Affinity Labeling of Lysine-149 in the Anion-Binding Exosite of Human α-Thrombin with an N^{α}-(Dinitrofluorobenzyl)hirudin C-Terminal Peptide†

Paul Bourdon,‡ John W. Fenton II,§ and John M. Maraganore*,‡

Biogen, Inc., 14 Cambridge Center, Cambridge, Massachusetts 02142, and Wadsworth Center for Laboratories and Research, School of Public Health Sciences, New York State Department of Health, Albany, New York 12202

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ABSTRACT: In order to define structural regions in thrombin that interact with hirudin, the N^{α}-dinitrofluorobenzyl analogue of an undecapeptide was synthesized corresponding to residues 54–64 of hirudin [GDFEEIPEEY($O^{35}SO_3$)L (DNFB-[^{35}S]Hir_{54-64})]. DNFB-[^{35}S]Hir_{54-64} was reacted at a 10-fold molar excess with human α-thrombin in phosphate-buffered saline at pH 7.4 and 23 °C for 18 h. Autoradiographs of the product in reducing SDS–polyacrylamide gels revealed a single ^{35}S-labeled band of M_r ~32 500. The labeled product was coincident with a band on Coomassie Blue stained gels migrating slightly above an unlabeled thrombin band at M_r ~31 000. Incorporation of the ^{35}S affinity reagent peptide was found markedly reduced when reaction with thrombin was performed in the presence of 5- and 20-fold molar excesses of unlabeled hirudin peptide, showing that a specific site was involved in complex formation. The human α-thrombin–DNFB-Hir_{54-64} complex was reduced, S-carboxymethylated, and treated with pepsin. Peptic fragments were separated by reverse-phase HPLC revealing two major peaks containing absorbance at 310 nm. Automated Edman degradation of the peptide fragments allowed identification of Lys-149 of human thrombin as the major site of DNFB-Hir_{54-64} derivatization. These data suggest that the anionic C-terminal tail of hirudin interacts with an anion-binding exosite in human thrombin removed 18–20 Å from the catalytic apparatus.

Thrombin (EC 3.4.21.5) has central, regulatory roles in thrombosis and hemostasis (Fenton, 1986a,b) mediating fibrin clot formation (Blomback, 1979) and stabilization (Lorand, 1964), platelet and endothelial cell activation (Tollefsen et al., 1974; Rodgers, 1988), and both positive and negative regulation of pathways leading to its own zymogen activation (Colman, 1969; Esmon et al., 1973; Esmon, 1987). Compared to other serine proteinases of the blood coagulation and fibrinolytic pathways, its zymogen, prothrombin, appears to have a more highly evolved genetic origin (Irwin et al., 1988) and is unique in that the majority of the propiece (prothrombin fragment F1.2) is lost upon activation with the consequence that various specificities of thrombin must be accommodated solely within the enzyme moiety consisting of the thrombin A and B chains (Degen et al., 1983; Rabiet et al., 1986). In addition to the catalytic site and adjacent regions (i.e., the classical enzyme active site), α-thrombin has a unique anion-binding exosite that is exposed upon prothrombin activation and is functionally independent of the enzyme active site (Fenton et al., 1988). This exosite is implicated in α-thrombin recognition of fibrin(ogen), binding to various cells, and adsorption on negatively charged surfaces (Fenton et al., 1988; Chang et al., 1979; Lewis et al., 1987).

As in recognition of substrate, inhibition of thrombin by hirudin appears to involve multiple sites of interaction discrete from the active-site pocket alone. Hirudin, a 65 amino acid residue protein from the medicinal leech, is a unique serine proteinase inhibitor as it lacks the conventional P_1[1] specificity pocket binding in complex formation; hirudin still binds to diisopropylphosphoryl-α-thrombin with a high affinity although decreased from that affinity determined for binding to unmodified enzyme (Stone & Hofsteenge, 1986). Further, site-directed mutagenesis of recombinant desulfatohirudin has failed to identify a critical residue for inhibitory function (Dodt et al., 1988). Kinetic analysis of the inhibition of thrombin by hirudin reveals the existence of more than a single binding site, one of lower affinity where occupancy fails to block active-site reactivity (Stone & Hofsteenge, 1986). Synthetic peptides derived from the C-terminus of hirudin (Bajusz et al., 1984; Krstenansky & Mao, 1987; Maraganore et al., 1989) are known to block thrombin activity toward fibrinogen but not toward small synthetic substrates. When the tyrosyl residue of synthetic peptides corresponding to Tyr-63 of natural hirudin is sulfated, anticoagulant potency is markedly increased to about 50-fold that of intact hirudin (Maraganore et al., 1989). Thus, structure–function studies of the thrombin–hirudin interaction reveal that the highly anionic, C-terminal structure of hirudin occupies the anion-binding exosite and that the apolar, disulfide-linked N-terminal structure (residues 1–50) interacts in or near the thrombin catalytic site.

