'S+XB)
resolution (Å)	3.1	3.1	3.0	3.5	2.4	2.4	3.5	3.0
data coverage (%)	88	88	88	83	85	91	83	85
R_{merge} ($\|F\|^2$) (%)	6.5	7.1	9.2	3.9	2.9	3.5	8.0	6.1
unit cell parameters								
a (Å)	129.75	128.08	128.30	130.38	71.00	71.27	126.45	126.31
b (Å)	51.94	51.76	51.11	52.13	72.41	72.20	48.39	48.43
c (Å)	63.32	62.94	62.97	63.50	72.80	72.32	52.86	52.96
β (deg)	101.21	100.74	100.44	100.52	100.96	100.32	96.26	96.40
volume (Å^3)	418557	409960	406041	424336	367449	366142	321482	321870
space group	*C*2	*C*2	*C*2	*C*2	*C*2	*C*2	*P*2	*C*2
Matthews constant, V_m	2.62	2.53	2.51	2.62	2.37	2.27	2.33	2.33
protein fraction	0.47	0.48	0.49	0.48	0.52	0.54	0.53	0.53
resolution range (Å)	7.0–3.1	7.0–3.1	7.0–3.0	7.0–3.5	7.0–2.4	7.0–2.4	9.0–3.5	7.0–3.0
R factor (%)	17.9	17.1	19.9	21.7	14.6	14.5	21.3	20.8
no. of reflections	4396	4572	5696	3556	11797	12963	6092	4192
no. of water molecules	105	117	73		210	207		53
rmsΔ bond length (Å)[a]	0.014	0.014	0.013	0.029	0.020	0.023	0.017	0.021
rmsΔ bond angle (Å)[a]	0.046	0.045	0.039	0.057	0.048	0.044	0.047	0.043
rmsΔ dihedral (Å)[a]	0.042	0.048	0.041	0.061	0.053	0.051	0.050	0.044
av thermal parameter (Å^2)	31	29	34	33	26	25	26	26

[a] The rms values are the respective root-mean-square deviations from the ideal values.

receptor peptide–thrombin complexes were prepared by placing an approximately 10-fold molar excess of the receptor peptides, NRS, NRP, NR'S, and *d*-FPR'S,[2] as solids over a frozen 1-mL sample of human α-thrombin (1.48 mg/mL in 0.75 M NaCl). The (PPACK+XA) peptide complex was made with PPACK-inhibited thrombin (0.94 mg/mL) and the XA peptide. The ternary complex of (NRP+H) and thrombin was prepared in a manner similar to that reported elsewhere (Maryanoff et al., 1993; Wu et al., 1993). The protein complexes were diluted to 2 mL with 0.1 M sodium phosphate buffer (pH 7.3) and concentrated to about 5 mg/mL using a Centricon-10 miniconcentrator in a refrigerated centrifuge. Initially, the crystals were grown by the hanging drop method; further work was carried out with sitting drops. The protein droplet consisted of 5 μL of the protein complex and 5 μL of the reservoir solution. Crystals could be obtained over a relatively broad range of conditions: 24–30% (w/v) PEG 4000 and 8000 and pH 6.5–8.5. The most suitable crystals of NRS, NRP, and (NRP+H) were those from 26% PEG 8000 and 0.1 M Tris buffer (pH 8.5). Seeding techniques using small crystals (Thaller et al., 1985) were essential to obtain larger diffraction-quality single crystals. Crystals of the NRS, NRP, and NR'S thrombin complexes could not be stored safely at room temperature for extended periods of time. Upon standing, they slowly became opaque, their morphology deteriorated, and they dissolved.

Intensity Data Collection. X-ray diffraction intensity data collection was carried out with a Siemens X-1000 multiwire area detector using graphite-monochromated Cu Kα radiation from a Rigaku RU200 rotating anode X-ray source with a normal focus (0.5 × 10 mm), operating at 50 kV and 130 mA. The dimensions of the crystals used for intensity data collection were typically in the range 0.3–0.6 mm, and in each case only one crystal was used for the measurements. The crystal-to-detector distance was 11.65 cm, the detector swing angle was −15.0°, and the scan range per frame was 0.2° for better diffracting (NRP+H) and (PPACK+XA) crystals; the latter two angles were −6.0° and 0.25°, respectively, for the other complexes. The average peak width of a reflection varied between 0.4 and 0.7°. The data frames were reduced to integrated intensities, scaled, and merged with the XENGEN package of programs (Howard et al., 1987).

[2] For abbreviations of the peptide sequences, refer to Table 1.

On the basis of unit cell dimensions, the complexes can be grouped into three classes (Table 2): (a) NRS, NRP, NR'S, and *d*-FPR'S; (b) (NRP+H) and (PPACK+XA); and (c) XA and (nR'S+XB). The unit cell dimensions are isomorphous in the respective classes; class b crystals diffracted X-rays to 2.4 Å, whereas the others diffracted to only about 3.0 Å. The unit cell dimensions of class b crystals are analogous to those of hirugen thrombin and similar complexes (Skrzypczak-Jankun et al., 1991; Qiu et al., 1992, 1993); class c crystals correspond to those of γ-thrombin (Rydel et al., 1993). Although XA crystals belong to space group *P*2 and not *C*2 as in γ-thrombin (Table 2), analysis of the diffraction data showed that the two molecules in the asymmetric unit give rise to pseudo-C_2 symmetry. The displacement of the independent molecules from *C*-centering (x, y, z; $^1/_2 + x, ^1/_2 + y, z$) corresponds to a rotation of 5° around the *b*-axis and a translation of 1.6 Å between the molecules along the *c*-axis. This produces a shift of about 1.20 and 0.73 Å in x and y directions from ideal *C*-centered symmetry. Essentials of the intensity data collections and the unit cell parameters are also summarized in Table 2.

Structure Solution. The first structure to be solved was that of the NRP receptor peptide complex by molecular replacement–rotation/translation methods. The thrombin coordinates of the hirugen thrombin complex (Skrzypczak-Jankun et al., 1991) were used as the search model (residues 1H–15 of the A-chain, 16–145 and 150–247 of the B-chain, chymotrypsinogen numbering; Bode et al., 1992). The rotation/translation search was carried out using the program XPLOR (Brunger, 1990a) and diffraction data in the resolution range 8.0–4.0 Å. The 3000 largest Patterson vectors between 20.0 and 3.0 Å of the model map were chosen for the rotation search. The best solution, which was eventually proved to be the correct one, had a peak height of 5.4 (10.4σ above the mean). The next best solution had a height of 4.1 (5.4σ above the mean). The eight highest peaks of the rotation function were then used in a Patterson correlation refinement (Brunger, 1990b), where the rotation angles are refined to maximize the correlation between the square of the normalized observed structure factors and the square of the structure factors of the search model placed in a triclinic unit cell, identical in dimensions to those of the crystal. The correlation for the highest solution was 0.30, and 0.07 for the next solution. The translation search with the best rotation gave a correlation

Table 3: Thrombin Receptor Peptide Interactions of NRS[a]

		distance (Å)	region	comment
Leu38rN	Tyr60AOH	2.7	active site	hydrogen bond
Asp39rOD1	His57ND1	3.5	active site	ion pair
Asp39rOD2	His57NE2	3.0	active site	hydrogen bond
Pro40rO	Gly219N	2.7	active site	hydrogen bond
Arg41rNH1	Glu53rOE1	4.6	intramolecular	ion pair
Phe43r	Pro37*	4.7	exosite	stacking
Leu45rCD2	Leu38rCD1	3.3	intramolecular	van der Waals
Leu45rCD2	Ile174CG1	3.2	active site	van der Waals
Asp50rOD2	Arg73*NH2	3.6	exosite	ion pair
Lys51rNZ	Glu39*OE1	3.5	exosite	ion pair
Tyr52rOH	Arg73*NH2	2.8	exosite	hydrogen bond
Tyr52r	Phe34*	3.4	exosite	stacking
Phe55r	Phe34*	3.4	exosite	stacking
Arg46rNH1	Glu97AOE2		solvent bridge	Arg46r–O_w480–O_w499–O_w443–Glu97A 3.1 Å 3.1 Å 3.0 Å 3.1 Å
Glu53rOE2	Arg35NH2		solvent bridge	Glu53r–O_w421–O_w455–Arg35 3.0 Å 2.8 Å 2.6 Å

[a] Asterisks designate symmetry-related molecules at ($^3/_2 - x$, $^1/_2 + y$, $-z$).

peak of 0.59 (11.6σ above the mean). Rigid-body refinement of this model produced a crystallographic R value of 0.40 for 5129 reflections in the resolution range 10.0–3.0 Å. The initial phases for the remainder of the complexes of class a crystals were approximated using the thrombin coordinates of the NRP complex.

