Binding of Fluorescent and Spin-Labeled C-Terminal Hirudin Analogs to Thrombin†

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Synthetic peptides based on the sequence of the negatively charged carboxyl tail of hirudin exhibit anticoagulant activity. Several antithrombin agents are being developed by chemical and structural optimization of these "hirupeptides". The present work demonstrates the design and use of novel spin-labeled and fluorescent-labeled C-terminal hirudin analogs to study the interactions of these antithrombin agents with thrombin in solution. Three labeled *hirulabels* were synthesized based upon the amino acid sequence of the antithrombin agent MDL 28050, X-NH-$(CH_2)_7$-CO-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH, where X = anthraniloyl, 1,5-dansyl, or 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl. The modifications did not significantly alter the potency of these inhibitors which showed K_i values of 100 nM. Their interactions with human and bovine thrombin were studied by ESR and fluorescence techniques. The spin-labeled hirupeptide was able to discern subtle differences in binding to human *versus* bovine thrombin. The 8-aminooctanoic acid spacer arm placed the nitroxide moieties near the active site, near regions of the autolysis loops which differentiates between human α- and γ-thrombin. It was also able to discern paramagnetic quenching and fluorescence energy transfer interactions, respectively, between covalently attached spin labels and fluorescent probes at the active site Ser 195 and the fluorophore on the hirupeptide.

Introduction

The serine protease thrombin (EC 3.4.21.5) is an important component of the vascular system. One of the principal catalytic functions of thrombin is the cleavage of the fibrinogen molecule at four unique arginyl–glycyl bonds yielding fibrin monomers which then associate to form a blood clot.[1,2] Thrombin also activates factor XIII to XIIIa, the transglutaminase responsible for cross-linking the associated monomers.[3] Thrombin-induced platelet activation is also of major importance in clot formation.[4] In addition, thrombin interacts with several other components of the vascular system such as Ca^{2+}, ATP, thrombomodulin, protein C, monocytes, and macrophages.[5–7] Since this unique protein is involved in so many varied functions and binds a very broad range of ligands, there must be several different classes of binding sites on the protein surface. It is therefore of interest to identify and understand how these different sites might communicate.

The most potent thrombin inhibitor currently known is the protein hirudin from the salivary glands of the European medicinal leech.[8] X-ray crystallography showed that hirudin spans both the active site and a noncatalytic anion binding exosite of thrombin.[9] Crystal structures of several thrombin–hirudin complexes have been reported all of which conclude that the C-terminus of hirudin makes several hydrophobic and charged interactions with the anion exosite region of the enzyme.[9–13] Synthetic peptides based on the sequence of the negatively charged carboxyl tail of hirudin possess anticoagulant activity, although they do not directly block the active site Ser 195.[14] Recently Krstenansky and co-workers[15] synthesized the antithrombin agent, MDL 28050, with the following amino acid sequence:

Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH

where Suc = succinyl and Cha = β-cyclohexylalanine.

The X-ray structure of the thrombin–MDL-28050 complex showed that the first stretch of this peptide (from Suc to Ile) associates with thrombin similar to hirudin 55–59, but the remaining residues accomplish the hydrophobic interaction in a different way by virtue of the substrate conformational readjustment.[13,15] The first stretch appears to be related to specific recognition associated with the thrombin exosite in all hirudin-based inhibitors.[13]

Several fluorescent probes and spin labels have been shown to be sensitive to tertiary structural changes at the active site of thrombin.[16] For example, conformational differences between human α- and γ-thrombins and between human α- and bovine α-thrombins were discerned.[17–19] The sensitivity of these labels can be further utilized by labeling the hirupeptides and probing their conformational interactions with the catalytic site of thrombin. The main aims of the present study are (i) to design and test novel hirulabels based on C-terminal hirupeptides; (ii) to study their interactions with thrombin exosite region; and (iii) potential measurement of distance between the exosite region and the catalytic site in solution. The N-terminus of a hirupeptide analog based on the MDL-28050 sequence were modified with spin label and fluorescent moieties shown in Figure 1, and their interactions with thrombin were investigated.