In order to identify the anion-binding exosite in thrombin, a proposed site for hirudin binding, we have prepared an N^{α}-dinitrofluorobenzyl derivative of a synthetic C-terminal undecapeptide of hirudin. Formation of a covalent complex was found to be specific by competitive binding experiments. Following peptide fragmentation of the complex, Lys-149[2] was determined to be the major site of derivatization. These data show that Lys-149 and surrounding structures define a substrate recognition site in thrombin some 18–20 Å removed from the catalytic site.

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* Address correspondence to this author.

‡ Biogen, Inc.

§ New York State Department of Health.

[1] Nomenclature of Schechter and Berger (1967).

[2] Chymotrypsin numbering as defined previously (Fenton, 1986b).

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Experimental Procedures

Materials. *t*-Boc[3] amino acids for solid-phase peptide synthesis were purchased from Peninsula Laboratories, Belmont, CA, and included Boc-L-leucyl-*O*-divinylbenzene resin, Boc-*O*-2,6-dichlorobenzyl-L-tyrosine, Boc-L-glutamic acid γ-benzyl ester, Boc-L-proline, Boc-L-isoleucine·$^1/_2H_2O$, Boc-L-phenylalanine, Boc-L-aspartic acid β-benzyl ester, and Boc-glycine. Human α-thrombin was purified as reported previously (Fenton et al., 1977). The human α-thrombin preparation (α309) was at a specific activity of 2678 units/mg, with active-site titration measurements showing 94% activity. SDS–PAGE analysis revealed the purity of α-thrombin to be 96%. Bovine thrombin was obtained from Sigma, St. Louis, MO. Sequenal grade dimethylformamide and dinitrodifluorobenzene (DNDFB) were purchased from Pierce Chemical Co., Rockford, IL. Dicyclohexylcarbodiimide (DCC) was from Sigma. All other reagents and solvents were of the finest grade commercially available.

Analytical Techniques. Amino acid analysis of Hir_{54-64} was performed by ion-exchange chromatography employing a Beckman System 6300 analyzer and standard preparation of samples by acid hydrolysis (Spackman et al., 1958). Automated Edman degradation was performed with an ABI 470A gas-phase sequencer equipped with a Model 900A data collection system. Phenylthiohydantoin (PTH) amino acids were separated on-line with an ABI 120A PTH analyzer. SDS–PAGE was performed with minigels (9.0 × 9.5 cm) using 12.5% acrylamide by the procedure of Laemmli (1970). Samples were applied to SDS–PAGE in the presence of 2-mercaptoethanol as reducing agent.

Peptide Synthesis. S-Hir_{53-64} was synthesized as reported previously (Maraganore et al., 1989). Hir_{54-64} was synthesized by standard solid-phase peptide methodologies employing an ABI 430A peptide synthesizer. Peptides were deprotected and uncoupled from the divinylbenzene resin by treatment with anhydrous HF, *p*-cresol, and ethyl methyl sulfide (10:1:1 v/v/v). Hir_{54-64} was purified by HPLC as reported previously (Maraganore et al., 1989). [^{35}S]Hir_{54-64} was prepared by using 2 mg of Hir_{54-64} dissolved in 0.08 mL of dimethylformamide in the presence of 2 mCi of $NaH^{35}SO_4$ (New England Nuclear, Boston, MA) previously dried under a gentle stream of N_2. Concentrated sulfuric acid (0.001 mL) was added to the solution, followed by 0.014 mL of a DCC solution (1 mg/0.8 mL of dimethylformamide). The reaction was on ice and was allowed to proceed for 1 min, during which time a cyclohexylurea precipitate formed. Water (0.5 mL) was added to stop the reaction. [^{35}S]Hir_{54-64} thus prepared was purified by HPLC employing an ABI 150A liquid chromatographic system and an Aquapore RP-300 C_8 column (0.46 × 10 cm). The column was equilibrated in 0.1% TFA/water and developed with an increasing gradient of acetonitrile concentration from 0 to 50% solvent B (0.085% TFA/70% acetonitrile) over 45 min at a flow rate of 1.0 mL/min. The effluent stream was monitored at 214 nm. [^{35}S]Hir_{54-64} was recovered as a single peak eluting prior to the position of Hir_{54-64} at 95% yield and then lyophilized to dryness. [^{35}S]Hir_{54-64} was at a specific radioactivity of ~1 × 10^5 cpm/μg.