The class b receptor complexes, (NRP+H) and (PPACK+XA), have unit cell parameters isomorphous with the hirugen thrombin complex (Skrzypczak-Jankun et al., 1991), so that the phases of the former were approximated using phases based on the thrombin coordinates of the latter. The ($2F_o - F_c$) electron and the ($F_o - F_c$) difference electron density maps were computed after restrained least-squares refinement in the resolution range 7.0–2.8 Å, initially with an overall B and followed with individual B values. The former map showed good density for most of the thrombin residues, while the ($F_o - F_c$) map clearly showed extra density for receptor residues[3] Leu38r–Arg41r in the active site, hirugen residues Asp55–Tyr63 in the exosite of the (NRP+H) complex, and PPACK and Lys51r–Phe55r of the receptor peptide in the active site and exosite, respectively, of the (PPACK+XA) complex.

Refinement. Refinement of all of the structures was carried out using the programs, PROLSQ (Hendrickson, 1985) and PROFFT (Finzel, 1987). Refinement was interrupted by a number interactive computer graphics sessions on an Evans and Sutherland PS390, using the program FRODO (Jones, 1982) for improving the fit of the model to the electron and difference density maps, simultaneously conforming the structure to allowed Ramachandran space. The refinements of the class b structures proceeded easily and smoothly, which was not always the case with the class a structures. The latter required considerable modeling and interpretation of the electron density maps. A notable exception was the NRS complex. The initial ($F_o - F_c$) map of the NRS complex showed difference density for most of the receptor peptide residues, and the trace of the main chain clearly confirmed the earlier deduction from some of the other structures that it extended from the active site of one thrombin molecule to the exosite of a neighboring thrombin molecule.

Solvent was included as water molecules in the final stages of refinement. The water molecules were located from ($F_o - F_c$) maps in two resolution ranges (lower resolution cutoffs of 7.0 and 9.0 Å) and the $2(F_o - F_c)$ map. The occurrence of a peak in all of the maps within 4.0 Å of either protein or neighboring water molecules and its suitable refinement (occupancy > 0.5, $B < 40$ Å^2) were used as acceptance criteria. Since class b crystals diffracted better, about 200 water molecules were assigned to density, while one-half this number was used in the class a crystal refinements (Table 2). The solvent interactions with the thrombin molecule are generally similar in all of the receptor peptide thrombin complexes and are also similar to those of other known thrombin complexes. Water-mediated interactions also occur between thrombin and the receptor peptide. In the NRS complex, there are two solvent bridges (Table 3) involving two and three water molecules, respectively. In the (NRP+H) complex there are three water-mediated interactions. Summaries of each of the refinements are presented in Table 2. The coordinates of the complexes have been deposited in the Brookhaven Protein Data Bank.

RESULTS

Receptor Peptide NRS. The NRS peptide represents the native thrombin receptor sequence between residues Leu38 and Glu60. In cocrystals of this peptide with α-thrombin, the electron density of the B-chain of the thrombin molecule was well-defined, except for a few longer side chains or parts thereof that appear to be disordered (Arg50, Lys70, Ile82, Lys109, Glu127, and Glu192). The density for the receptor peptide is also generally good, extending to Trp56r (Figure 1). The peptide has a twisted, three-stranded, antiparallel, S-like secondary structure. The plane of the backbone of the first three residues (including the extended Leu38r side chain) and the beginning of the second strand are approximately at right angles to the plane of the longer second and third peptide segments (Arg41r–Arg46r and Asn47r–Phe55r) (Figure 1). Although there are breaks in the electron density at the turns (Pro40r–Arg41r and Pro48r–Asn49r), the path of the chain is unambiguous because of the sharpness of the reverse turns. Furthermore, the side groups of Leu44r and Trp56r have little density and are flexibly disordered. The disorder of Leu44r may be the result of the close proximity of the side chains of Arg77A and Asn78 of thrombin in the complex (~3.5–4.0 Å).

The three antiparallel strands are Leu38r–Pro40r, Arg41r–Leu45, and Asp50r–Pro54r; they are about 6–7 Å apart and

[3] Amino acids or peptide sequences designated with an "r" correspond to receptor peptides.

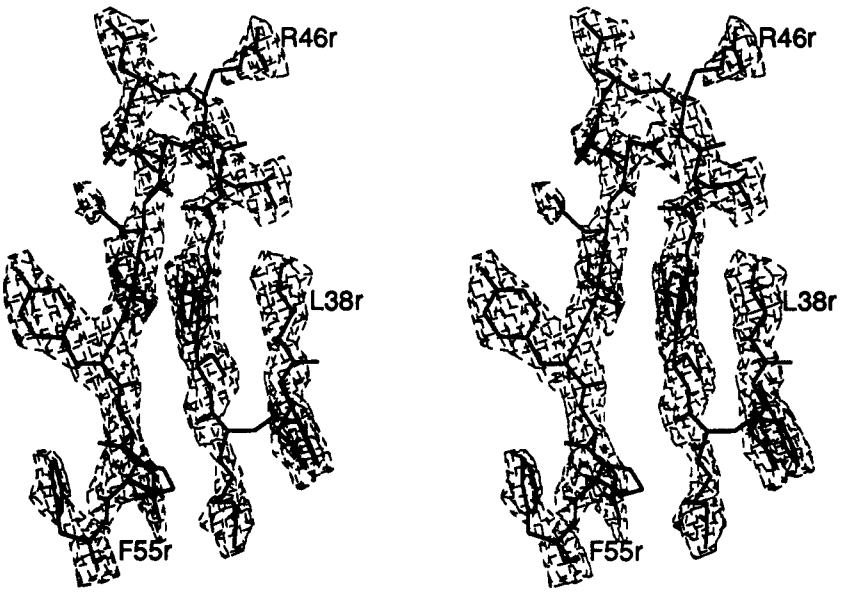

FIGURE 1: Stereoview of the electron density of the NRS receptor peptide. Conformation of that in the thrombin complex; basket contour at 1σ.

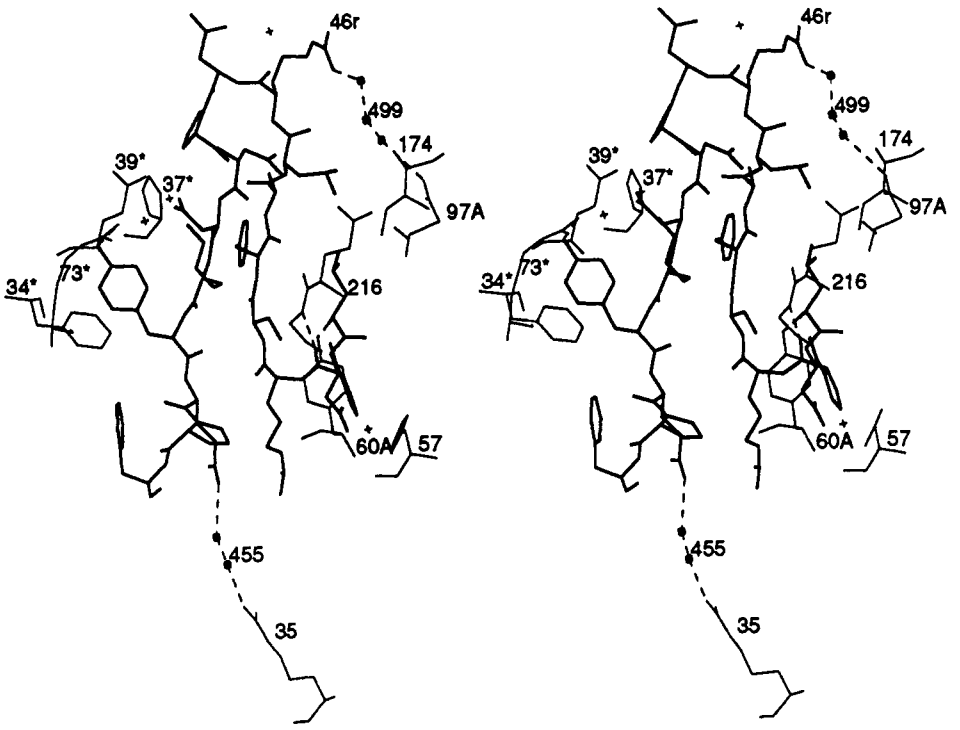

FIGURE 2: Stereoview of the intermolecular interaction of the NRS peptide with two thrombin molecules. The NRS peptide is in bold lines; thrombin is in thin lines; crosses (4) are located between ion pairs; asterisks represent water positions; hydrogen bonds are broken lines; symmetry-related thrombin numbering is designated with stars.