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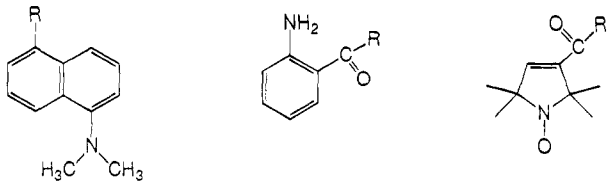

Figure 1. (a) Hirulabels used in these studies. The label structures from left to right are anthraniloyl, 1,5-dansyl, CTPO.

Materials and Methods

Materials. Human prothrombin concentrate was a gift of Dr. John W. Fenton II, New York State Department of Health, Albany. Human α-thrombin was prepared by activation with *Echis carinatus* snake venom.[20,21] Bovine thrombin was a gift of Dr. James Cavanaugh, Armour Research Labs, Kankakee, Illinois. Thrombin (M_r = 36 600) concentration was determined spectrophotometrically using $\epsilon_{290} = 1.73$ mL mg^{-1} cm^{-1}. Boc-L and Boc-D amino acids and *p*-methylbenzhydrylamine resin were purchased from Peptides International (Louisville, KY). Boc-Cha[22] was from Bachem (Torrance, CA), and Boc amino alcohols were from Advanced ChemTech (Louisville, KY). The fluorosulfonylphenyl nitroxide spin labels were synthesized according to published procedures.[23] The fluorescent label, 2,5-dansyl fluoride (B. L. Zhao and L. J. Berliner, unpublished results) was prepared from 2,5-dansyl chloride (Molecular Probes, Eugene, Oregon). All other chemicals were reagent grade and were used without further purification.

Methods. The C-terminal "11-mer" peptides were synthesized by solid-phase techniques using an Applied Biosystems Model 430-A peptide synthesizer as previously reported.[11] The peptides were purified by preparative HPLC and were analyzed using analytical HPLC, amino acid analysis, and fast-atom bombardment mass spectrometry.

Anthraniloyl-11-mer. The labeled analog was made by coupling 20 mg of the purified peptide, H-Aoc-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH, in 40 μL of DMF with 5 equiv of isatoic anhydride in 6 mL of THF/acetonitrile (1:1) containing 240 μL of triethylamine overnight or until the reaction was completed as determined by TLC (ethyl acetate/acetone/hexane (2:1:1)). The mixture was evaporated *in vacuo* and purified by preparative HPLC. The identity of the materials was confirmed by amino acid analysis and fast atom bombardment mass spectrometry: AAA [Asx 1.03; Glx 4.13; Pro 1.93; Ala 1.02; Ile 0.89; Tyr 1.00]; FAB-MS $(MH)^+ = 1605$.

Dansyl-11-mer. The fluorophore label was prepared by coupling 20 mg of the purified peptide, H-Aoc-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH, in 40 μL of DMF with 5 equiv of dansyl chloride in 6 mL of THF/acetonitrile (1:1) containing 240 μL of triethylamine overnight or until the reaction was completed as determined by TLC (ethyl acetate/acetone/hexane (2:1:1)). The mixture was evaporated *in vacuo* and purified by preparative HPLC. The identity of the materials was confirmed by amino acid analysis and fast atom bombardment mass spectrometry: AAA [Asx 1.04; Glx 4.13; Pro 1.95; Ala 1.03; Ile 0.91; Tyr 0.95]; FAB-MS $(MH)^+ = 1718$.

CTPO-11-mer Spin Label. The labeled analog was made by coupling 20 mg of the purified peptide, H-Aoc-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH, in 40 μL of DMF with 5 equiv of the preformed symmetrical anhydride of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical anhydride in 6 mL of THF/acetonitrile (1:1) containing 240 μL of triethylamine overnight or until the reaction was completed as determined by TLC (ethyl acetate/acetone/hexane (2:1:1)). The mixture was evaporated *in vacuo* and purified by preparative HPLC. The identity of the materials was confirmed by amino acid analysis and fast atom bombardment mass spectrometry: AAA [Asx 1.01; Glx 4.02; Pro 2.03; Ala 0.99; Ile 0.96; Tyr 0.98]; FAB-MS reduced $(MH)^+ = 1653$.