Synthesis of DNFB-Hir_{54-64} and DNFB-[^{35}S]Hir_{54-64}. N^{α}-(Dinitrofluorobenzyl)-Hir_{54-64} (DNFB-Hir_{54-64}) was prepared by reaction of Hir_{54-64} (10 mg) with 1 molar equiv of dinitrodifluorobenzene (DNDFB), 1.5 mg, in dimethylformamide (0.5 mL) for 18 h at 25 °C. N^{α}-(Dinitrofluorobenzyl)-[^{35}S]Hir_{54-64} was prepared by reaction of [^{35}S]Hir_{54-64} (2.0 mg) with 1 molar equiv of DNDFB (0.3 mg) in dimethylformamide (0.2 mL) for 18 h at 25 °C. The extent of N-alkylation in DNFB-Hir_{54-64} and DNFB-[^{35}S]Hir_{54-64} was monitored by analytical HPLC using the same system and gradient reported above for purification of [^{35}S]Hir_{54-64}. Synthesis of DNFB-Hir_{54-64} and DNFB-[^{35}S]Hir_{54-64} was at 68.9% and 20.6% yield, respectively. N-Alkylated peptides were used without further purification and stored at 4 °C in the dimethylformamide solvent.

Reaction of DNFB-hirudin Peptides with Thrombin. Human or bovine thrombins (12.5 μg) were reacted with DNFB-[^{35}S]Hir_{54-64} (5 μg) in phosphate-buffered saline (0.03 mL) for 18 h at 25 °C. Separately, the same reactions were performed in the presence of S-Hir_{53-64} (25 and 100 μg). The reaction mixtures were reduced and applied to SDS–PAGE as described above. The gels were stained with Coomassie Blue, dried, and exposed to X-OMAT AR film (Kodak, Rochester, NY) for 18 h at −70 °C. Autoradiographs were developed with an RP X-OMAT processor (Kodak).

In order to prepare the Hir_{54-64}–thrombin complex for protein structural analysis, human α-thrombin (1.0 mg) was reacted with a 20-fold molar excess of DNFB-Hir_{54-64} (0.87 mg) in 1.0 mL of 0.5 M sodium borate, pH 8.5, for 4 h at 25 °C. The extent of covalent complex formation was determined as ~50% by SDS–PAGE analysis as described above. The reaction mixture was applied to a column (1.5 × 45 cm) of Sephadex G-50 (fine) equilibrated and developed in 8 M urea and 20 mM Tris at pH 7.7. Molecular sieve chromatography allowed separation of thrombin and the Hir_{54-64}–thrombin complex from free, *N*-alkyl-Hir_{54-64}. A pool containing absorbance at both 280 and 310 nm was isolated in 8.0 mL eluting in the column exclusion volume.

Protein Structural Analysis of the Hir_{54-64}–Thrombin Complex. Hir_{54-64}–thrombin prepared as described above was reduced and S-carboxymethylated by a modification (Maraganore & Heinrikson, 1986) of the method of Hirs (1967), except urea was used instead of guanidinium chloride as the chaotrope. The reduced, S-alkylated complex (~1 mg in 8.0 mL) was dialyzed twice against 2-L volumes of 3% acetic acid. The recovered protein was treated with porcine pepsin (2% w/w) (Boehringer-Mannheim, Indianapolis, IN) for 4 h at 37 °C. Pepsin fragments thus obtained were separated by reverse-phase HPLC employing an ABI 150A system and an Aquapore RP-300 C_8 column (0.46 × 10 cm) equilibrated in 0.1% TFA/water. The column was developed with a linear gradient of increasing acetonitrile concentration from 0 to 60% solvent B (0.085% TFA/70% acetonitrile) over 90 min at a flow rate of 1.0 mL/min. The effluent stream was monitored at both 214 and 310 nm for absorbance. The major peaks (1 and 2) containing absorbance at 310 nm were resolved and isolated by this procedure. Peptide in peaks 1 and 2 (P-1 and P-2) were further purified separately by HPLC employing the same column and solvent compositions. The column was developed with a linear gradient of increasing acetonitrile concentration from 20% to 50% solvent B over 90 min at 1.0 mL/min. The effluent stream was monitored at both 214 and 310 nm. Purified P-1 and P-2 thus isolated were subjected

[3] Abbreviations: DNDFB, dinitrodifluorobenzene; DCC, dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography; DNFB-Hir_{54-64}, N^{α}-(dinitrofluorobenzyl)glycylaspartylphenylalanylglutamylglutamylisoleucylprolylglutamylglutamyltyrosylleucine; DNFB-[^{35}S]Hir_{54-64}, N^{α}-(dinitrofluorobenzyl)glycylaspartylphenylalanylglutamylglutamylisoleucylprolylglutamylglutamyl($O^{35}SO_3$-tyrosyl)leucine; S-Hir_{53-64}, *N*-acetylasparaginylglycylaspartylphenylalanylglutamylglutamylisoleucylprolylglutamylglutamyl(OSO_3-tyrosyl)leucine; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; *t*-Boc, *tert*-butoxycarbonyl.

