9-5-1992

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### Recommended Citation

Vitali, J., Martin, P. D., Malkowski, M. G., Robertson, W. D., Lazar, J. B., Winant, R. C., Johnson, P. H., and Edwards, B. F. P. The structure of a complex of bovine alpha-thrombin and recombinant hirudin at 2.8 Å resolution, J. Biol. Chemistry 267: 17670-17678, 1992. https://doi.org/10.1016/s0021-9258(19)37095-4

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# The Structure of a Complex of Bovine α-Thrombin and Recombinant Hirudin at 2.8-Å Resolution*

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Crystals of the complex of bovine α-thrombin with recombinant hirudin variant 1 have space group C222₁ with cell constants $a = 59.11$, $b = 102.62$, and $c = 143.26$ Å. The orientation and position of the thrombin component was determined by molecular replacement and the hirudin molecule was fit in $2|F_o| - |F_c|$ electron density maps. The structure was refined by restrained least squares and simulated annealing to $R = 0.161$ at 2.8-Å resolution. The binding of hirudin to thrombin is generally similar to that observed in the crystals of human thrombin-hirudin. Several differences in the interactions of the COOH-terminal polypeptide of hirudin, specifically of residues Asp-55h, Phe-56h, Glu-57h, and Glu-58h, and a few differences in the interactions of the hirudin core, specifically of residues Asp-5h, Ser-19h, and Asn-20h, with thrombin from human thrombin-hirudin suggest that there is some flexibility in the binding of these 2 molecules. Most of the residues in the 9 subsites that bind fibrinopeptide A₇₋₁₆ to thrombin also interact with the NH₂-terminal domain of hirudin. The S1 subsite is a notable exception in that only 1 of its 6 residues, namely Ser-214, interacts with hirudin. The only difference between human and bovine thrombins that appears to influence the binding of hirudin is the replacement of Lys-149E by an acidic glutamate in the bovine enzyme.

α-Thrombin (EC 3.4.21.5) is a serine protease with a high specificity for arginine bonds that plays a central role in thrombosis and hemostasis. It is the product of prothrombin cleavage by factor Xa in the final step of the blood clotting cascade (reviewed by Mann, 1987; Davie et al., 1991). During clotting, α-thrombin converts fibrinogen into fibrin by removing fibrinopeptide A from the Aα-chain and fibrinopeptide B from the Bβ-chains of fibrinogen. Thrombin also functions as a major regulator of hemostasis (Fenton, 1986; Davie et al.,

1991, and references therein). Free thrombin accelerates clotting by activating platelets and key enzymes in the clotting cascade, such as factor V, factor VIII, factor XI, and factor XIII, whereas thrombin bound to thrombomodulin attenuates clotting by activating protein C, which then inactivates factors Va and VIIIa (Esmon, 1987; Dittman and Majerus, 1990).

α-Thrombin consists of two polypeptide chains, A and B, connected through a single disulfide bond. Bovine α-thrombin has 49 amino acid residues in the A-chain and 259 residues in the B-chain (Magnusson et al., 1975; MacGillivray and Davie, 1984; Walz et al., 1986) whereas human α-thrombin has 36 residues, after the loss of a tridecapeptide during activation from prothrombin, in the A-chain, of which 27 are identical with those in bovine thrombin, and 259 in the B-chain, of which 225 are identical (Butkowski et al., 1977; Degan et al., 1983; Walz et al., 1986). The B-chain contains a carbohydrate at Asn-60G and the active site residues His-57, Asp-102, and Ser-195.[1]

The most potent natural inhibitor of thrombin known is hirudin, which is a small protein containing 65 residues and 3 disulfide bonds that is isolated from the glandular secretions of the leech Hirudo medicinalis (Markwardt, 1970). The dissociation constants for the noncovalent, equimolar complex of hirudin with bovine thrombin or with human thrombin are 3.0 pM and 0.3 pM, respectively (Dodt et al., 1988, 1990). Hirudin blocks or greatly attenuates thrombin activity toward fibrinogen, platelets, clotting factors, thrombomodulin, and monocytes. Because hirudin reacts only with thrombin, is well tolerated by the immune system, and has no hemorrhagic side effects, it is a potentially valuable drug for thromboembolic disorders (Markwardt, 1991). The three-dimensional structures of the amino-terminal domain of two recombinant hirudins have been determined in solution by NMR methods (Clore et al., 1987; Folkers et al., 1989; Haruyama and Wuthrich, 1989). In both cases, residues 1h, 31h–36h, and 49h–65h had no defined structure in solution.

The crystal structures of the complexes of human α-thrombin with recombinant hirudin variants 1 (Grütter et al., 1990) and 2 (Rydel et al., 1990, 1991) have been recently determined. These studies reveal a mode of binding that has not been previously observed for a protease inhibitor. The unique features are as follows.

1) The contact area between hirudin and thrombin in the

[1] The residue numbers for thrombin in this paper are assigned by homology with chymotrypsin (Bode et al., 1989). A suffix denotes an insertion in the thrombin sequence relative to that of chymotrypsin. Sequence numbers for hirudin and fibrinopeptide residues are identified by the suffix "h" or "f," respectively. The prime symbol (') denotes a residue number in a symmetry-related molecule. "Sn" is used for the enzyme subsite that binds the $n^{th}$ residue on the amino-terminal side of the scissile bond (Schechter and Berger, 1967).

complex is large, which probably accounts for the high affinity and selectivity of hirudin for thrombin. 2) The first 3 residues of hirudin bind at the active site of thrombin, but the primary specificity site of thrombin is not occupied by hirudin. 3) The last 16 residues of hirudin are in an extended conformation and bind at an anion binding exosite on the surface of thrombin that extends from the active site and is probably the secondary fibrinogen binding site.

We undertook the structural analysis of the complex between bovine $\alpha$-thrombin and recombinant hirudin variant 1 for three reasons. The first was to gain more insight into the interactions between hirudin and thrombin at the molecular level using a crystal form different from that used in the earlier studies. The second was to investigate how the species variation of the thrombin enzyme, bovine *versus* human, affects the binding of hirudin at the molecular level. The third was to determine the structure of a bovine thrombin that was wholly in the $\alpha$-form. All previously solved crystal forms of bovine thrombin have contained significant amounts of proteolytic derivatives (Martin *et al.*, 1992). In this paper, we report the determination of the crystal structure of the complex between bovine $\alpha$-thrombin and recombinant hirudin variant 1 and a comparison of this structure with those of the human $\alpha$-thrombin-hirudin complexes (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990) and the bovine $\alpha$-thrombin-FPA$_{7-16}$[2] complex (Martin *et al.*, 1992) reported earlier.