An estimate of the binding constants of these analogs with α-thrombin was determined from clotting inhibition measurements on a BBL fibrometer.[21]

Spin labeling of thrombin with fluorosulfonylphenyl nitroxides was typically carried out with a 20-fold molar excess of label in 7% (v/v) acetonitrile, 0.05 M Tris, 0.75 M NaCl, pH 7.2, room temperature, for 1 h. The extent of labeling, as estimated from the residual esterase activity, was typically 95–99%. Fluorescent labeling of thrombin was carried out with a 10-fold excess of 2,5-dansyl fluoride in the same buffer as above but using 10–15% (v/v) 2-propanol as cosolvent. The reaction was allowed to proceed for 10 h at room temperature followed by 3 days at 4 °C,[24] where the extent of inhibition was about 60–65%. In all cases, unreacted label was removed by exhaustive dialysis in the cold room against the same buffer, but at pH 6.5. Fluorescence measurements were performed on a Perkin-Elmer MPF-44 or LS-50 instrument with a constant-temperature controller. ESR spectra were measured on a Varian E-4 spectrometer equipped with an E-257 variable temperature accessory at 25 ± 0.5 °C. Typical instrument settings were as follows: frequency, 9.15 GHz; microwave power, 20 mW; scan range, 100 G; time constant, 0.25 s; and modulation amplitude, 1.0 G.

Results and Discussion

The fluorescent and spin-labeled hirupeptide inhibitors were synthesized by solid-phase methods, and clotting inhibition assays showed that these labels had relatively strong K_i values (*ca.* 100 nM) which were in the same range (IC_{50} = 0.029–0.15 μM) reported for the unmodified synthetic inhibitor, MDL-28050.[13]

Figure 2 depicts ESR spectra of CTPO-11-mer label alone (a) and complexed with human (b) and bovine thrombins (c), respectively. The "bound" spectra 2b and c were quite mobile, with correlation times in the fast tumbling regime (10^{-12}–10^{-10} s). In order to "enhance" the difference in spin label motion relative to that of the protein, spectra were measured for the sample in saturated sucrose which slows down both protein and label motion. The resultant spectra, shown in Figure 2d–f, are now a reflection of nitroxide motion in a highly viscous medium where it is quite clear in spectra 2e and 2f that binding to both human (2e) and bovine thrombin (2f) results in CTPO-11-mer nitroxide immobilization (shown by arrows) *versus* the free, unbound label in saturated sucrose (Figure 2d). The anisotropic hyperfine extrema, $2T_{||}$ (denoted by arrows in Figure 2) was greater with bovine ($2T_{||}$ = 64 G) *versus* human thrombin ($2T_{||}$ = 61 G) (Table 1). Thus the nitroxide moiety is partially immobilized, probably on some hydrophobic region on the thrombin surface, which is however different in the two thrombin species.

The fluorescence excitation and emission spectra of anthraniloyl-11-mer and dansyl-11-mer alone were also measured and complexed with human or bovine thrombin in 50 mM Tris, 0.75 M NaCl buffer, pH 6.5 at 25 ± 0.5 °C. The excitation and emission maxima for anthraniloyl-11-mer alone were 315 and 413 nm, respectively, and for dansyl-11-mer they were 330 and 546 nm, respectively. Surprisingly there was essentially no change in either λ^{max}_{em} or the fluorescence emission quantum yield upon complete binding with thrombin, suggesting that the bound fluorophore moiety was not sensing any environmental differences in the complex *versus* bulk solution, i.e., no major conformational changes in the thrombin structure aside from any small changes noted in the crystal structure results.[13] In essence this was also consistent with the relatively high