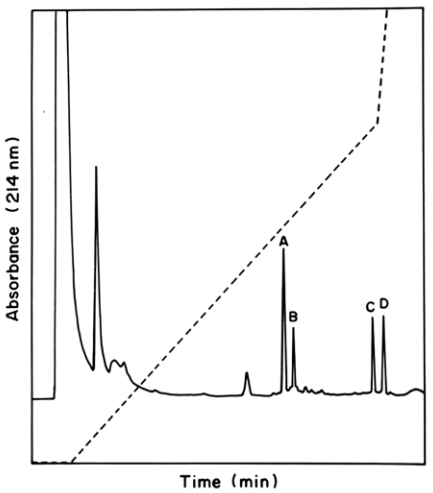

FIGURE 1: Analytical HPLC separation of the reaction mixture from DNFB-[^{35}S]Hir$_{54-64}$ synthesis. Reaction of [^{35}S]Hir$_{54-64}$ with DNDFB allowed N$^{\alpha}$-alkylation of the peptide (peaks C and D) as well as desulfation of the parent and the N$^{\alpha}$-alkylated peptide, (peaks B and D, respectively). DNFB-[^{35}S]Hir$_{54-64}$ was obtained at 20.6% yield.

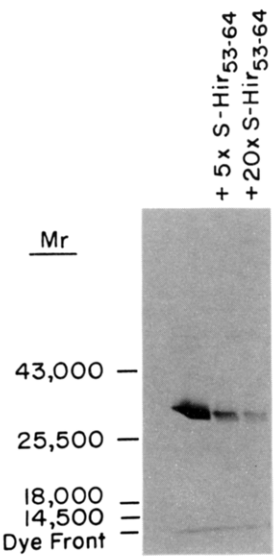

FIGURE 2: Analysis of specificity in DNFB-hirudin peptide–thrombin complex formation. Autoradiogram showing incorporation of DNFB-[^{35}S]Hir$_{54-64}$ in a thrombin band at M_r = 32 500. In the presence of increasing concentrations of cold S-Hir$_{53-64}$, the extent of the covalent complex is reduced incrementally to <10%.

to automated Edman degradation as described above. Identification of Lys-149 as the site of DNFB-Hir$_{54-64}$ alkylation was by absence of a recoverable PTH-lysine at cycle 10 in sequence analysis of P-1.

Results

Synthesis of N$^{\alpha}$-(Dinitrofluorobenzyl)-[^{35}S]Hir$_{54-64}$. [^{35}S]Hir$_{54-64}$, a Tyr-sulfated synthetic undecapeptide [Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-(OSO$_3$-Tyr)-Leu] derived from residues 54–64 of hirudin, was prepared as reported previously (Maraganore et al., 1989). Modification of the peptide α-NH$_2$ group to yield an N$^{\alpha}$-dinitrofluorobenzyl derivative was achieved by reaction of [^{35}S]Hir$_{54-64}$ with stoichiometric quantities of dinitrodifluorobenzene (DNDFB) (Marfey & Tsai, 1975). As shown in Figure 1, HPLC analysis of the reaction mixture shows resolution of four major peptide peaks labeled A–D. Peak A was identified as unreacted [^{35}S]Hir$_{54-64}$ while the peptide in peak B was identified as desulfated [^{35}S]Hir$_{54-64}$ or Hir$_{54-64}$. Both peaks C and D were found to contain absorbance at 310 nm while radioactivity was recovered in peak C alone. These data are consistent with assignment of the peptide in peak C as the desired product, DNFB-[^{35}S]Hir$_{54-64}$, and that in peak D as the desulfated derivative. Desulfation of [^{35}S]Hir$_{54-64}$ is proposed to occur due to acid catalysis by HF released during alkylation of the peptide with the DNDFB reagent. Synthesis of DNFB-[^{35}S]Hir$_{54-64}$ by this procedure was at 20.6% yield.