## MATERIALS AND METHODS

*Preparation of Proteins*—Thrombin was prepared as previously described (Martin *et al.*, 1983) and had approximately 2000 NIH clotting units per mg of protein. It was stored at $-20$ °C in ammonium phosphate before being set up for crystallization. Recombinant hirudin was purified from *Escherichia coli* cells transformed with the pBR-CRM-CTAP-Hir plasmid (GenBank M88535), which codes for hirudin fused to a CTAP leader sequence (Waleh *et al.*, 1992). After the leader sequence had been removed with cyanogen bromide, the hirudin molecule was purified by high performance liquid chromatography on reverse phase columns (Winant *et al.*, 1991). The recombinant protein, which lacks the sulfate group on Tyr-63h that is responsible for a 3–10-fold increase in the affinity of leech hirudin for thrombin, had the same affinity as desulfated hirudin from natural sources (Stone and Hofsteenge, 1986; Winant *et al.*, 1991).

*Crystallization*—The complex was prepared by mixing bovine $\alpha$-thrombin and recombinant hirudin at a molar ratio 1:1.5. The crystals were grown by the hanging drop method at 22 °C from reservoirs containing 29% PEG 4000, 250 mM sodium phosphate buffer, pH 4.7, 94 mM NaCl, and 0.005% NaN$_3$. The initial thrombin-hirudin concentration in the drop was 7 mg/ml. Polyacrylamide gel electrophoresis (Laemmli, 1970) on a crystal dissolved in 1% sodium dodecyl sulfate solution (Martin *et al.*, 1992) showed that no conversion of the $\alpha$-thrombin to the $\beta$, $\epsilon$, or $\gamma$ proteolytic derivatives had occurred during the crystallization. This conversion has occurred at least partially in all other crystals of bovine $\alpha$-thrombin with substrate analogs or inhibitors that we have grown in our laboratory. The crystals are orthorhombic, cell constants $a = 59.11$, $b = 102.62$, $c = 143.26$ Å, space group C222$_1$ with 1 molecule per asymmetric unit. The crystals of the human thrombin-hirudin complexes are tetragonal, P4$_3$2$_1$2 (Rydel *et al.*, 1990; Grütter *et al.*, 1990).

*Data Collection*—Intensity data were measured at 22 °C on the two largest crystals (approximate dimensions 0.40 × 0.20 × 0.05 mm) using a Siemens area detector with a Rigaku RU200H rotating anode x-ray source and Supper graphite monochromator operated at 40 kV and 70 mA. The radiation used was CuK$\alpha$. Two "sweeps" were made for the first crystal, one for the second. Each covered 110° in $\omega$ with 440 frames of data. In the first crystal, the $\phi$ angle was incremented by 70° between sweeps. Each frame was measured for 5 min to ensure that it contained at least 1 million total counts. 19008 measurements with $I > \sigma$ were merged, scaled, and averaged with the XENGEN

package (Howard *et al.*, 1987) to give 7688 unique reflections. The overall, unweighted, absolute $R_{sym}$ was 0.099. The fraction of observed reflections for the shells $\infty$–7.0 Å, 7.0–3.3 Å, 3.3–3.0 Å, 3.0–2.8 Å, and 2.8–2.6 Å was 92%, 84%, 50%, 24%, and 13%, respectively.

*Structure Solution*—The orientation and position of the thrombin component in the crystal was determined by the molecular replacement method using the package MERLOT (Fitzgerald, 1988), molecule 2 of bovine $\alpha$-thrombin in the complex with fibrinopeptide A$_{7-16}$ (Martin *et al.*, 1992) as the probe, and 4$\sigma$ data between 7.0 and 3.5 Å. The rotational search was carried out with the fast rotation function of Crowther (1972) using the program CROSUM, a radius of integration of 20.0 Å, and grid interval of 2.5° in $\alpha$, and 5.0° in $\beta$ and $\gamma$. Three translation functions (Crowther and Blow, 1967) were computed with the program TRNSUM covering the entire unit cell and with step size along $a$, $b$, and $c$ of 0.02 times the cell edge. The molecular replacement solution was refined with the $R$-factor search of Ward *et al.* (1975) using the program RMINIM and subsequently with rigid body least-squares using the program ROTLSQ (W. A. Hendrickson, Columbia University) and 7.0 to 3.0 Å data with $|F| \geq 10\sigma$.

The structure of the hirudin component was determined from $2|F_o| - |F_c|$ electron density maps. The refinement of the complex was carried out using restrained least-squares procedures with the programs PROLSQ and PROFFT (Agarwal, 1978; Hendrickson and Konnert, 1979; Finzel, 1987) and by simulated annealing with the program XPLOR (Brunger, 1988). The slow cool annealing protocol was used with XPLOR. The dynamics temperature started out at 4000 K and decreased in intervals of 25 K to a final temperature of 0 K with 25 steps (0.5 fs/step) of Verlet dynamics at each temperature. The PROLSQ refinement was carried out using data 7.0 to 3.0 Å with $|F| > 2\sigma$. All the available data beyond 7.0 Å were used for the XPLOR and PROFFT refinements.

*Structure Analysis*—Hydrogen bonds were calculated in QUANTA (Polygen Corporation, Waltham, MA) using as criteria a distance of 3.5 Å or less between the donor and acceptor atoms and a value of 90° or more for the angles centered at these atoms. A hydrophobic contact was assumed to exist between 2 apolar residues if they had a carbon-carbon distance of 4.0 Å or less.

## RESULTS AND DISCUSSION

*Structure Solution*—The orientation and position of the thrombin component in the crystal was strongly indicated by the molecular replacement searches. It corresponded to the top peak in all searches with a high signal to noise ratio. The top peak in the rotational search at $(\alpha, \beta, \gamma) = (97.50°, 55.00°, 230.00°)$ was 1.7 times larger than the next highest peak. The top peak in each translation function, corresponding to the position of thrombin in the unit cell at $(x, y, z) = (0.22, 0.22, 0.15)$, was 2.5 to 2.7 times larger than the next highest peak. The $R$ value after ROTLSQ was 0.357.

Initial refinement of the thrombin component using PROLSQ brought the crystallographic $R$-factor to 0.282. Residues 1h–31h and 37h–42h of hirudin were then fit into the electron density of a $2|F_o| - |F_c|$ Fourier map. The NMR model of hirudin (Folkers *et al.*, 1989; Protein Data Bank entry 5HIR) facilitated the fitting process for the amino-terminal domain. Nine alternate cycles of PROLSQ refinement followed by graphics intervention allowed us to build the rest of the hirudin molecule and refit parts of the thrombin component. The complex was further refined with XPLOR to $R = 0.171$. The final refinement of the structure was carried out with PROFFT. Toward the end of the PROFFT refinement, water molecules were included at 129 peaks that were greater than 2.9$\sigma$ in the $|F_o| - |F_c|$ Fourier map, greater than 0.7$\sigma$ in the $2|F_o| - |F_c|$ Fourier map, and within hydrogen bonding distances from appropriate atoms. The $R$ value at the conclusion of the refinement was 0.161 (Tables I and II). The fit of parts of the final model to the electron density is illustrated in Fig. 1.