are located between and associate with the active site of one thrombin molecule and the fibrinogen recognition binding exosite of a crystallographically adjacent molecule (Figures 2 and 3). The extended conformation of the Arg41r side chain, along with Phe55r–Trp56r, makes the last two strands appear longer (Figures 1–3). The crystal packing is additionally complicated by a receptor peptide–receptor peptide association due to the nearby presence of two receptor peptide residues [Asn47r and Phe55r* (of a symmetry-related molecule) ~3.8 Å], and Arg46r is about 4.5 Å from the Glu77–Asn78 of a third thrombin molecule (Figure 3). The three strands correspond to (a) active-site binding, (b) an agonist peptide, and (c) the hirudin-like domain. Although the LDPR sequence of the peptide interacts with the active site, there is a sharp turn at Pro40r–Arg41r (Figure 1) that does not resemble the productive binding mode of PPACK in PPACK thrombin (Figure 4) or those of other thrombin active-site inhibitors (Bode et al., 1989, 1992; Banner & Hadvary, 1991; Brandstetter et al., 1992; Martin et al., 1992; Qiu et al., 1992; Stubbs et al., 1992). The Lys51r–Phe55r sequence of the peptide binds in the fibrinogen exosite (Figure 5a) similar to hirugen (Figure 5b), and especially similar to hirulog1, in their respective thrombin complexes (Skrzypczak-Jankun et al., 1991). Thus, the crystal structure of the NRS complex is an infinite network of bimolecular strands of receptor peptide cross-linked thrombin molecules extended along the *b*-direction of the crystals (Figure 3).

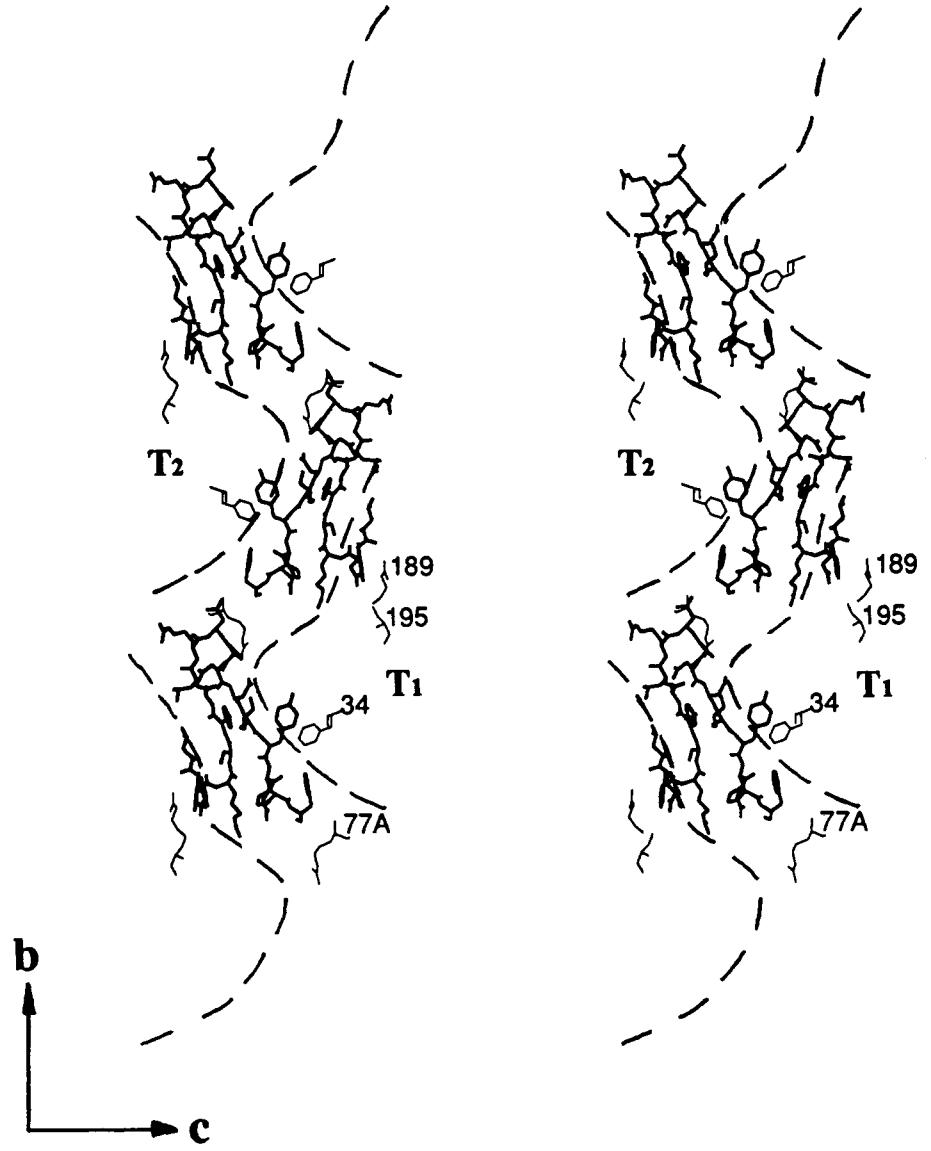

FIGURE 3: Stereoview of the NRS bimolecular receptor peptide interaction. Thrombin surfaces are approximated with broken lines; active-site and fibrinogen exosite residues are numbered; symmetry-related thrombin molecules are designated T1 and T2.

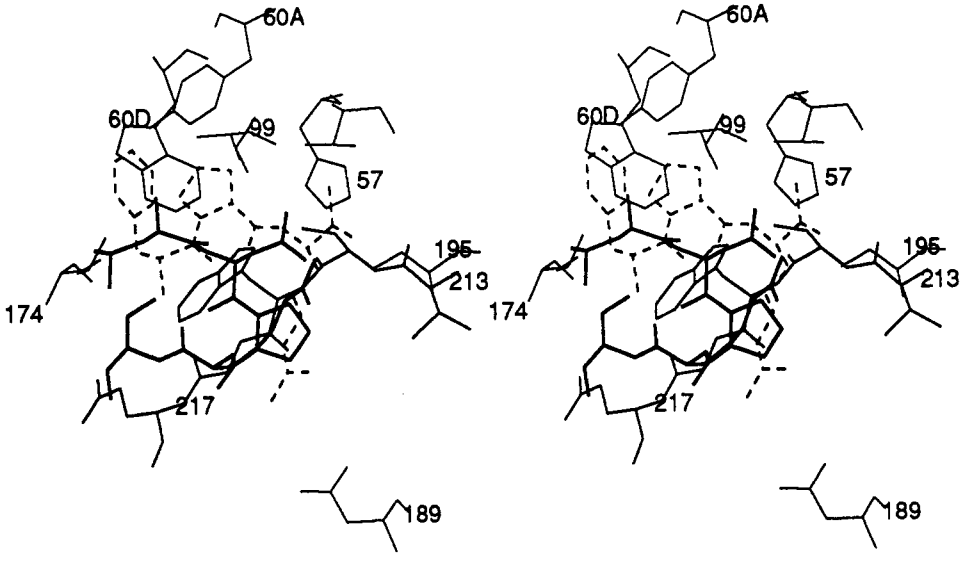

FIGURE 4: Stereoview of the active-site region of the NRS complex. NRS peptide is in bold lines; PPACK is in broken lines; thrombin is numbered.