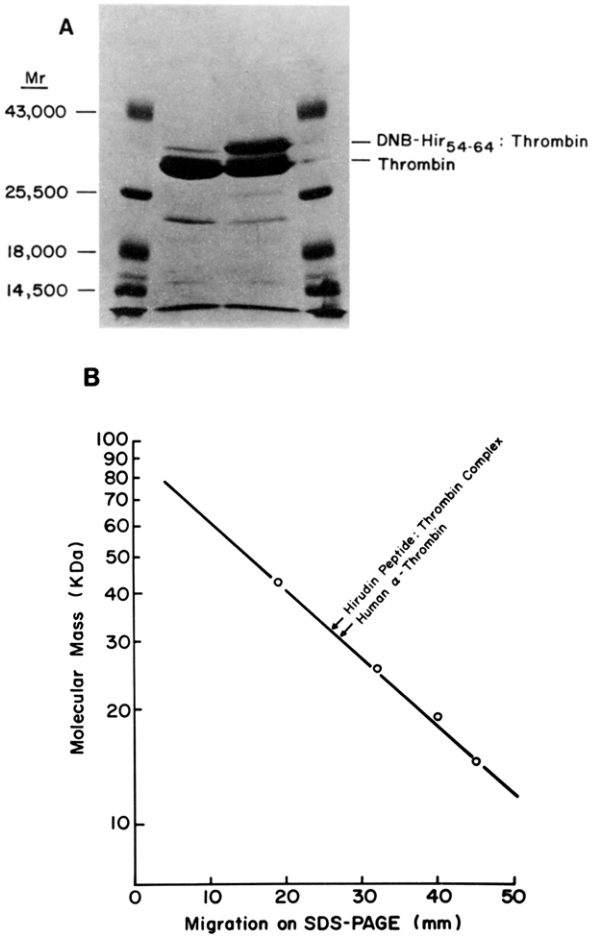

FIGURE 3: Evidence for stoichiometric alkylation of human α-thrombin with DNFB-Hir$_{54-64}$. Reaction of human α-thrombin with DNFB-Hir$_{54-64}$ was at ~50% yield (A) and the complex was found to migrate on SDS–PAGE at M_r ~32 500 (B). (A, from left) Lanes 1 and 4, molecular weight standards; lane 2, purified human α-thrombin; lane 3, human α-thrombin reacted with DNFB-Hir$_{54-64}$. As purified thrombin migrates at M_r ~31 000 and the mass of the ϵ-NH$_2$-alkylated affinity-peptide reagent is 1581 Da, these data suggest that modification of thrombin with the hirudin peptide is stoichiometric.

Reaction of Thrombin with DNFB-[^{35}S]Hir$_{54-64}$. Human α-thrombin reacted with DNFB-[^{35}S]Hir$_{54-64}$ was analyzed by SDS–PAGE and autoradiography. As shown in Figure 2, autoradiographs reveal incorporation of hirudin peptide associated, ^{35}S radiolabel coincident with a band on a corresponding Coomassie Blue stained gel migrating as a protein of M_r ~32 500 (Figure 3). Unreacted human α-thrombin was determined to migrate as a protein of M_r ~31 000 on SDS–PAGE gels (Figure 3A). The increase in apparent molecular mass of the [^{35}S]Hir$_{54-64}$–thrombin complex is an increment of ~1500 Da (Figure 3B), consistent with stoichiometric modification of thrombin with DNFB-[^{35}S]Hir$_{54-64}$ (M_r = 1581 as alkylated form).

The specificity of DNFB-[^{35}S]Hir$_{54-64}$ for α-thrombin was examined by reaction of thrombin with the DNFB-peptide in the presence of S-Hir$_{53-64}$ at 5- and 20-fold molar excess (Figure 2). In the presence of increasing concentrations of cold S-Hir$_{53-64}$, the extent of covalent derivatization of α-