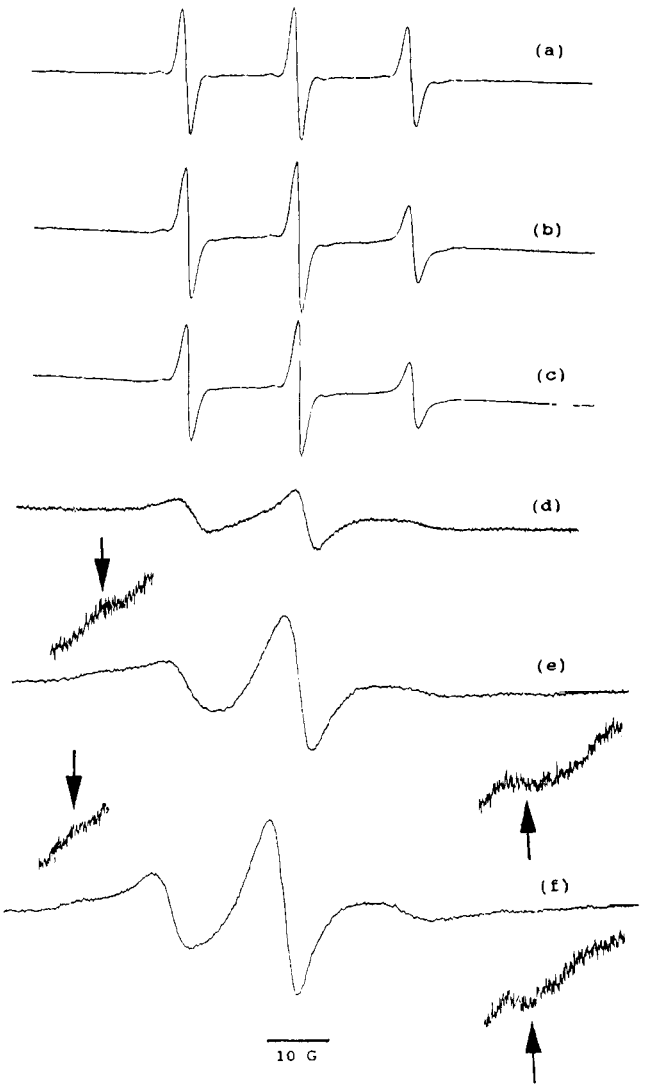

Figure 2. ESR spectra of CTPO-11-mer peptide. The top three spectra represent CTPO-11-mer either free (a) or complexed with human (b) and bovine (c) thrombin. The bottom three spectra represent CTPO-11-mer peptide in the presence of saturating sucrose: alone (d), bound to human thrombin (e), and bovine thrombin (f), respectively. The arrows indicate the positions of the hyperfine extrema. Conditions were 60 μM thrombin, 30 μM CTPO peptide, 50 mM Tris, 0.75 M NaCl, pH 6.5 with 2% (v/v) CH_3CN and 25 ± 0.5 °C.

degree of immobilization of the bound spin-labeled peptide analog. Specifically, if the fluorophore moiety of anthraniloyl- or dansyl-11-mer physically overlaps the nitroxide moiety of Ser 195 sulfonylphenyl spin label, one should observe contact quenching of fluorescence.[25] As shown in Figure 3, upon incubation of 1 μM anthraniloyl-11-mer with 36 μM m-**VII** spin-labeled human α-thrombin, a 10–15% decrease in the fluorescence quantum yield was observed. In order to prove that this significant, yet relatively small quenching was due to intramolecular collisional overlap with the spin label, spin-labeled thrombin was first treated with 5-fold molar excess of sodium ascorbate (which chemically reduces the nitroxide to the diamagnetic hydroxylamine), followed by dialysis in oxygen-depleted (degassed) buffer. Note that the intensity of 1 μM anthraniloyl-11-mer complexed with 36 μM ascorbate-reduced m-**VII**–thrombin was almost identical to that of the fluorescent 11-mer complexed with thrombin (Figure 3). Similar results were also obtained for m-**IV**-labeled