In this cocrystal, the receptor peptide is not cleaved by thrombin at Arg41r–Ser42r as anticipated, most likely because the three-stranded structure prevents the peptide from easily achieving a more extended, productive cleavage–binding

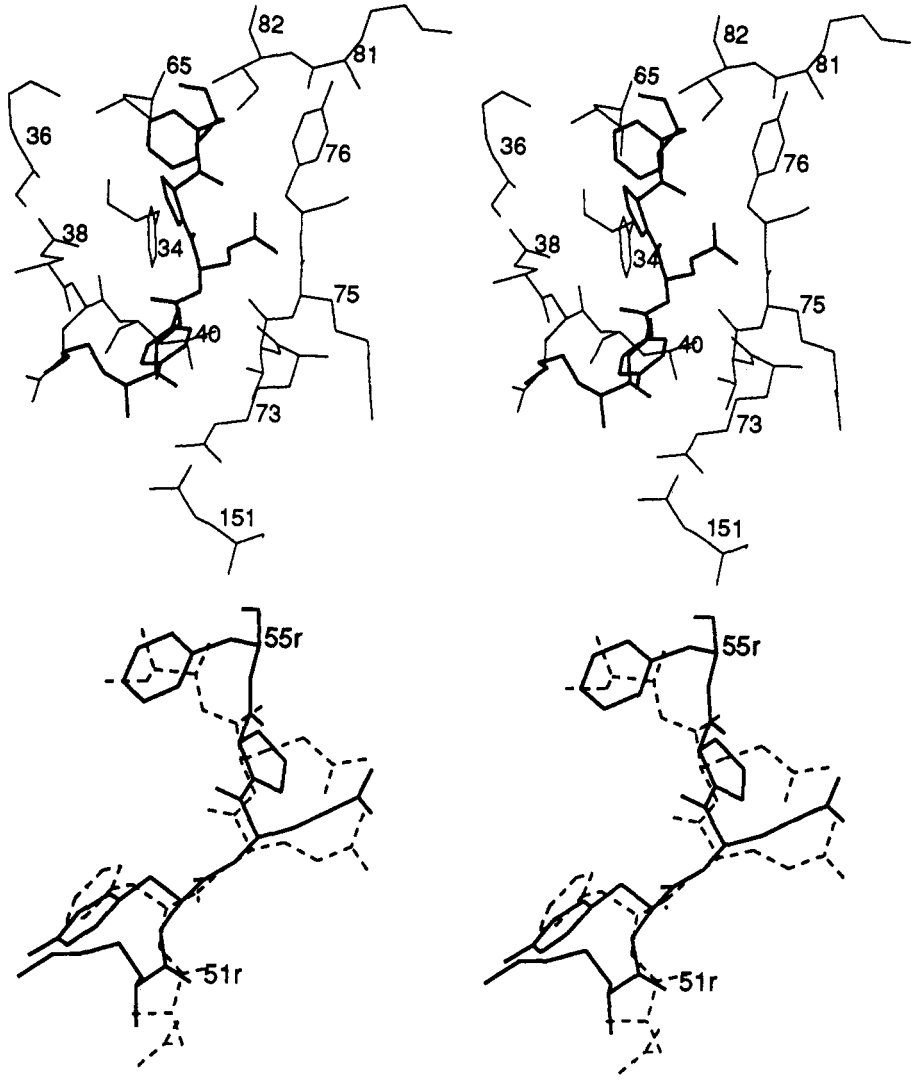

FIGURE 5: Stereoview of the NRS exosite binding structure. (a, top) Interaction with the fibrinogen recognition exosite (neighbor of thrombin shown in Figure 4); NRS peptide is in bold lines; thrombin is numbered. (b, bottom) Comparison of the thrombin binding of KYEPF of the receptor peptide and DFEEI of hirugen; hirugen is in broken lines; platelet receptor peptide is numbered.

conformation. The side chain of Leu38r occupies a hydrophobic site formed by Ile174 and Trp215 (Figure 4), but in addition, it appears to participate in an intrareceptor peptide, hydrophobic leucine clustering with Leu45r (Figures 1 and 2, Table 3). The dominant interaction with the active center is a salt bridge between Asp39r and His57 of the catalytic site (Figure 2, Table 3) and near Ser195; the interaction also apparently causes the side chain of the Glu192 of thrombin to become disordered. Moreover, the amino terminal of the receptor peptide forms a hydrogen bond with the hydroxyl group of the Tyr60A (2.7 Å) of the 60(A–I) insertion loop of thrombin. Clustering of the leucine side chains is a direct consequence of the turn at Pro40r, which forms a hydrogen bond with Gly219 (Figure 2, Table 3). The arginyl side chain at the P1 scissile bond assumes an unproductive conformation pointing toward the solvent and is not located in the S1 specificity pocket of thrombin (Figure 4). Instead, Arg41r makes an ion pair with Glu53r (Figure 2, Table 3). This unusual receptor peptide thrombin association appears to be the result of a stable folding conformation of the peptide, involving the intramolecular hydrophobic clustering of leucine side chains separated by five residues and a terminal salt bridge that produces a three-strand β-like structure. An approximately linear extended conformation is observed for the 20-residue noncleavable β-homoarginine hirulog3 inhibitor complexed with thrombin (Qiu et al., 1992). Since the NRS peptide does not achieve the extended conformation in the cocrystal complex, the twisted S-like structure appears to be more stable under the conditions of crystallization.

The Asp50r–Phe55r residues of the third strand of the S-conformation of the receptor peptide bind to the fibrinogen binding exosite of a neighboring thrombin molecule. Both ion-pair and hydrophobic interactions stabilize the exosite binding (Figure 5). The side chains of Asp50r–Lys51r form salt bridges with Arg73* and Glu39*, respectively, of a symmetry-related molecule (Asp50rOD2–Arg73*NH2, 3.6 Å; Lys51rNZ–Glu39*OE1, 3.5 Å). The hydroxyl group of Tyr52r makes a hydrogen bond with Arg73*NH2 (2.8 Å), Phe55r stacks at right angles to the plane of Phe34*, and Glu53r may be connected to Arg35, mediated through a bridge of two water molecules (Figure 2, Table 3). Although there is some main-chain density for Trp56r, the remainder of the carboxylate terminal (WEDEE) is completely disordered. Similar behavior was observed with the carboxylate terminal of hirulog1, where only the DFEEI sequence of hirugen bound in the fibrinogen binding exosite (Skrzypczak-Jankun et al., 1991). This particular hirudin-based pentapeptide sequence is equivalent in position to KYEPF of the receptor peptide in the exosite (Figure 5b), both of which bind and interact with thrombin in a very similar manner. The binding of KYEPF

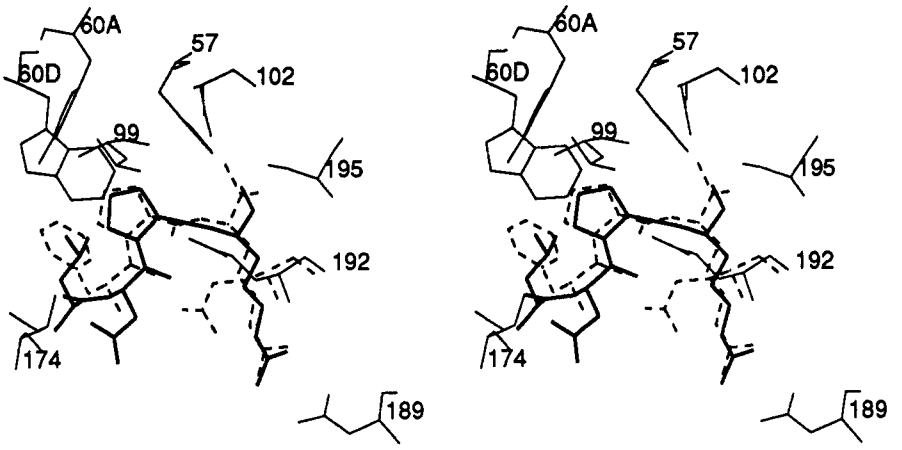

FIGURE 6: Stereoview of the active-site region in the (NRP+H) complex. Cleaved receptor peptide fragment (LDPR) is in bold lines; PPACK is in broken lines; thrombin is numbered; two conformations are observed for Glu 192.

is consistent with deletional and alanine-scanning mutagenesis of the receptor peptide, which suggested that Tyr52r, Glu53r, and Phe55r provide important specific interactions for thrombin binding (Vu et al., 1991b).