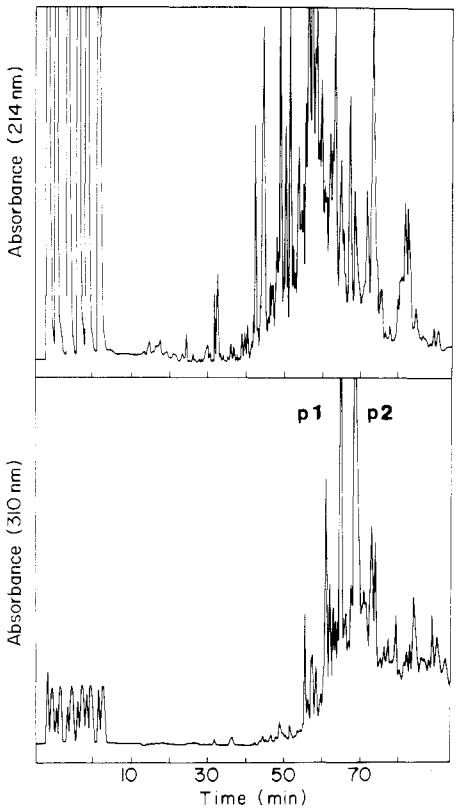

FIGURE 4: HPLC separation of pepsin fragments of the Hir_{54-64}–thrombin complex. Pepsin fragments of the reduced, *S*-(carboxymethyl)cysteinyl-Hir_{54-64}–thrombin complex were purified by reverse-phase HPLC as described under Experimental Procedures. The effluent was monitored for absorbance at 214 nm (top) and 310 nm (bottom). Two major peaks of 310-nm absorbance containing peptides P-1 and P-2 were isolated.

thrombin by DNFB-[^{35}S]undecapeptide was incrementally reduced to <10%. The ability of S-Hir_{53-64} to block covalent complex formation suggests that DNFB-[^{35}S]Hir_{54-64} modifies a discrete hirudin peptide binding site in thrombin. Thus, these data appear to exclude the possibility of random lysyl alkylation in thrombin with the DNFB-peptide.

Protein Structural Analysis of the Hir_{54-64}–Thrombin Complex. In order to identify the site of human α-thrombin modification by DNFB-[^{35}S]Hir_{54-64}, an unsulfated derivative, DNFB-Hir_{54-64}, was reacted at 20-fold excess with thrombin in 0.5 M sodium borate at pH 8.5. Reaction was found to allow derivatization of thrombin at ~50% yield. Following removal of unreacted DNFB-peptide by molecular sieve chromatography equilibrated and developed in an 8 M urea-containing buffer, the Hir_{54-64}–thrombin complex was reduced, S-alkylated, and treated with pepsin. Peptic fragments were isolated by reverse-phase HPLC with simultaneous detection of the effluent stream at 214 and 310 nm (Figure 4). Detection at 310 nm was to allow identification of thrombin peptide fragments alkylated with the DNFB-derivatized hirudin peptide. Two major peaks (1 and 2) containing absorbance at 310 nm were resolved and isolated. An additional HPLC purification was performed to assure a level of purity in the peptide samples (designated P-1 and P-2) suitable for sequence analysis.

Automated Edman degradation of the peptic fragment P-1, isolated in peak 1, revealed a sequence of human thrombin corresponding to pepsin cleavage at the Leu_{144}–Lys_{145} bond (Table I). Sequence analysis of fragment P-1 was performed in triplicate from two separate preparations of peptic fragments of the reduced *S*-alkyl-Hir_{54-64}–thrombin complex. Recovery

Table I: Results from Sequence Analysis of Pepsin Fragments[a]

P-1			P-2	
amino acid identified	expt 1, recovery (pmol)	expt 2, recovery (pmol)	amino acid identified	recovery (pmol)
Lys	63	859	Gln	220
Glu	81	629	Ala	624
Thr	50	358	Gly	190
Trp	17	276	Tyr	235
Thr	33	289	Lys	581
Ala	55	474	Gly	127
Asn	22	369	Arg	111
Val	51	491	Val	264
Gly	*b*	296	Thr	121
Lys[c]			Gly	157
Gly	46	267	Trp	20
Gln	24	209	Gly	57
Pro	10	104	Asn	97
Ser	5	22	Leu	111
Val		23	Lys	85
			Glu	85
			Thr	52
			Trp	4
			Thr	2
			Ala	38
			Asn	21
			Val	20
			Gly	10

[a] Automated Edman degradation was performed as described under Experimental Procedures. [b] Gly was identified but not quantitated. [c] Lys at cycle 10, corresponding to Lys-149, was not identified.

of lysine at cycle 10, corresponding to Lys-149 of human thrombin, was not possible despite an average repetitive yield (in experiment 2, Table I) of 98.1% in sequence analysis from Lys-145 through Val-153. The absence of PTH-Lys at cycle 10 appears to be due to its ϵ-NH_2 modification with the hirudin peptide affinity-labeling reagent. These data are consistent with the site of DNFB-Hir_{53-64} modification at Lys-149 of human thrombin.

Analysis of fragment P-2, isolated in peak 2, revealed the sequence of a peptide derived from pepsin cleavage at the Leu_{130}–Glu_{131} bond (Table I). Sequence analysis allowed assignment of residues 131–148 of the thrombin structure including lysyl residues at positions 135 and 145. It was not possible, however, to assign the sequence past 23 cycles of Edman degradation including Lys-149. Nevertheless, it is reasonable to expect partial cleavages of protein substrates with pepsin and to expect that the peptide in peak 2 is likewise modified at the ϵ-NH_2 of Lys-149. Indeed, sequence analysis of P-2 shows that the Leu_{144}–Lys_{145} bond, cleaved by pepsin to yield fragment P-1, is intact.