Except for a few residues, both thrombin and hirudin are well defined in the electron density maps contoured at the 1$\sigma$ level. There is no density for the first 13 residues at the NH$_2$

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[2] The abbreviations used are: FPA$_{7-16}$, residues 7–16 of fibrinopeptide A from the A$\alpha$-chain of human fibrinogen; CTAP, connective tissue activating protein III; PPACK, D-phenyl-L-prolyl-L-arginyl chloromethyl ketone.

TABLE I

*Refinement statistics from PROFFT*

| | |
|---|---|
| Total number of atoms | 2996 |
| Number of solvent atoms | 129 |
| Number of observations (7–2.5 Å) | 6958 |
| Root mean square deviations | |
| Bond distances | 0.020 Å |
| Angle distances | 0.042 Å |
| 1–4 distances | 0.040 Å |
| 360 peptide planes | 0.011 Å |
| 40 aromatic planes | 0.006 Å |
| Chiral volumes | 0.151 Å³ |
| Single torsion contacts | 0.22 Å |
| Multiple torsion contacts | 0.33 Å |
| Possible (X...Y) H-bonds | 0.36 Å |
| Planar torsion angles | 2.8° |
| Staggered torsion angles | 24.1° |
| Orthonormal torsion angles | 29.8° |

TABLE II

*Final R-factors from PROFFT as a function of resolution*

| $D_{min}$ | Reflections | $R_{shell}$ | $R_{sphere}$ |
|---|---|---|---|
| Å | | | |
| 6.0[a] | 398 | 0.198 | 0.198 |
| 5.0 | 805 | 0.169 | 0.178 |
| 4.0 | 1659 | 0.135 | 0.151 |
| 3.5 | 1470 | 0.150 | 0.150 |
| 3.0 | 1864 | 0.174 | 0.156 |
| 2.8 | 409 | 0.225 | 0.158 |
| 2.5 | 353 | 0.270 | 0.161 |

[a] Reflections beyond 7.0 Å are not included.

terminus of the A-chain nor for several atoms in the side chains of Glu-14L and Arg-15 at the COOH terminus of the A-chain. In the B-chain of thrombin, the density for Thr-147 through Ala-149D of the autolysis loop is fragmented, and there is no density for the side chain of Leu-245 at the COOH terminus. In hirudin, there is fragmented density for Gln-49h and Ser-50h, weak, but continuous density for the main chain of His-51h, Asn-52h, Glu-61h, and Glu-62h, no density for the CB atoms of Glu-61h and Glu-62h, and little density for Gln-65h.

*Structure of Hirudin*—The secondary and tertiary structural features that we observe for hirudin are generally in agreement with the published descriptions of the complexes with human thrombin (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990). In the complex with bovine α-thrombin, hirudin variant 1, whose sequence is shown in Fig. 2, has a compact NH₂-terminal domain, comprised of residues 1h through 48h, and an extended COOH-terminal polypeptide chain, comprised of residues 49h through 65h (Figs. 3 and 4). The compactness of the NH₂-terminal domain is largely due to the three disulfide bridges formed between Cys-6h and Cys-14h, Cys-16h and Cys-28h, and Cys-22h and Cys-39h (Fig. 3). This domain is also stabilized by 24 intramolecular hydrogen bonds of which 13 involve only main chain atoms (Fig. 2) and the remaining 11 involve side chain atoms (Table III). Finally, there are close contacts between the side chains of the hydrophobic residues Leu-13h and Pro-46h, Val-21h and Tyr-3h, Ile-29h and Val-40h, Leu-30h and Cys-6h. The ability of reduced and denatured hirudin to refold at extremely high concentrations and in the presence of a high concentration of guanidine hydrochloride (Johnson *et al.*, 1991) may result from the highly compact nature of the hirudin core and its stabilization by numerous intramolecular contacts. Table IV summarizes the secondary structural elements of hirudin. The COOH terminus of the hirudin core folds back to the NH₂ terminus with the main chain hydrogen bond from Asn-12h N to Thr-

45h O (Fig. 2), the two hydrogen bonds from Lys-47h NZ to Thr-4h OG1 and Asp-5h O (Table III), and the hydrophobic contact between Pro-46h and Leu-13h. Gln-49h through Pro-60h form two extended stretches of chain, each approximately 15 Å long, with a bend at Gly-54h. A type IV reverse turn for Glu-61h through Leu-64h (Table IV) gave the best fit for the existing density.

In solution, only residues 2h through 30h and 37h through 48h of hirudin are ordered (Folkers *et al.*, 1989; Haruyama and Wuthrich, 1989). A superposition of the $C_\alpha$ atoms of the structure determined by NMR (Folkers *et al.*, 1989) with residues 5h–30h and 37h–48h of hirudin in the crystal complex using the program ALIGN (G. Cohen, NIH; Satow *et al.*, 1986) gave a root mean square difference of 0.83 Å. When all atoms were included in the superposition, the root mean square difference was 1.39 Å. These figures are comparable with the deviations from the average NMR structure (Folkers *et al.*, 1989), suggesting that the overall structure of the NH₂-terminal domain of hirudin changes little upon association with thrombin and subsequent crystallization (Fig. 3). A few large changes of 5 Å or more relative to the NMR structure occur in the side chains of Asp-5h, which forms salt bridges with Arg-221A; Asn-12h, which is located in the interior of hirudin; Lys-27h, which is on the surface of the complex; and Thr-41h and Glu-43h, which interact with a symmetry-related hirudin molecule. Gly-42h, which also has intermolecular contacts, exhibits no large differences from the NMR structure. Residues 31h through 36h, which were disordered in the NMR studies, are well-defined in our crystal structure due to the only other intermolecular contacts involving hirudin residues present in the structure, namely the close contacts of residues 34h, 35h, 36h, and 38h with thrombin residues belonging to a symmetry-related complex. Residues 49h–65h, which also had no fixed structure in the NMR experiments, are generally well-defined in our crystal structure as the result of numerous interactions with thrombin (see below).

*Structure of Bovine Thrombin*—The model of bovine α-thrombin in this structure includes residues 1H through 247. It is closely similar with the structures of molecules 2 and 3 of bovine α-thrombin in the complex with FPA₇₋₁₆ (Martin *et al.*, 1992). When these 2 thrombin molecules are superimposed onto the thrombin molecule in the hirudin complex with the program ALIGN, the rms difference between corresponding $C_\alpha$ atoms is 0.55 Å and 0.56 Å, respectively.