Receptor Peptides NRP and NR'S. The peptides NRP and NR'S are similar to the native NRS receptor peptide, except for the Ser42Pro and Arg41Argh substitutions, respectively, and the presence of four additional residues at the carboxylate terminal in NRP (Table 1). The two complexes are, in general, isostructural and very similar to the NRS complex. The interconnecting peptide, however, between the active and exosite regions of neighboring thrombin molecules is only poorly defined in the electron density. Conversely, LDPRP has good density at the active center, KYEPF is well-resolved in the fibrinogen exosite, and their specific associations with thrombin are the same as those of the NRS complex. Again, as with the NRS peptide, the Trp56r–Glu64r carboxylate-terminal peptide is disordered and has no electron density.

The flexibility of the interconnecting peptide between the active site and exosite raised a noteworthy possibility: the receptor peptide may be binding with only one thrombin molecule in these crystals. Even though Leu38r–Asp39r is in an antiparallel stance with respect to Ser214–Gly216 of thrombin (Figure 4), similar to that of substrates and inhibitors, the receptor chain makes a reverse turn at Pro40r–Arg41r (or Argh41r), which changes the path of the peptide to a direction away from the fibrinogen exosite. Consequently, another turn is required to reach the exosite in the same molecule by the shortest path. If this turn occurred as near as Leu44r to the first turn, the distance to KYEPF at the exosite would be about 33 Å, whereas the length of the Leu44r–Asp50r peptide stretch in an extended conformation is only 27 Å. Therefore, intramolecular active site–exosite bidentate binding cannot take place in these structures by virtue of the turn at Pro40r–Arg41r. However, if the P1–P4 residues of the receptor peptide were to bind in a productive way at the active center, then the Ser42r–Asp50r peptide would be more than sufficient in length to reach the KYEPF sequence binding region in the exosite of the same thrombin molecule; a heptapeptide bridges the two sites in the bidentate hirulog3 thrombin complex (Qiu et al., 1992), and it appears that an even shorter bridge would suffice.

Receptor d-FPR'S. The LDPR of NRS was substituted with *d*-PheProArgh in this receptor peptide, so that it too is not cleavable. Since PPACK (Bode et al., 1992) and its β-homoarginyl analog (Qiu et al., 1992) interact with thrombin in a productive binding mode in the active site, the *d*-FPR'S peptide was expected to do likewise. The structure, however, proved to be very similar to those of the NRP and NR'S complexes, except that the electron density of the peptide chain at the active site extended further to include two additional residues (Phe43r–Leu44r); the beginning of the chain was essentially the same as that of NRS. Binding at the exosite was again restricted to the KYEPF sequence. The two new residues in the active-site region clearly revealed a two-stranded β-like conformation for the receptor peptide.

Receptor (NRP+H). The structure of this ternary hirugen–receptor peptide–thrombin complex is isomorphous with hirugen thrombin crystals (Skrzypczak-Jankun et al., 1991), with hirugen binding in the normal way in the fibrinogen binding exosite. In addition, it is also similar to the hirulog1 thrombin complex, in that the scissile bond, which is Arg41r–Pro42r, is cleaved; moreover, LDPR remains bound in the active site, showing that its binding constant is favorable for the association. In this case, the LDPR binds in a productive manner similar to PPACK in PPACK thrombin (Figure 6), so that it represents the cleavable substrate binding mode of the receptor peptide. The Arg41r side chain is in the S1 specificity site and makes a hydrogen-bonded ion pair with Asp189. The extended apolar S2 subsite of thrombin is filled with Leu38r and Pro40r in a similar way as *d*-PhePro of PPACK, the side chain of Asp39r points into the solvent region and does not interact with His57, as in the nonproductive binding mode, and the thrombin side group of Glu192 has an ordered conformation in this structure, which is the same as that in hirugen thrombin and points out into the solvent region. By contrast, in PPACK thrombin, Glu192 points toward the active site (Figure 6); the orientation of Glu192 in the (NRP+H) cocrystal may better accommodate the like-charged groups of Asp39r and Glu192. Even though it extends into solvent, one of the Asp39r carboxylate oxygen atoms (OD1) is involved in a water-mediated hydrogen-bonded interaction with Arg221ANH2 (3.5 and 2.9 Å, respectively). The latter is in agreement with NMR solution studies of a N-terminal platelet receptor peptide (Glu30–Arg41) bound to bovine α-thrombin (Ni et al., 1992). Hydrogen bonds are also present in the crystal structure between the Asp39r oxygen atom, its amide nitrogen, and the main chain of thrombin (Asp39rOD1–Gly219N, 3.1 Å; Asp39rN–Gly216O, 2.8 Å). One of the terminal carboxylate oxygen atoms of the LDPR in the complex interacts with Lys60F by hydrogen bonding through a solvent molecule (3.1 and 3.3 Å, respectively), and two nitrogen atoms of the Arg41r side chain make hydrogen-

bonded interactions with Phe227O and Tyr225O. The binding of hirugen in this complex in the fibrinogen exosite is the same as that of the hirugen thrombin complex (Skrzypczak-Jankun et al., 1991).

Receptor Peptide (PPACK+XA). Crystals of this complex are also isomorphous with hirugen thrombin. The binding of PPACK in the active site is the same as that in PPACK thrombin. The binding in the fibrinogen exosite is like that of the other complexes with a KYEPF pentapeptidyl unit; the last two residues, however, are not as well-defined as the others. The remainder of the peptide appears to be disordered.

Receptor Peptides XA and (nR'S+XB). The class c crystals resulting from these two peptides proved to be those of γ-thrombin (Rydel et al., 1993), an autolysis product of α-thrombin. The XA and (nR'S+XB) structures were refined to the point where the structures of the active-site region and the γ-thrombin cleavage sites were well-defined. In both crystals, nothing was found to be bound in either the active site or the disrupted fibrinogen exosite. This is to be expected with the latter because the exosite has a cleavage at Arg77A and most likely an excision at Lys145 and Lys149E of the flexible autolysis loop of thrombin (Rydel et al., 1993). The fact that the nR'S peptide did not bind at the active site suggests that the binding affinity of the peptide is weak; the same applies to XB, supporting the inference that the two must be covalently linked in order to achieve high-affinity binding.

DISCUSSION

The active-site interactions of thrombin with peptide substrates and inhibitors, on the basis of crystallography, show that all follow the same canonical binding mode, where the substrate forms an antiparallel β-strand with thrombin (Bode et al., 1989; Martin et al., 1992; Qiu et al., 1992). Hirudin is a special type of inhibitor (Fenton et al., 1988), which binds to thrombin in a manner unique among serine protease inhibitors. The binding occurs at two distinct sites: the active site (Rydel et al., 1990; Grutter et al., 1990) and the fibrinogen recognition exosite (Rydel et al., 1990, 1991). Moreover, hirudin does not utilize the S1 specificity site, forms a parallel rather than antiparallel β-strand with thrombin, and is not hydrolyzed during the inhibition process. A useful approach for evaluating the relationships between the catalytic site and the fibrinogen anion binding exosite of thrombin has centered around synthetic hirulog peptides, which contain an active-site-specific sequence and an anion binding exosite sequence bridged by a linker peptide (Maraganore et al., 1989, 1990; Di Maio et al., 1990; Skrzypczak-Jankun et al., 1991; Kline et al., 1991; Qiu et al., 1992). The bidentate binding hypothesis for the platelet receptor peptide–thrombin interaction (Vu et al., 1991b) was based on the expectation that the overall binding would be similar to that of hirulog thrombin complexes.

NRP Complex. The fact that the Arg–Pro peptide bond is less favorable for thrombin-catalyzed cleavage was the rationale behind the Ser42rPro substitution in the NRP peptide. The peptide was expected to bind to the active site of thrombin and span to the fibrinogen exosite, similar to hirulog3 (Qiu et al., 1992). It was thus the first structure determination of the work reported here. However, the crystal structure of the NRP–thrombin complex revealed only partial electron density for the receptor, corresponding to only LDPRP in the active site and KYEPF at the fibrinogen site. Furthermore, a comparison with other active-site binding substrates and inhibitors showed a mode of binding of LDPRP different from that normally associated with productive cleavage, but similar to LDPRS of NRS (Figure 4). Conversely, the KYEPF sequence bound in the fibrinogen exosite in the normal way, similar to hirudin and hirugen (Figure 5), while the bridging residues (Phe43r–Pro48r) and the carboxylate terminal (Trp56r–Glu60r) had no electron density and were disordered in crystals. The carboxyl terminal of the hirulog1 thrombin complex was disordered in a similar manner (Skrzypczak-Jankun et al., 1991).