Table 1. ESR and Fluorescent Parameters of Labeled 11-mer–Thrombin Complexes

ESR Hyperfine Extrema for CTPO-11-mer–Thrombin Complexes

thrombin species	$2T_{11}$, G
human	61.0
bovine	64.0

Quenching of Fluorescence Emission of Anthraniloyl-11-mer–Sulfonyl Fluoride-Labeled Thrombins[a]

	I^{rel}_{413nm} ± 0.02[c]	
spin label[b]	paramagnetic	diamagnetic
m-**IV**	0.83	0.98
m-**VII**	0.88	1.00
p-**III**	1.00	1.02
p-**V**	1.00	1.01
m-**IV** (bovine thrombin)	0.80	1.02

[a] Fluorescence parameters were λ_{ex} = 315 nm, λ_{em} = 413 nm. [b] Spin labels are designated m-**IV** (m-CO-6-NH), 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)-*m*-(fluorosulfonyl)benzamide; m-**VII** (m-NCO-6-NH), N-[*m*-(fluorosulfonyl)phenyl]-4-*N*-(2,2,6,6-tetramethylpiperidine-1-oxyl)urea; p-**III** (p-CO-6-OH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)benzoate; p-**V** (p-CO-5-NH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)benzamide. Diamagnetic indicates the ascorbate reduced nitroxide spin-labeled sample. [c] I^{rel}_{413nm} = intensity of emission at 413 nm relative to that of the control (no thrombin or with native thrombin).

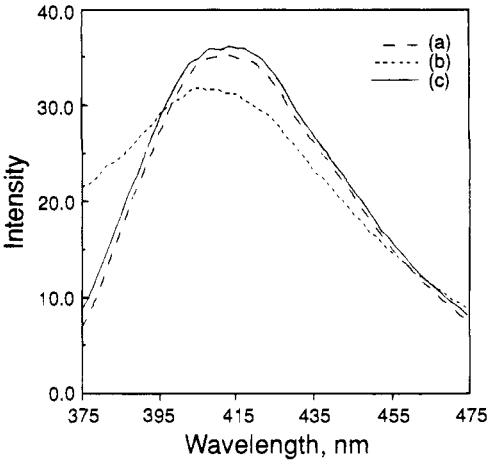

Figure 3. Extrinsic emission fluorescence spectra of anthraniloyl peptide alone (a) and bound to human m-**VII**–thrombin (b) paramagnetic nitroxide and (c) diamagnetic hydroxylamine. Thrombin concentration was 36.5 μM and anthraniloyl peptide was 1 μM. Conditions were: 36.5 thrombin, 1 μM anthraniloyl peptide, 1% CH_3CN.

thrombin; however, upon binding to either p-**III**- and p-**V**-labeled thrombin no change in the emission intensity of the fluorescent peptide was observed (Table 1)! It is pertinent to note that the nitroxide moiety of both m-**IV** and m-**VII** labels were previously shown to occupy the α–γ difference site in human thrombin whereas the p-**III** and p-**V** labels occupy a topographically distinct "apolar site".[26] Furthermore, m-**VII**-labeled bovine α-thrombin also gave similar results (data not shown). Although the total collisional quenching was only *ca.* 15% in the cases noted above, this is still strong evidence for steric overlap in the tumbling loci of the two labels. One can also understand this from the spectra of the 11-mer analogs where it was evident that the label was undergoing broad, rapid motion while only spending a small fraction of its time in regions that overlap the nitroxide label on Ser 195.

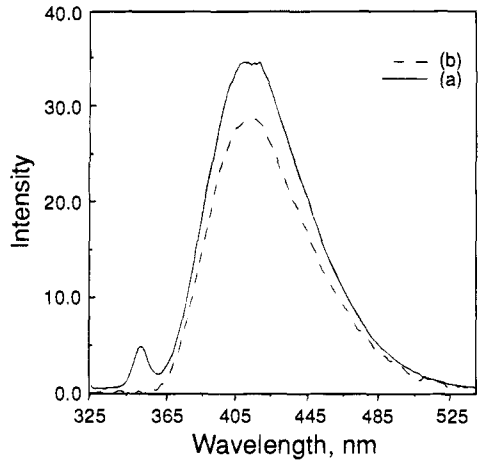

Figure 4. Anthraniloyl-11-mer emission spectra (a) 1 μM anthraniloyl peptide alone; (b) 1 μM anthraniloyl peptide incubated with 15 μM 2,5-dansyl human α-thrombin. The decrease in emission intensity is caused by energy transfer to the 2,5-dansyl thrombin acceptor. All other conditions were as in Figure 3.