Residues 60A through 60D (the YPPW loop) occupy similar positions in the hirudin and FPA₇₋₁₆ complexes of bovine α-thrombin. The movement of this loop in the fibrinopeptide complex relative to its position in human PPACK-thrombin has been attributed to the presence of the fibrinopeptide in the active site (Martin *et al.*, 1992). In this connection, it may be noted that Val-1h and Leu-13h of hirudin occupy similar positions with Val-15f and Leu-9f of the fibrinopeptide (Fig. 5) interacting, as will be discussed later, with the residues of the YPPW loop (Fig. 6).

Twenty thrombin residues are identified by ALIGN as having significantly different $C_\alpha$ positions in the hirudin complex as opposed to the FPA₇₋₁₆ complex of bovine α-thrombin. These residues include those that are not well defined either in the present structure or in the fibrinopeptide structure, or in both, such as Thr-147 through Ala-149D of the autolysis loop, the termini of the A-chain, and the COOH terminus of the B-chain. Glu-149E of the autolysis loop interacts with a symmetry-related molecule in crystals of the hirudin complex and is displaced by 2.5 and 2.4 Å relative to molecules 2 and 3, respectively, of FPA₇₋₁₆ thrombin. Also, small displacements ranging from 1.3 to 1.9 Å are observed
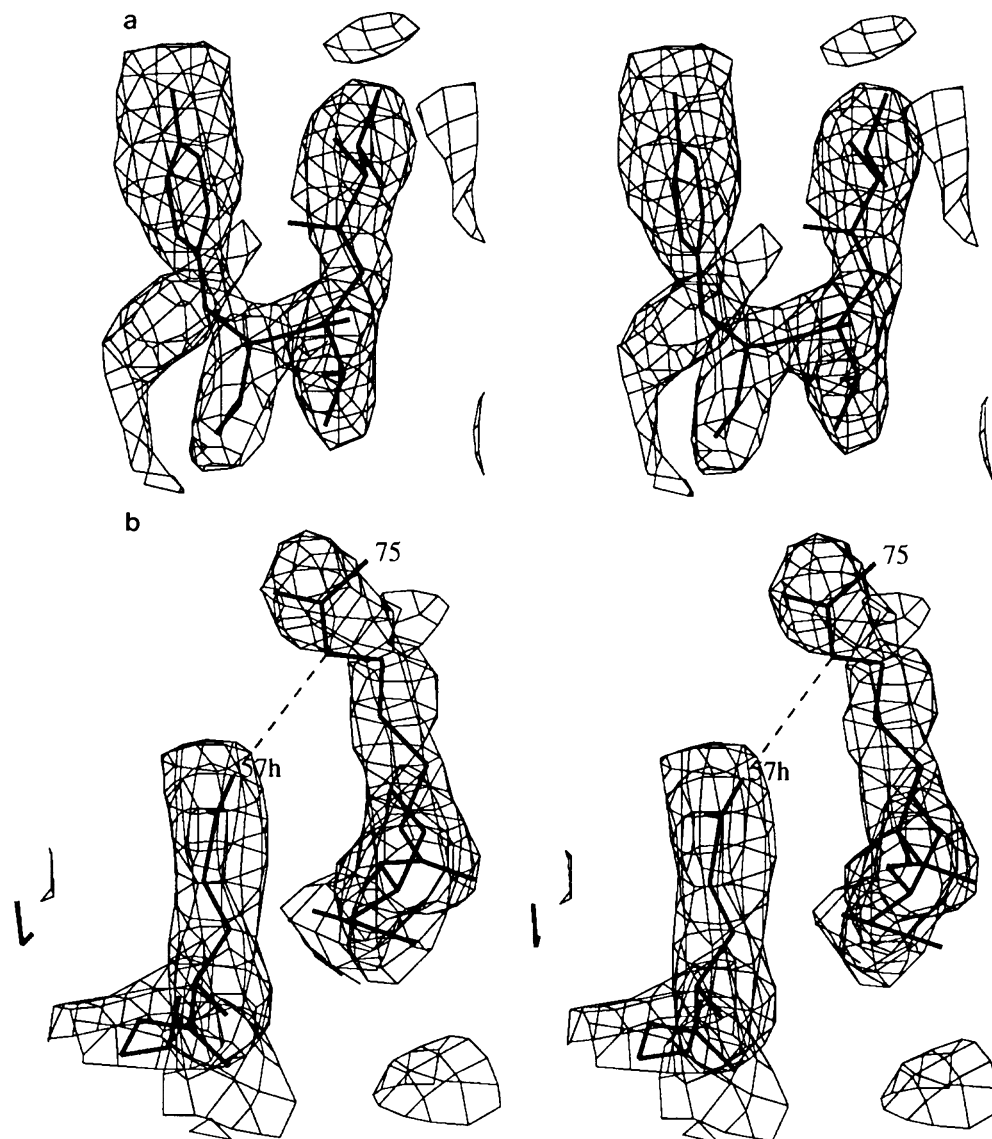
FIG. 1. **Stereo views of the** $2|F_o| - |F_c|$ **electron density map for residues 1h through 3h of hirudin, contoured at** $1.4\sigma$ **(a), and residues 57h of hirudin and 75 of thrombin, contoured at** $1.2\sigma$ **(b).** The salt bridge Arg-75 NE. . .Glu-57h OE1 is illustrated in *b*. Any unaccounted density in these figures corresponds to residues that have been omitted for clarity.

for Gly-186A and Ser-36A through Gln-38. These residues are on the surface of thrombin but do not interact with symmetry-related molecules, although Gln-38 interacts with Phe-56h in its own complex (Fig. 7).

*Interactions between Hirudin and Thrombin*—The earlier crystallographic studies (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990) showed that the mode of binding of hirudin to thrombin is unique among other inhibitors of serine proteases. Most inhibitors interact with their target enzymes, mainly in the region of the active site. In contrast, hirudin interacts with thrombin over an extended area, both within and far from the active site. Of the 65 residues of hirudin, 27 actually contact thrombin. The total solvent-accessible surface area buried in the interaction between thrombin and hirudin is 3775 Å$^2$ or 2.6 times the surface area buried in the interaction between trypsin and the 14 residues of bovine pancreatic trypsin inhibitor that actually contact trypsin (1452 Å$^2$; Ruehlmann *et al.*, 1973; Janin and Chothia, 1976; Huber and Bode, 1978). The solvent-accessible surface area buried in the interaction between thrombin and the 10 residues of FPA$_{7-16}$

is 1318 Å$^2$ (Martin *et al.*, 1992).