The close proximity of the active site of one molecule to the exosite of a neighboring molecule in the crystal structure of the NRP complex suggested an intriguing possibility at that time. From the orientations of the two observed parts of the receptor chain between or within the molecules (Figures 2–5), attempts to trace the peptide chain from the active site to the exosite of the same molecule in a 1:1 interaction with thrombin failed uniformly, indicating the improbability of the chain traveling the usual binding path to achieve bidentate ligation (Rydel et al., 1991; Qiu et al., 1992). Therefore, it was concluded that the receptor peptide is most likely involved in a bimolecular association, even though the bridging residues are disordered and not observed.

Further support for this notion came from cross-linker studies of the solutions from which crystals were obtained. Mother liquors used for crystallization were rapidly diluted with an equal volume of 75 mM sodium phosphate (pH 7.3), 0.1875 M NaCl, and 10% PEG 8000 containing 0, 0.2, or 2 mM disuccinimidyl suberate, a bifunctional cross-linking agent. After 5 min at room temperature, the reaction was quenched with excess glycine, and the products were analyzed by immunoblot with thrombin antisera (Hung et al., 1992). A band migrating at the molecular weight of the B-chain monomer of thrombin was detected in all samples, and higher molecular weight bands consistent with cross-linked thrombin dimers and multimers were detected in samples exposed to cross-linker.

These data suggest that thrombin dimers form under the conditions of crystallization. However, no evidence for thrombin dimers was found by the addition of cross-linker to more physiological solutions containing thrombin and receptor peptides. If the thrombin receptor functioned as a monomer, a linear relationship between receptor density and signaling would be expected, while a nonlinear cooperative-type relation would occur if dimerization or oligomerization was important. The dependence of receptor density on a cell surface and response in a *Xenopus* oocyte expression system was linear (Figure 7), militating against a role for receptor oligomerization in signaling. Any physiological significance for the unusual bridging mode of thrombin–receptor peptide binding observed under the conditions used to generate the NRS crystals thus remains to be identified.

The autolysis loop of thrombin (Lys145–Lys149E) was observed to have an ordered and well-defined structure in the NRP complex (Figure 8). Earlier work had shown that this loop was flexible in other thrombin crystals (Skrzypczak-Jankun et al., 1991; Qiu et al., 1992, 1993), and in the cases where it was ordered (PPACK–thrombin and hirudin–thrombin), the conformation could be attributed to crystal-packing interactions (Rydel et al., 1991). The electron density for the main chain of the autolysis loop was good except for Asn149B–Val149C, which is at a sharp turn (Figure 8). Since there are no intermolecular crystallographic interactions between neighboring molecules in the region, the folding must be a stable conformation native to the loop, but the conformation is very different from the crystal-packing-induced ones of PPACK–thrombin and hirudin–thrombin (Figure 9) (Rydel et al., 1991). The presence of a defined conformation for the

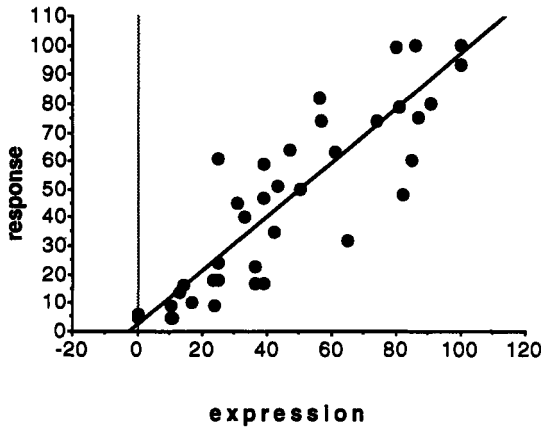

FIGURE 7: Relation between the density of thrombin receptors on the cell surface and the magnitude of the cellular response to thrombin. *Xenopus* oocytes were microinjected with between 1 and 50 ng of thrombin receptor cRNA per oocyte and then cultured for 24 h. Surface density was determined as the surface binding of receptor antibodies (Ishii et al., 1993). Thrombin responses were measured as ^{45}Ca release induced by 30 pM thrombin (Vu et al., 1991a). Data are expressed as the % of maximum (that seen at 50 ng of cRNA/oocyte) and represent 10 experiments.

autolysis loop in thrombin–receptor peptide complexes may be related to a heretofore unsuspected biological role of this loop.

The structure of the autolysis loop in the NRP and NR'S complexes may correspond to a stable conformation or it may be the result of a conformational change accompanying complex formation. The latter would be in agreement with the finding that a peptide including receptor residues Tyr52r–Tyr69r altered chromogenic substrate specificity and the fluorescence emission intensity of a probe attached to the active site of thrombin (Liu et al., 1991). Similar changes were observed when hirugen was bound to thrombin. The region of conformational change upon hirugen binding to the fibrinogen exosite has been fixed by determining the structure of a PPACK thrombin that crystallizes isomorphously with hirugen thrombin[4] (unpublished results from A.T.'s laboratory). Circular dichroism measurements indicate that PPACK binds to thrombin without producing a conformational change (Konno et al., 1988). The foregoing crystal structures are essentially identical, except for relatively small shifts (1.0–1.5 Å) involving residues Ala190–Gly197 of the active site that change the nature of the center, particularly the distribution of water molecules; this stretch corresponds to two back-to-back type II β-turns. The thrombin structure of the receptor peptide complexes closely resembles that of hirugen thrombin in this region, suggesting that KYEPF binding to the exosite causes a similar conformational change. Thus, the ordered conformation of the autolysis loop most likely is not the result of KYEPF binding at the exosite and probably does not alter chromogenic substrate specificity. Rather, the active-site conformational changes involving Ala190–Gly197 may account for the ability of KYEPF-containing peptides to change thrombin substrate specificity. The conformational change of the Ala190–Gly197 region includes a disordering of the Glu192 side group. This would suggest that the binding of KYEPF of the receptor to thrombin randomizes the position of Glu192, such that the nearby presence of the negatively charged Asp39r interacting with His57 can be accommodated.

[4] This PPACK thrombin structure has a disordered autolysis loop just like hirugen thrombin.

NR'S Complex. The structure of the hirulog3 thrombin complex (Qiu et al., 1992) revealed the utility of using β-homoarginine as a nonhydrolyzable substitution at the P1 site of substrates or inhibitors. The lack of thrombin cleavage is due to the presence of a methylene insertion preceding the carbonyl of the scissile bond, thus displacing it by a bond length. With this in mind, the receptor NR'S sequence with β-homoarginine was synthesized and was the next receptor peptide thrombin structure to be determined. The approach proved to be a disappointment in the quest for a bidentate interaction because the results were practically the same as those with the NRP peptide, including the disorder of the bridging residues. The active-site binding was the nonproductive binding mode, and it further verified that a sharp reverse turn occurs at Pro40r–Ser42r. Binding at the fibrinogen exosite was the same KYEPF sequence, in the same manner, as in the NRP complex.

d-FPR'S Complex. An additional β-homoarginine complex was crystallized, where LDPR' was replaced with the *d*-PheProArgh analog of PPACK, since PPACK conforms so well to the active center of thrombin and is such a potent inhibitor. Again, the binding mode was similar to those of NRP and NR'S at both sites with disordered bridging residues, but the electron density at the active site extended further to Leu44r. This was the first positive evidence that the peptide was in a three-stranded antiparallel conformation, since KYEPF, binding at the fibrinogen exosite of a neighboring molecule, was antiparallel to the second strand.

(NRP+H) Complex. In yet another attempt to bind the NRP peptide in a productive way at the active site, crystals of the NRP complex were grown in the presence of hirugen. The structure of the resultant ternary complex proved to be highly informative, in that a cleaved receptor fragment of LDPR bound in a productive manner in the active site of a hirugen thrombin complex (Figure 6). This result clearly established that the Arg–Pro bond of the NRP peptide can be cleaved by thrombin; a similar cleavage was observed with hirulog1 (Skrzypczak-Jankun et al., 1991; Wittig et al., 1992). Therefore, the three-strand receptor peptide S-structure is most likely in equilibrium with an extended or, possibly, a partially unfolded structure, with the amino-terminal peptides in an extended conformation in solution. The fibrinogen exosite of the (NRP+H) complex is occupied by hirugen, and the binding is identical to the hirugen thrombin complex.