Discussion

An affinity-labeling reagent (Wofsy et al., 1962) consists of a ligand moiety, which binds to a site with sufficient affinity to permit labeling; a reactive group, which is capable of forming a covalent bond with a residue at or near the binding site(s); and a detection chromophore or radioactive group. Criteria for which a reagent specifically modifies reactive residues within a high-affinity binding site have been established (Baker, 1967; Singer, 1967; Shaw, 1970). In order to define molecular detail in the interaction of hirudin and its peptide derivatives with human thrombin, the undecapeptide corresponding to residues 54–64 of the hirudin HV1 variant (Tripier, 1988) was employed as the ligand of an affinity-labeling reagent. A reactive DNFB group was conjugated at the NH_2 terminus of the peptide and is capable of covalent derivatization of amino groups in thrombin at or near the hirudin peptide binding site. In addition, the DNFB group

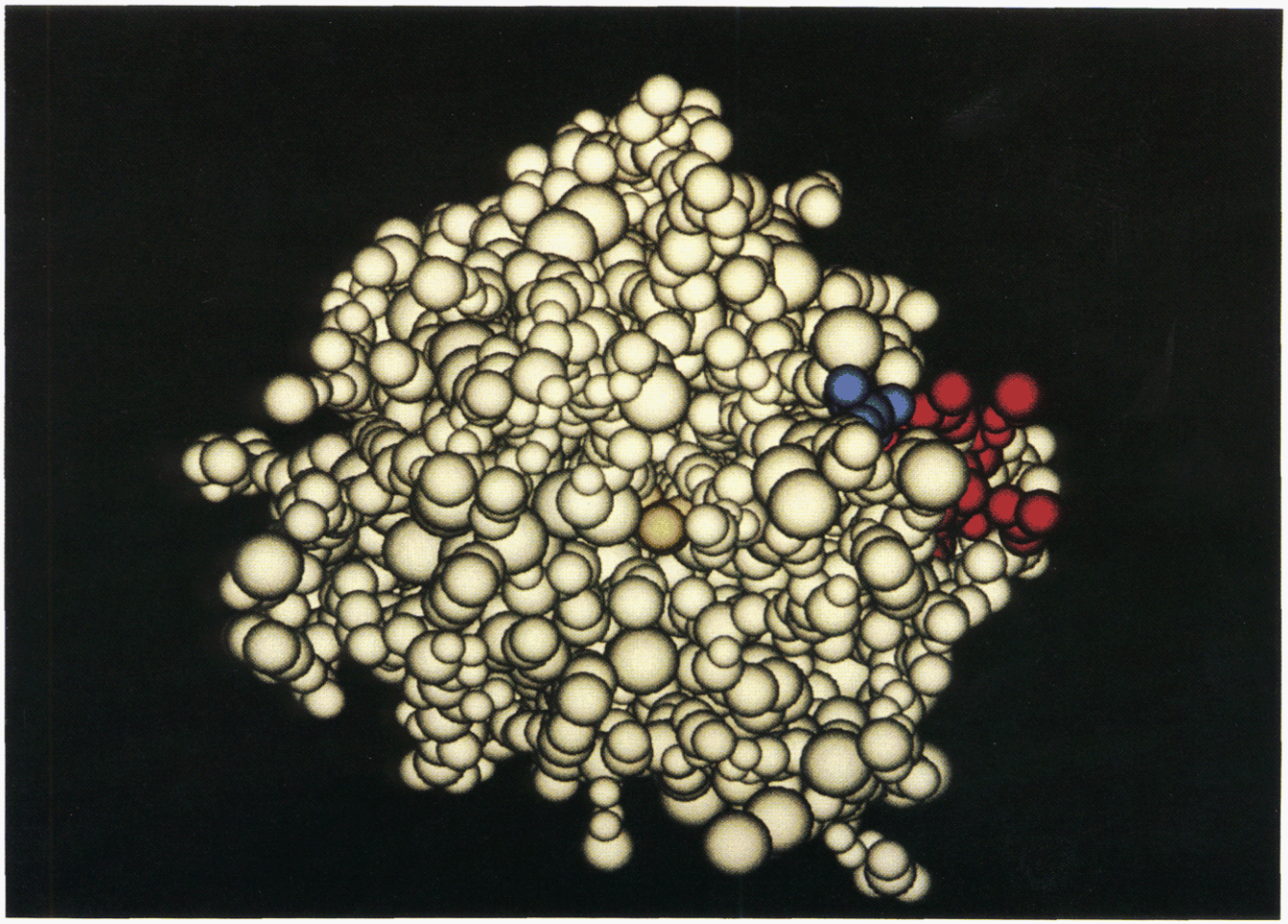

FIGURE 5: Model of the three-dimensional structure of thrombin (36) showing positions of Lys-149 (blue), neighboring cationic residues (red), and Ser-195 (yellow). Lys-149 is a glutamic acid in the bovine structure.