The NH$_2$-terminal 3 residues of hirudin, Val-1h, Val-2h, and Tyr-3h, bind in the active site to Ser-214 through Gly-219 (Fig. 6) as a parallel $\beta$-structure that contains 4 hydrogen bonds, namely Val-1h N→Ser-214 O (2.79 Å), Gly-216 N→ Val-1h O (2.75 Å), Tyr-3h N→Gly-216 O (2.69 Å), and Gly-219 N→Tyr-3h O (3.04 Å). As noted in previous studies (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990), this interaction is quite novel in that other serine protease inhibitor complexes (Huber and Bode, 1978; Read and James, 1986; Bode *et al.*, 1989) and the fibrinopeptide complex (Martin *et al.*, 1992) have an antiparallel $\beta$ structure. The side chains of Val-1h and Tyr-3h project into the "apolar binding site" (Berliner and Shen, 1977; Bing *et al.*, 1981; Sonder and Fenton, 1984) where Val-1h makes close contacts with Trp-60D and Tyr-60A, and Tyr-3h makes close contacts with Trp-215. The hydrophobic nature of the region about Trp-215 explains the increase in $K_i$ that accompanies the hydroxyl-group ionization of Tyr-3h upon nitration (Winant *et al.*, 1991) and the decrease in $K_i$ upon substitution of Tyr-3h with phenylalanine or tryptophan (Lazar *et al.*, 1991).

The interactions of the NH₂-terminal tripeptide of hirudin with thrombin are similar in the present structure and in human thrombin-hirudin (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990). However, in the human complexes, the amino-terminal group of hirudin forms two hydrogen bonds to thrombin. One hydrogen bond is to the carbonyl oxygen of Ser-214, as in the present structure. The second is to the catalytic Ser-195 in the structure done at pH 4.5 (Rydel *et al.*, 1991) and to the catalytic His-57 in the structure done at neutral pH (Grütter *et al.*, 1990). The latter hydrogen bond is not possible in the bovine thrombin-hirudin crystals which were grown at pH 4.7. At this pH, His-57 is most likely protonated and cannot function as a hydrogen bond acceptor. However, we also do not observe a hydrogen bond to Ser-195 in the present structure. This observation suggests that a second hydrogen bond for the amino-terminal group is not essential for the binding of hirudin to thrombin.

Beyond Tyr-3h, the interactions of the NH₂-terminal do-



FIG. 2. **Amino acid sequence and main chain hydrogen bonds of hirudin.** *Arcs* represent reverse turns.

main of hirudin with thrombin involve three salt bridges, three hydrogen bonds, and two hydrophobic pairs. The salt bridges are Arg-173 NH1...Glu-17h OE2 (3.52 Å), Arg-221A NH1...Asp-5h OD1 (2.93 Å), and Arg-221A NH2...Asp-5h OD1 (3.08Å). The three hydrogen bonds are Lys-224 NZ→ Ser-19h O (3.33 Å), Lys-224 NZ→Asn-20h OD1 (3.38 Å), and Val-21h N→Glu-217 OE2 (3.11 Å). The two hydrophobic interactions involve Leu-13h with Pro-60C and Val-21h with Ile-174 (Fig. 6).

In contrast to the present structure, Asp-5h and Arg-221A in human thrombin-hirudin (Rydel *et al.*, 1991) interact with only one salt bridge contact and Lys-224 NZ is hydrogen bonded only to Ser-19h OG. Since these residues are on the surface of the complex and do not interact with symmetry-related molecules, it is possible that there is some flexibility in the binding of residues 4h through 48h of the hirudin core to thrombin.

Modifications within the first 5 amino-terminal residues of hirudin can reduce the affinity with thrombin by 2 to 6 orders of magnitude (Lazar *et al.*, 1991). These changes affect direct interactions between hirudin and the active site and the apolar binding site of thrombin. However, the large reduction in affinity suggests that these changes also disrupt other stabilizing interactions (Lazar *et al.*, 1991), such as the salt link between Asp-5h and Arg-221A and the two hydrogen bonds from Lys-47h to Thr-4h and Asp-5h in the present structure (Table III).

It is interesting that the NH₂-terminal domain of hirudin interacts with most of the residues which define the 9 subsites that bind fibrinogen to thrombin. Thus, Trp-60D that contacts Val-1h is part of S2, Gly-216 that interacts as a parallel β-strand with Val-1h and Tyr-3h corresponds to S3, Arg-173 that makes a salt bridge with Glu-17h corresponds to S6, Pro-60C that interacts with Leu-13h is part of S8 but was inadvertently omitted by Martin *et al.* (1992), Tyr-60A that also contacts Val-1h is part of S8 and S9, and Ile-174 and Trp-215, which interact with Val-21h and Tyr-3h, respectively, are part of S9. The S1 subsite, which is defined by the 6 thrombin residues that interact with Arg-16f, is the major exception in that only Ser-214 also interacts with hirudin. Subsites S4, S5, and S7 do not exist in that no thrombin residues interact with the corresponding residues in FPA₇₋₁₆.

Martin *et al.* (1992) discuss the interactions of fibrinogen with the apolar binding site of thrombin in terms of a hydrophobic cage about Phe-8f that is formed by thrombin residues Tyr-60A, Pro-60C, Trp-60D, Leu-99, Ile-174, and Trp-215 and fibrinogen residues Leu-9f, Gly 13f, and Val-15f. When the structures of the hirudin and FPA₇₋₁₆ complexes with
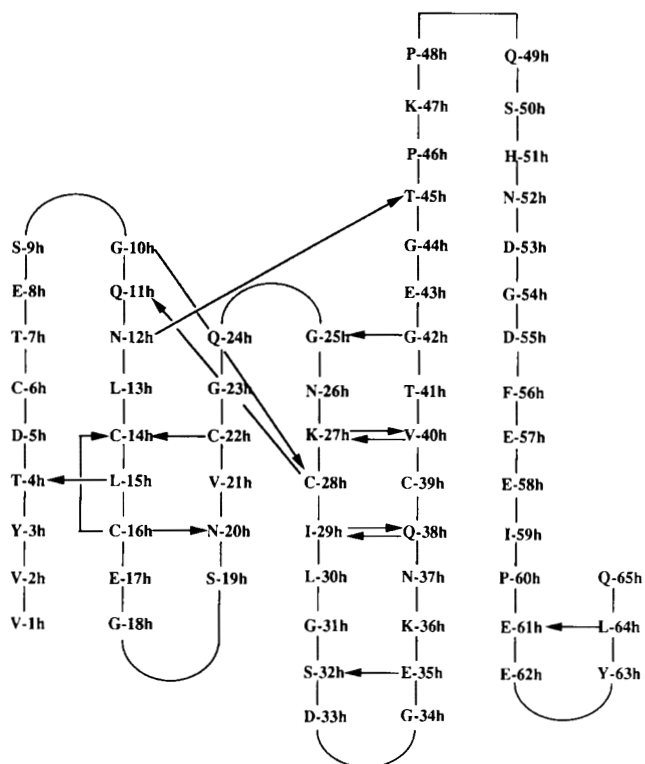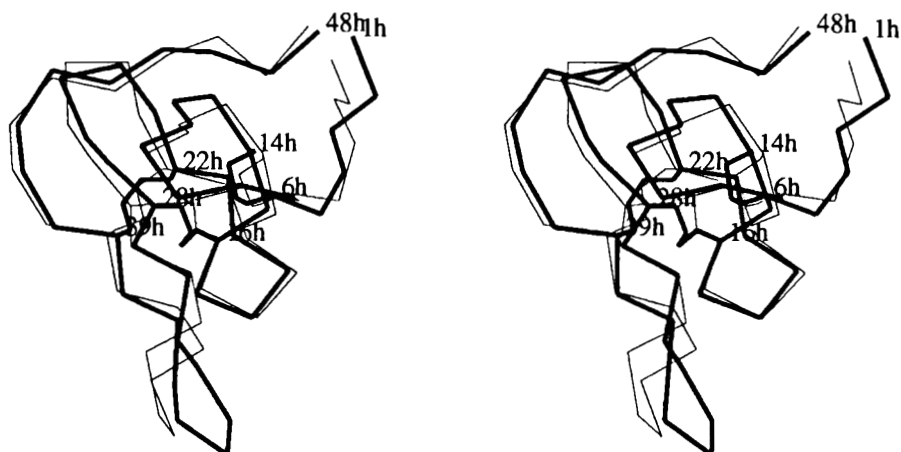
FIG. 3. **A stereo view of the Cα structure of the NH₂-terminal domain of hirudin in the crystal (*thick lines*) and in solution (*thin lines*).** The three disulfide bridges of this domain are also shown. The solution structure has been superimposed on the crystal structure on the basis of the Cα coordinates of residues 5h through 30h and 37h through 48h with the program ALIGN.