In order to refine the interlabel distance measurement further, a fluorescence energy transfer experiment was attempted between an active site-directed fluorescent donor, 2,5-dansyl fluoride, whose excitation spectrum (λ^{max}_{ex} = 390 nm) overlaps with emission band of an acceptor anthraniloyl moiety (λ^{max}_{em} = 415 nm) on the *hirupeptide*. If the two fluorophores reside within 15–30 Å of one another, the resultant resonance energy transfer which appear qualitatively as an apparent quenching of the emission of the 2,5-dansyl donor and the sensitized emission of the acceptor. Since the excitation spectrum of 2,5-dansylthrombin partially overlaps the excitation band of the anthraniloyl group, one could monitor only the anthraniloyl emission spectrum unambiguously. Figure 4 shows the anthraniloyl-11-mer emission intensity at 413 nm before and after complexation with 2,5-dansyl α-thrombin. The resultant 15% energy transfer between the two labels qualitatively confirms a region of overlap between them.

By making consecutive changes in the spacer arm length, one can deduce more precisely where the region of maximum overlap exists between hirulabels and the active site. Further work in this direction is under progress. In summary, these labels may serve as novel probes for studying the conformational interactions between thrombin and a variety of new anticoagulant synthetic peptide analogs.

References

(1) Henschen, A.; Lottspeich, F.; Kehl, M.; Southan, C. Covalent Structure of Fibrinogen. *Ann. N.Y. Acad. Sci.* **1983**, *408*, 28–43.
(2) Scheraga, H. A. Chemical Basis of Thrombin Interaction With Fibrinogen. *Ann. N.Y. Acad. Sci.* **1986**, *485*, 124–133
(3) Lorand, L.; Konishi, K. Activation of Fibrin Stabilizing Factor of Plasma by Thrombin. *Arch. Biochem. Biophys.* **1984**, *105*, 58–67.
(4) Davey, M. G.; Luscher, E. F. Actions of Thrombin And Other Coagulant And Proteolytic Enzymes On Blood Platelets *Nature* **1967**, *216*, 857–858.
(5) Conery, B. G.; Berliner, L. J. Binding Subsites In Human Thrombins. *Biochemistry* **1983**, *22*, 369–375.
(6) Esmon, C. T.; Owen, W. G. Identification Of An Endothelial Cell Factor For Thrombin-catalyzed Activation Of Protein C. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2249–2252.
(7) Bar-Shavit, R. A.; Kahn, A.; Wilner, G. D.; Fenton, J. W. Monocyte Chemotaxis: Stimulation By Specific Exosite Region In Thrombin. *Science* **1983**, *220*, 728–731.
(8) Markwardt, F. Hirudin As An Inhibitor Of Thrombin. *Methods Enzymol.* **1970**, *19*, 924–932.
(9) Rydel, T. J.; Ravichandran, K. G.; Tulinsky, A.; Bode, W.; Huber, R.; Roitsch, C.; Fenton, J. W., II. The Structure Of A Complex Of Recombinant Hirudin And Human α-thrombin. *Science* **1990**, *249*, 277–280.
(10) Rydel, T. J.; Tulinsky, A.; Bode, W.; Huber, R. Refined Structure Of The Hirudin-thrombin Complex. *J. Mol. Biol.* **1991**, *211*, 583–601.
(11) Grutter, M. G.; Priestle, J. R.; Rahuel, J.; Grossenbacher, H.; Bode, W.; Hofsteenge, J.; Stone, S. R. Crystal Structure Of The Thrombin-hirudin Complex. A Novel Mode Of Serine Protease Inhibitor. *EMBO J.* **1990**, *9*, 2361–2365.