absorbs at the far-UV and near-visible spectra, allowing specific detection. Alternatively, a hirudin peptide containing a ^{35}S-sulfated tyrosyl residue corresponding to sulfated Tyr_{63} of hirudin was used where it was necessary to detect small amounts of the hirudin peptide–thrombin complex.

Specificity of the DNFB-[^{35}S]Hir_{54-64} reaction with human α-thrombin was demonstrated by protection of thrombin labeling of the ^{35}S reagent in the presence of the hirudin peptide S-Hir_{53-64}, which lacks both radioactivity and the N^{α}-DNFB moiety. The reaction of the affinity reagent with thrombin was found to yield a stoichiometric covalent complex based on SDS–PAGE analysis. Whereas other affinity-labeling reagents may modify more than a single residue (Fenton & Singer, 1971; Bing et al., 1977), only Lys-149 was presently identified among peptic digests of the hirudin peptide–thrombin complex. This finding implies that the reagent must assume a relatively well-defined orientation upon binding to the human enzyme.

The absence of Lys-149 in bovine thrombin may explain why hirudin peptides exhibit a 10-fold decreased reactivity toward this enzyme. However, a direct role for Lys-149 in the interaction of intact hirudin, the protein, with human thrombin is yet to be demonstrated. Indeed, hirudin inhibits fibrinogenolytic activity of human and bovine thrombins with a comparable potency (Maraganore et al., 1989). A recent study by Chang (1989) involving chemical modification of thrombin lysyl residues with S-DABITC [4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid] in the presence or absence of hirudin shows that Lys-149 was one of six lysine residues protected from alkylation as a result of complex formation. Interestingly, examination of a three-dimensional model for bovine thrombin (Furie et al., 1982) shows that lysyl residues protected from S-DABITC modification in the hirudin–thrombin complex are randomly distributed throughout the thrombin structure. Excluding Lys-149, protection of an additional five lysyl residues via hirudin complex formation may derive as a result of conformational change from the hirudin–thrombin interaction (Konno et al., 1988) or due to involvement of additional structure in thrombin which participates in hirudin binding vs hirudin peptide binding, as might be expected. Additional data regarding the loci of hirudin binding to thrombin derive from studies of Noe et al. (1988), who found that sequence-specific antibodies toward residues 62–73 of thrombin inhibit competitively hirudin binding to thrombin.

Further examination of a model for bovine thrombin structure reveals that the site of DNFB-Hir_{54-64} alkylation in human thrombin, Lys-149, corresponding to Glu-149 of the bovine enzyme, is at least 18–20 Å removed from the β-hydroxylate of Ser-195 (Figure 5). Surrounding Lys-149 in both human and bovine structures exists a packed cluster of five additional cationic residues including Lys-145, Lys-185a,[4] Lys-186, Arg-187, and Arg-222, all or at least some of which may define additional determinants for hirudin peptide binding. Certainly, a total of six cationic valences of human thrombin would appear suited for interaction with six anionic pairs in

[4] Lys-185a is in fact Lys-191 in the human thrombin B-chain sequence. It is designated as "Lys-185a" as it does not align directly with chymotrypsin.

C-terminal hirudin peptides (corresponding in hirudin numbering to Asp-55, Glu-57, Glu-58, Glu-61, Glu-62, and sulfato-Tyr-63). If in fact hirudin peptide binding is localized to a cluster of these cationic residues in thrombin, then these amino acids may define a functional domain comprising the anion-binding exosite. The finding that N^{α}-DNFB-Hir_{54-64} alkylates Lys-149 does not as yet allow designation of a specific ion pair in the hirudin peptide–thrombin interaction, only that the NH_2 terminus of such peptides lies in proximity to this cationic side chain. Nevertheless, our experiments support the hypothesis (Fenton, 1989; Fenton et al., 1989a,b) that the mechanism of hirudin peptide inhibition is by antagonism by anion-binding exosite function, i.e., macromolecular ligand recognition, by binding to a site removed from the thrombin catalytic center.

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