FIG. 4. **A stereo view of the Cα structure of the hirudin-thrombin complex.** *Thick lines* represent hirudin, *thin lines* represent thrombin.
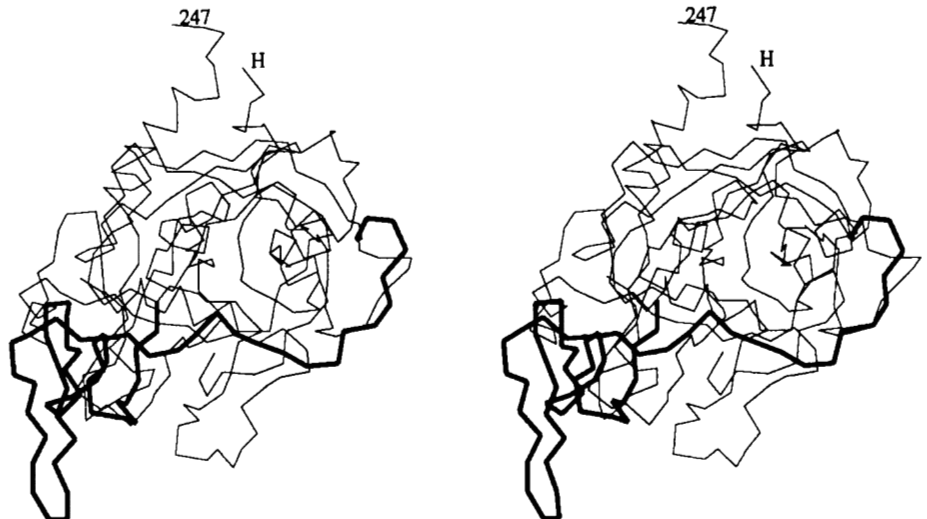
TABLE III

*Hydrogen bonds involving side chain atoms in the $NH_2$-terminal domain of hirudin*

| Donor | Acceptor | Distance |
|---|---|---|
| | | Å |
| Thr-7h N | Gln-11h OE1 | 3.06 |
| Gln-11h NE2 | Thr-7h OG1 | 3.20 |
| Asp-12h ND2 | Gly-23h O | 3.21 |
| Asp-12h ND2 | Asn-26h O | 2.87 |
| Asn-20h ND2 | Glu-17h O | 3.14 |
| Gln-24h NE2 | Asp-12h OD1 | 3.15 |
| Lys-27h NZ | Thr-41h O | 3.03 |
| Gly-31h N | Asn-37h OD1 | 3.40 |
| Cys-39h N | Glu-17h OE1 | 3.47 |
| Lys-47h NZ | Thr-4h OG1 | 3.09 |
| Lys-47h NZ | Asp-5h O | 2.62 |

TABLE IV

*Secondary structural elements of hirudin*

| Residues | Secondary structure |
|---|---|
| Thr-4h through Cys-6h; Cys-14h through Cys-16h | Parallel β ladder[a,b] |
| Cys-14h through Cys-16h; Asn-20h through Cys-22h | Anti-parallel β ladder[b] |
| Asn-26h through Leu-30h; Asn-37h through Thr-41h | Anti-parallel β ladder |
| Thr-45h through Pro-48h | Polyproline II helix |
| Glu-8h through Gln-11h | II reverse turn[c,d] |
| Glu-17h through Asn-20h | II' reverse turn[c,d] |
| Gly-23h through Asn-26h | II reverse turn[c,d] |
| Ser-32h through Glu-35h | II reverse turn[c,e] |
| Glu-61h through Leu-64h | IV reverse turn[c,e] |

[a] The Cys-6h N→Leu-15h O hydrogen bond of 3.63 Å is slightly longer than our cutoff of 3.5 Å but it has been included as part of the ladder.

[b] Thr-4h through Cys-6h, Cys-14h through Cys-16h, and Asn-20h through Cys-22h form an approximate, mixed β pleated sheet.

[c] Chou and Fasman, 1977.

[d] Reverse turn without a hydrogen bond.

[e] Reverse turn with a hydrogen bond.

thrombin are superimposed with ALIGN (Fig. 5), Leu-13h and Val-1h occupy the same positions relative to thrombin as Leu-9f and Val-15f of the fibrinopeptide. Further, Tyr-3h is close to Phe-8f and intersects the peptide bond between Gly-13f and Gly-14f. Leu-13h and Val-1h replace Leu-9f and Val-15f in the cluster, and Tyr-3h replaces Phe-8f and Gly-13f. The hydrophobic cluster in the hirudin structure contains an additional residue, Val-21h (Fig. 6), which occupies essentially

the same position as Gly-12f of the fibrinopeptide (Fig. 5) but has a bulky side chain that can interact with Ile-174.