In this complex, Glu192 has an ordered conformation but moves to a position pointing toward solvent, suggesting the tolerance of nearby Asp39r (Figure 6). In the thrombin–protein C–thrombomodulin system, a P3 aspartate in protein C is normally inhibitory to thrombin activity; studies with mutant proteins suggest that thrombomodulin induces a conformational change in thrombin, such that Glu192 moves to accommodate the P3 aspartate of protein C (Ehrlich et al., 1990; LeBonniec & Esmon, 1991). Since the same conformation of the Glu192 of (NRP+H) is found in hirugen thrombin, where the active site is unoccupied, the conformational change is the result of exosite binding rather than the nature of the P3 position of substrate. Both crystallographic and NMR solution studies (Ni et al., 1992) show that the P3 aspartate is near Arg221A, thereby neutralizing the charge of Asp39r.

NRS Complex. With few logical alternatives remaining to be pursued, the cleavable native NRS receptor peptide was complexed with thrombin. Since it has the scissile Arg41r–Ser42r bond, the expectation was to reproduce the productive binding mode of LDPR observed in the termolecular

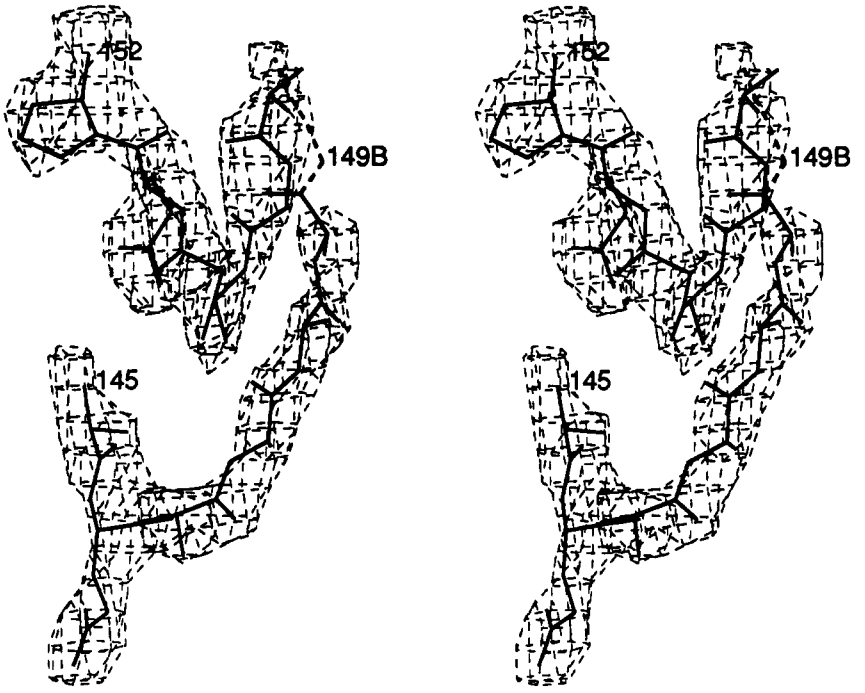

FIGURE 8: Stereoview of the electron density of the autolysis loop of thrombin in NRP. Break of about two residues at Ala149A–Asn149B; basket contour at 1σ.

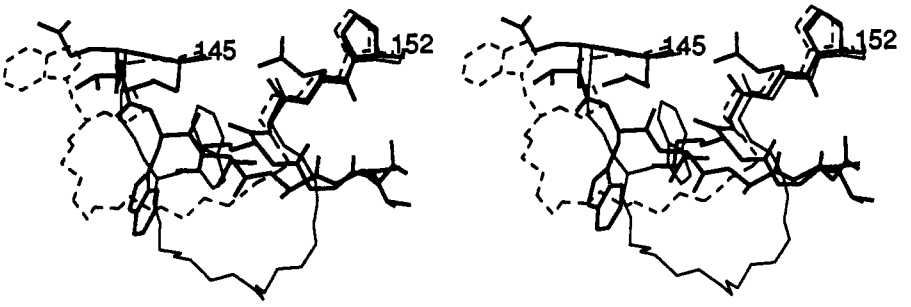

FIGURE 9: Stereoview comparing known autolysis loop conformations. NRP complex is in bold lines; hirudin thrombin is in light lines; PPACK thrombin is in broken lines.

(NRP+H) complex. Therefore, it was indeed surprising that the receptor peptide showed good intact electron density for most of the 19 residues of the Leu38r–Trp56r sequence. Moreover, it was located between two neighboring thrombin molecules, revealing beyond a doubt that the receptor peptide was localized at the active site of one and the fibrinogen exosite of the other. Since the leucine cluster appears to be a dominant factor in producing the antiparallel β-strand conformation of the receptor peptide, in order to address its importance in thrombin receptor function, mutant receptors in which Leu38, Leu44, and Leu45 were changed to Gly and Ala were examined. Signaling by these mutants in response to thrombin was indistinguishable from that of wild-type receptor (Figure 10), indicating that they are unimportant for receptor function.

Receptor Peptide Solution Conformation. The three-strand antiparallel S-structure of the different receptor peptides in the crystals suggests that it may be a solution folding conformation of the isolated peptides. Preliminary NMR solution studies of the NRS peptide show long-range NOEs, indicating a population of folded structures having a hairpin-like reverse turn (F. Ni, personal communication). Since bidentate binding probably occurs first to the fibrinogen exosite (Jackman et al., 1992; Vu et al., 1991b; Liu et al., 1991), such peptides would have to unfold in order to span both thrombin sites. Present results indicate that, under the conditions employed to generate the NRS, NRP, NR'S, and *d*-FPR'S

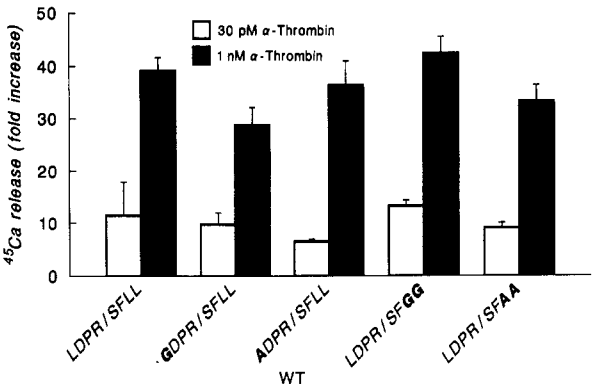

FIGURE 10: Thrombin receptor with mutations designed to eliminate the Leu–Leu interactions of the NRS thrombin complex. Alanine or glycine substitutions were introduced into the LDPR/SFLL sequence of the receptor as indicated in bold type. Mutant or wild-type receptors were expressed in *Xenopus* oocytes, and responses to submaximal (30 pM) or maximal (1 nM) thrombin were determined as agonist-induced ^{45}Ca release (Vu et al., 1991a). Data shown represent the mean of triplicate determinations. This experiment was replicated twice.

cocrystals, an intermolecular bimolecular complex forms and crystallizes more readily with the folded structure. Conversely, since LDPR can bind in a productive way as in the (NRP+H) complex, the three-strand peptide structure must be in

equilibrium with a more extended conformation that can lead to bidentate binding and catalysis. The NMR observations are in agreement with at least a small concentration of a folded structure.

The failure to observe crystals with bidentate binding was somewhat unexpected, given the many functional studies suggesting this mode of thrombin receptor interaction and the precedents set for the interaction in hirulog thrombin crystals. All of the crystals described above were grown by thawing frozen thrombin in the presence of peptide added as a powder; this was done in an effort to bind peptide to thrombin as it thawed to rapidly inhibit protease activity. Since such a protocol may produce highly concentrated local regions that may be the source of the intermolecular thrombin complex formation, docking of the peptide was additionally attempted under more "physiological" conditions, where crystals were grown from a solution of peptide and frozen thrombin and, alternately, with thrombin also in solution. Crystals of hirugen, hirulog1, and hirulog3 thrombin complexes were grown by the former procedure. The NRP peptide was used for a solution/solid crystallization because it should be cleaved only very slowly (Wittig et al., 1992). The resulting crystals grown under these conditions were γ-thrombin (monoclinic, a = 125.5, b = 48.3, c = 52.2 Å, β = 95.1°, space group $C2$). Amino-terminal sequencing of peptides purified from the mother liquor of the crystals revealed the sequences XVE, XQP, and NPNDK, where X is an unidentified amino acid. XVE and XQP must derive from thrombin and presumably represent the B-chain amino terminus (Ile16) and the amino terminus generated upon autolysis at the VGK/GQPS γ-cleavage site (Lys149E). NPNDK must have been produced by cleaving the receptor peptide after the agonist peptide stretch (SFLLR/NPNDK). These data confirm that both thrombin and receptor peptide were cleaved; no uncleaved NRP peptide was recovered.