(12) Skrzypczak-Jankun, E.; Carperos, V.; Ravichandran, K. G.; Tulinsky, A.; Westbrook, M.; Maraganore, J. M. The Structure Of The Hirugen And Hirulog 1 -Thrombin Complexes Of α-Thrombin. *J. Mol. Biol.* **1991**, *221*, 1379–1393.
(13) Qui, X.; Yin, M.; Padmanabhan, K. P.; Krstenansky, J. L.; Tulinksy, A. Structures of Thrombin Complexes With a Designed and a Natural Exosite Peptide Inhibitor. *J. Biol. Chem.* **1993**, *268*, 20318–20316.
(14) Krstenansky, J. L.; Mao, S. J. T. Antithrombin Properties Of C-terminus Of Hirudin Using Synthetic Unsulfated N-α-acetyl $Hirudin_{45-65}$. *FEBS Lett.* **1987**, *211*, 10–16.
(15) Krstenansky, J. L.; Broersma, R. J.; Owen, T. J.; Payne, M. H.; Yates, M. T.; Mao, S. J. T. Development of MDL28050, a Small Synthetic Antithrombin Agent Based on the Functional Doman of the Leech Protein Hirudin. *Thromb. Haemostasis* **1990**, *63*, 208–214.
(16) Berliner, L. J. *Thrombin: Structure and Function*; Plenum Press: New York, 1992.
(17) Berliner, L. J.; Shen, Y. Y. L. Active Site Fluorescent Labeled Dansyl And Anthraniloyl Human Thrombins. *Thromb. Res.* **1977**, *12*, 15–25.
(18) Berliner, L. J.; Bauer, R. S.; Chang, T.-L.; Fenton, J. W., II; Shen, Y. Y. L. Active Site Topography Of Human Coagulant (α-) and Non-coagulant (γ-) Thrombins. *Biochemistry* **1981**, *20*, 1831–1837.
(19) Nienaber, V. L.; Berliner, L. J. Subtle Differences In Active Site Structure Between Bovine And Human Thrombins: ESR And Fluorescence Studies. *Thromb. Haemostasis* **1990**, *65*, 40–45.
(20) Franza, B. R., Jr.; Aronson, D. L.; Finlayson, J. S. Activation Of Human Prothrombin By A Procoagulant Fraction From The Venom *Echis carinatus*. Identification Of A High Molecular Weight Intermediate With Thrombin Activity. *J. Biol. Chem.* **1975**, *250*, 7057–7068.
(21) Fenton, J. W.; Fasco, M. J.; Stackrow, A. B. Human Thrombins: Production, Evaluation And Properties Of α-thrombin. *J. Biol. Chem.* **1977**, *252*, 3587–3598.
(22) Abbreviations used: Cha, β-cyclohexyl-L-alanine; Aoc, 8-aminooctanoic acid; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy; ESR, electron spin resonance; m-**IV** (m-CO-6-NH), 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)-*m*-(fluorosulfonyl)benzamide; m-**VII** (m-NCO-6-NH), *N*-[*m*-(fluorosulfonyl)phenyl]-4-*N*-(2,2,6,6-tetramethyl-piperidine-1-oxyl)urea; p-**III** (p-CO-6-OH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)benzoate; p-**V** (p-CO-5-NH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)benzamide.
(23) Wong, S. S.; Quiggle, K.; Triplett, C.; Berliner, L. J. Spin-labeled Sulfonyl Fluorides As Active Site Probes Of Protease Structure. II. Spin Label Syntheses And Enzyme Inhibition. *J. Biol. Chem.* **1974**, *249*, 1678–1682.
(24) A control was almost fully active after 20 h at room temperature.
(25) Birks, J. B. *Photophysics of Aromatic Molecules*; Wiley-Interscience: London, 1970.
(26) Berliner, L. J. Structure-function Relationships In Human α- and γ-Thrombins. *Mol. Cell. Biochem.* **1984**, *61*, 159–172.