The COOH-terminal peptide of hirudin binds on the surface of thrombin (Fig. 4) between the two loops formed by Phe-34 through Leu-41 and Lys-70 through Glu-80. This region is rich in positively charged side chains and has been considered in the earlier crystallographic studies (Rydel *et al.*, 1990, 1991; Grütter *et al.*, 1990) to be the secondary binding site for fibrinogen. In the first segment of extended chain, Asn-52h OD1 forms a hydrogen bond with Asn-143 ND2 (3.34 Å) and Asp-53h OD2 forms a salt bridge with Arg-73 NH2 (2.61 Å). In the second segment, Asp-55h makes two salt bridge contacts with Arg-73, namely Arg-73 NH2...Asp-55h OD2 (4.93 Å) and Arg-73 NH1...Asp-55h OD1 (4.94 Å). The N atom of Phe-56h makes a hydrogen bond with Gln-38 OE1 (3.12 Å). Its phenyl side chain is sandwiched between the ring of Phe-34 and the CB-CG2 bond of Thr-74, making close contacts with Phe-34 and Leu-40. The stacking of the two phenylalanine rings has the characteristic edge-on pattern (Burley and Petsko, 1985). Glu-57h is engaged in two hydrogen bond contacts, Glu-57h N→Thr-74 O (3.74 Å) and Tyr-76 N→Glu-57h OE1 (3.78 Å), and a salt bridge, Arg-75 NE...Glu-57h OE1 (3.46 Å) (Fig. 1). Leu-65, Ile-82, and Tyr-76 form a hydrophobic cavity on the surface of thrombin that accommodates the side chains of the hydrophobic residues at the COOH terminus of hirudin –Ile-59h, Pro-60h, Tyr-63h, and Leu-64h. Ile-59h contacts Tyr-76 and Ile-82, Pro-60h contacts Tyr-76, and Tyr-63h contacts Leu-65 and Ile-82, as well as Ile-59h and Pro-60h.

The fact that the hydroxyl group of Tyr-63h is not involved in any stabilizing interactions with thrombin explains the insignificant difference in binding energy when Tyr-63h in recombinant hirudin is replaced by phenylalanine (Betz *et al.*, 1991). Iodination or nitration of Tyr-63h reduces the pK of the hydroxyl group and increases the affinity of hirudin for human thrombin by 3–10-fold, as reported by Winant *et al.* (1991), who suggested that the negatively charged hydroxyl group on the modified tyrosine ring effectively mimics the acidic sulfatotyrosine residue in leech hirudin. In the present structure, the hydroxyl group of Tyr-63h is located 4.95 Å and 4.72 Å, respectively, from the hydroxyl group of Tyr-76 and the amide nitrogen of Ile-82 that make hydrogen bonds with sulfate oxygens on Tyr-63h in the complex of sulfated hirugen with human α-thrombin (Skrzypczak-Jankun *et al.*, 1991). Single bond rotations cannot bring these groups within hydrogen bonding distance without creating steric conflicts with

FIG. 5. A stereo view of the residues of hirudin (*thick lines*) that are near residues of fibrinopeptide A (*thin lines*) bound to thrombin.
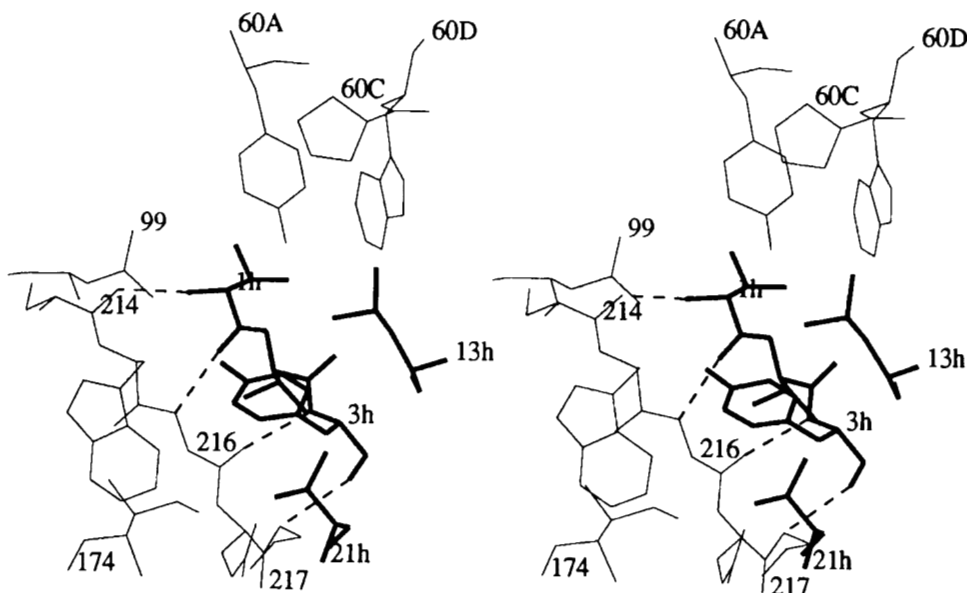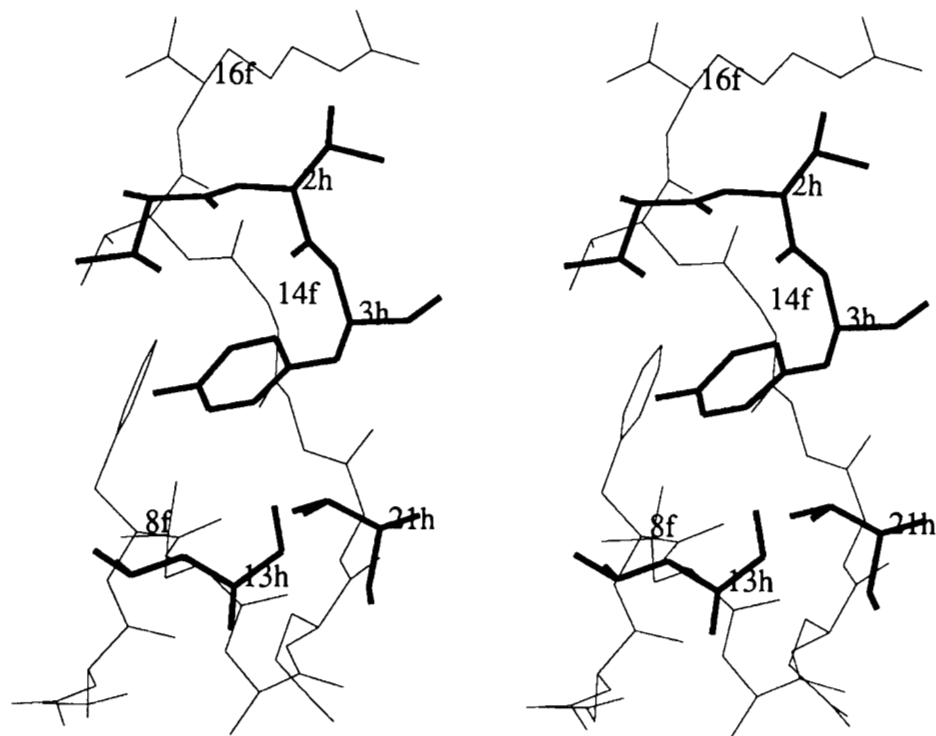


FIG. 6. **A stereo view of the interactions of the NH$_2$-terminal tripeptide of hirudin with thrombin.** Residues 13h and 21h of hirudin that interact with the apolar binding site of thrombin are also shown. *Thick lines* represent hirudin, *thin lines* represent thrombin, and *dashed lines* represent hydrogen bonds.

nearby atoms. However, in the present structure, the amino group of Lys-81 can be brought within 3.0 Å from the hydroxyl group of Tyr-63h by single bond rotations, and it is possible that these two groups form a favorable ion pair in the nitrated or iodinated structure. This proposal is supported by an alternative interpretation of the electron density of the sulfated hirugen structure (Skrzypczak-Jankun *et al.*, 1991) that places the side chain of Lys-81 in the density attributed to two waters involved in a hydrogen bonding network with the third sulfate oxygen.