Presumably the LDPRP sequence of the NRP peptide docked productively with the active center and then underwent subsequent cleavage of the Arg41r–Pro42r peptide bond. Since γ-thrombin was ultimately produced, the Pro42r–Ser64r exosite binding peptide was released, exposing the exosite-uninhibited α-thrombin that autolytically converted to β-thrombin and subsequently to γ-thrombin (Rydel et al., 1993). It thus appears that the docking of LDPR, as in the active center of the (NRP+H) complex, and KYEPF with the exosite of thrombin in the other complexes represents the interactions that likely exist in the physiological bidentate binding mode, but that efficient cleavage of the receptor peptides docked in this mode prevents trapping them in crystals. The same crystals as those of the NR′S complex are grown when the NR′S peptide and thrombin are either both in solution or when thrombin is frozen, indicating that the intermolecular receptor peptide–thrombin association of this uncleavable substrate is not an artifact of the crystallization procedure, but is inherent to a solution conformation of the receptor peptides.

The finding of the receptor peptide-derived NPNDK sequence in the mother liquor shows that α- or γ-thrombin can cleave the receptor peptide, not only at the activating LDPR/S site (Vu et al., 1991b) but also at the SFLLR/NPN site. Whether such a second cleavage occurs under physiological conditions and whether it plays a role in receptor inactivation or other receptor functions also remain to be determined.

Fibrinogen Recognition Site. Precedence for only binding the KYEPF pentapeptide sequence of receptor peptides in the fibrinogen recognition site region of thrombin (Figure 5) has been set with the structural determination of the hirulog1 thrombin complex (Skrzypczak-Jankun et al., 1991). hirulog1 has an Arg–Pro bond as the scissile bond that is cleaved by thrombin (Maraganore et al., 1990), leaving a carboxylic acid PPACK in the active site [like LDPR of the (NRP+H) complex] but only the Asp55–Ile59 pentapeptide sequence (DFEEI) bound in the exosite. Comparison of the structures of a number of different exosite thrombin complexes suggests that the thrombin exosite peptide binding region of hirudin can be divided into two stretches (Asp55–Ile59 and Pro60–Glu65, hirudin numbering) (Qiu et al., 1993). The first appears to have fairly well-defined structural requirements for binding, whereas the other is a hydrophobic patch with only nonpolar matching requirements. The structurally specific region is about 20 Å long (about a pentapeptide length) and interacts similarly in different thrombin peptide complexes (Figure 5); sequence substitutions, however, are tolerated: DFEEI in hirugen, KYEPF in receptor peptide, YEPI in MDL 28050 (designed thrombin inhibitor; Qiu et al., 1993), and DFEEF in hirullin (different leech hirudin; Qiu et al., 1993). Binding at the hydrophobic patch of the exosite has been found to be variable and highly dependent on peptide sequence, where the binding conformations of the peptides can even change in order to assume more optimal nonpolar interactions with thrombin (Qiu et al., 1993). Thus, fibrinogen-like recognition requirements may be confined to a much smaller region of the thrombin exosite than previously thought. The minimal common underlying binding motif appears to be a small pentapeptide or a facsimile. Consequently, shorter peptide sequences of substrates, cofactors, and different inhibitors may bind at somewhat displaced positions within a larger exosite region of thrombin, thus accounting for the variability of the response of thrombin to different exosite binding molecules (Tulinsky & Qiu, 1993). For instance, mutation of Arg73 affects interactions with fibrinogen, thrombin platelet receptor, and thrombomodulin, whereas mutation of Arg75 only affects thrombomodulin binding (Wu et al., 1991).

Table 4: Comparison of Exosite Interactions[a]

thrombin	NRS inhibitor	distance (Å)	hirugen	distance (Å)
Phe34CE1	Tyr52rCD2	3.4	Phe56CD2	3.5
Phe34CE2	Tyr52rCE1	3.7	Phe56CE1	3.6
Arg73NH2	Tyr52rOH	2.7		
Leu65CD2	Phe55rCZ	3.9		
	Phe55rCD1		Ile59CD1	3.6
Arg67CZ	Phe55rCD2	4.1		
	Phe55rCG2		Ile59CG2	3.9
Ile82CD1	Phe55rCG	3.7		
	Phe55rCG2		Ile59CG2	3.9

[a] See Table 1 for numbering of receptor residues; hirugen begins at Asn53 in hirudin.

Precedence for the binding of the specific KYEPF sequence of the receptor peptides with thrombin has also been set indirectly with the structural determinations of a designed exosite inhibitor (MDL 28050) and the carboxylate terminal of the natural inhibitor hirullin P18 from the leech *Hirudinaria manillensis* (Qiu et al., 1993). The positions of Tyr52r and Pro54r of the receptor peptide are the same as the MDL 28050 equivalents, while hirullin has a phenylalanine corresponding to Phe55r. Since hirugen has Glu53r, the YEPF motif is compatible with, and predictably involved in, thrombin exosite binding (Vu et al., 1991b; Qiu et al., 1993). The substitution of Tyr for Phe in hirugen is mild, as is the Phe for Ile exchange of hirullin (Table 4). The Pro for Glu substitution would appear more drastic if it were not for the fact that the equivalent

Glu58 of hirudin and its derivatives are directed into solvent in the thrombin complex and do not interact with the host. Thus, a proline substitution in MDL 28050 and the receptor peptide are easily accommodated by thrombin without requiring the alteration of the main-chain conformation of the inhibitor or the structure of the host molecule (Qiu et al., 1993). The appearance of either an acidic residue (as in hirudin) or a basic one (as Lys51r) is consistent with the observed alternate salt-bridged binding modes between the side group of the inhibitor and either Lys149E or Glu39 of thrombin.

γ-Thrombin Crystals. The class c crystals (Table 2) were isomorphous with those of γ-thrombin (Rydel et al., 1993), and structural analysis of the crystals indicated likewise. The fact that autolysis takes place in attempts to prepare and crystallize the XA and (nR′S+XB) thrombin complexes (Table 1) showed that the peptides are not efficient inhibitors. As exosite complexation renders α-thrombin stable toward autolysis, sufficient α-thrombin with an exposed fibrinogen exosite must be present in crystallization solutions of the XA and XB complexes to produce and maintain β- and γ-autolytic cleavage. This is in agreement with the production of γ-thrombin crystals in the solution/solid crystallization experiment using the NRP peptide; in addition, the Leu38r–Arg46r peptide may not be a good inhibitor. The experiment using the XA peptide was aimed at exploring the response of the thrombin-produced receptor amino terminal to thrombin, which is so functionally important in platelet activation. The results are in agreement with the ready release of the segment following thrombin cleavage through the bidentate binding mode. The attempt to produce the (NR′S+XB) complex was to demonstrate a cleaved bidentate substrate-like ternary thrombin complex with two fragments of receptor peptide, which might even span the enzyme as in the hirulog3 thrombin complex (Qiu et al., 1992). The inability to accomplish either of the forgoing appears to be related to relatively weak binding constants of the exosite peptides and is also consistent with a requirement for connected bidentate binding of receptor peptides for effective binding to thrombin (Vu et al., 1991b; Liu et al., 1991).

Concluding Remarks. In summary, the two components of the proposed bidentate docking of the thrombin receptor with thrombin were captured in crystals of receptor-based peptides with thrombin. However, generation of a single crystal that included both components was not possible, apparently because of efficient cleavage of the receptor peptides bound productively in the active center of thrombin, even peptides with the poorly cleavable Arg–Pro bonds. The alternate intermolecular bridging mode of peptide thrombin binding revealed in these studies appears to be the result of a solution conformation of the peptides. Receptor mutations that removed important determinants of the S-like receptor peptide structure underlying the intermolecular bridging mode did not significantly alter receptor function, and a comparison of receptor density to the responsiveness of a cell did not support a role for receptor oligomerization in signaling. The physiological role of the intermolecular binding, if any, thus remains to be defined. It is possible that the S-like structure has a role in receptor functions other than its initial activation or in promoting receptor specificity by inhibiting cleavage by other proteases in its complex environment *in vivo*.

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