There are several differences in the polar interactions of the COOH-terminal polypeptide of hirudin with thrombin between the present structure and that of human thrombin-hirudin (Rydel *et al.*, 1991). First, the carboxyl atoms of Asp-

55h are approximately 2 Å closer to the guanidinium group of Arg-73 in the human structure. Second, the hydrogen bond Phe-56h N→Gln-38 OE1 is not present in the complex with the human enzyme. Third, Glu-57h forms a salt bridge with Arg-75' of a symmetry-related complex in human thrombin-hirudin crystals, although Glu-57h is also close to the Arg-75 residue in its own complex and a salt bridge was predicted to occur in solution. In the present crystal structure, Glu-57h is not near any symmetry-related molecules and forms the predicted intramolecular salt bridge with Arg-75. Fourth, Glu-58h forms an ion pair with Arg-77A in human thrombin-hirudin whereas it does not interact with any thrombin residue in the present structure. With the exception of Glu-57h in human thrombin-hirudin, these residues are on the surface
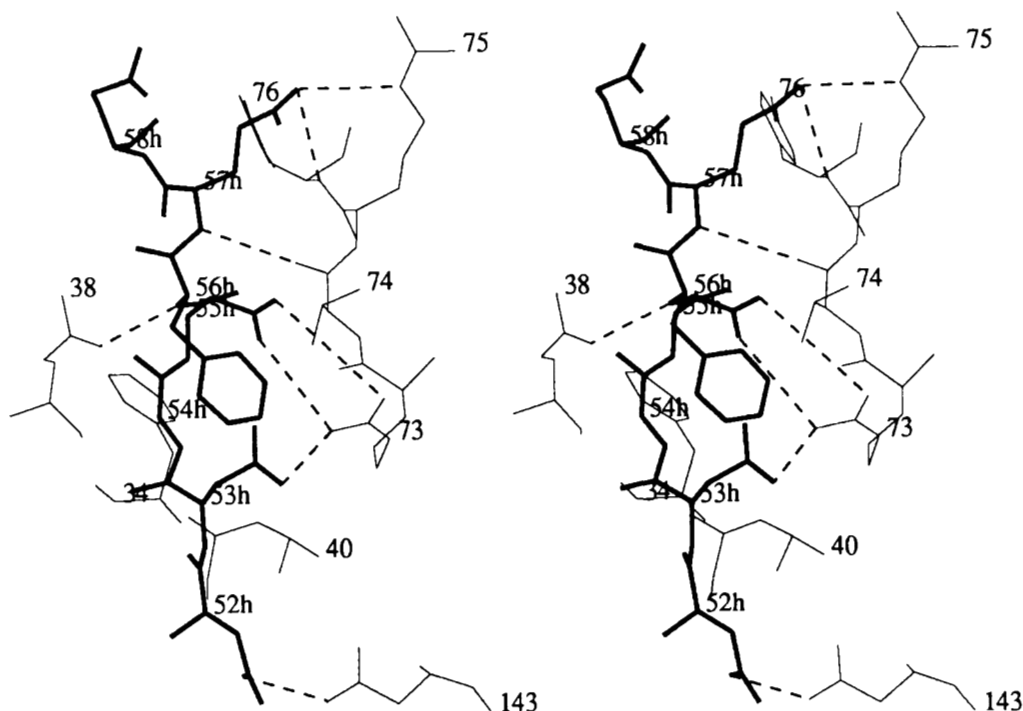
FIG. 7. **A stereo view of the interactions of residues 52h through 58h of hirudin with thrombin.** *Thick lines* represent hirudin, *thin lines* represent thrombin, and *dashed lines* represent hydrogen bonds.

of the complex and do not interact with symmetry-related molecules.

Hirudin binds more tightly to human as compared to bovine thrombin (Dodt *et al.*, 1988, 1990), and hirudin-related carboxyl-terminal fragments are consistently more effective inhibitors of human α-thrombin by almost 1 order of magnitude (DiMaio *et al.*, 1990; Maraganore *et al.*, 1989). Consequently, Dodt *et al.* (1990) speculated that the observed species differences in the complexes of hirudin with human and bovine α-thrombins are exclusively due to differences in the interaction with the carboxyl-terminal hirudin region. However, we cannot attribute the differences that we observe in the binding of the COOH-terminal peptide of hirudin to bovine thrombin to any differences in structure or sequence from human thrombin. The 2 thrombin molecules are closely similar in structure (Martin *et al.*, 1992), and the residues involved in the interactions with the COOH-terminal peptide of hirudin are common in the human and bovine complexes. It is more likely that these differences indicate some flexibility in the binding of the COOH-terminal polypeptide of hirudin to thrombin. Additional support for this conclusion is provided by the structure of human α-thrombin complexed with sulfated hirugen (Skrzypczak-Jankun *et al.*, 1991), which exhibits several differences in the interactions of the COOH-terminal peptide with thrombin from both human and bovine thrombin-hirudin complexes. In particular, only one of the NH groups of Arg-73 interacts with the carboxylate oxygens of Asp-55h and, as with human thrombin-hirudin, the distances are approximately 2 Å closer than in the present structure. The salt bridge interaction of Glu-57h with Arg-75 is intermolecular in this structure, as in human thrombin-hirudin (Rydel *et al.*, 1991), while Glu-58h appears not to interact with thrombin, as in the present structure. Gln-38 interacts with Glu-57h in the hirugen structure, with Phe-56h in our structure and with no residue in human thrombin-hirudin. In human thrombin-hirudin (Rydel *et al.*, 1991), Lys-149E makes a salt bridge contact with Asp-55h. In the bovine enzyme,

Lys-149E is replaced by an acidic glutamate that cannot interact with Asp-55h. This is the only difference between the two crystal structures that we can with certainty attribute to the species variation of the thrombin enzyme.

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