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# Crystal structure of the thrombin – hirudin complex: a novel mode of serine protease inhibition

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**Thrombin is a serine protease that plays a central role in blood coagulation. It is inibited by hirudin, a polypeptide of 65 amino acids, through the formation of a tight, noncovalent complex. Tetragonal crystals of the complex formed between human α-thrombin and recombinant hirudin (variant 1) have been grown and the crystal structure of this complex has been determined to a resolution of 2.95 Å. This structure shows that hirudin inhibits thrombin by a previously unobserved mechanism. In contrast to other inhibitors of serine proteases, the specificity of hirudin is not due to interaction with the primary specificity pocket of thrombin, but rather through binding at sites both close to and distant from the active site. The carboxyl tail of hirudin (residues 48–65) wraps around thrombin along the putative fibrinogen secondary binding site. This long groove extends from the active site cleft and is flanked by the thrombin loops 35–39 and 70–80. Hirudin makes a number of ionic and hydrophobic interactions with thrombin in this area. Furthermore hirudin binds with its N-terminal three residues Val, Val, Tyr to the thrombin active site cleft. Val1 occupies the position P2 and Tyr3 approximately the position P3 of the synthetic inhibitor D-Phe-Pro-ArgCH$_2$Cl. Thus the hirudin polypeptide chain runs in a direction opposite to that expected for fibrinogen and that observed for the substrate-like inhibitor D-Phe-Pro-ArgCH$_2$Cl.**

*Key words:* blood clotting/hirudin/thrombin/X-ray crystallography

## Introduction

Saliva of leeches contains a number of substances that prevent the clotting of ingested blood in the digestive tract of these animals (Sawyer, 1986). One of the compounds involved in this process, hirudin, is an efficient protease inhibitor that is entirely specific for thrombin (Walsmann and Markwardt, 1981). Hirudin consists of a polypeptide chain of 65 or 66 residues with three disulphide bridges (Bagdy *et al.*, 1976; Dodt *et al.*, 1984, 1985); its three-dimensional structure has recently been determined by two-dimensional NMR techniques (Clore *et al.*, 1987; Folkers *et al.*, 1989; Haruyama and Wuthrich, 1989).

The serine protease thrombin plays a central role in the blood coagulation process. It activates platelets as well as the blood coagulation factors V, VIII and XIII that are essential for the formation of the blood clot. Moreover, polymerization of fibrin monomers that arise from limited proteolysis of fibrinogen by thrombin form the basis of this clot (Jackson and Nemerson, 1980).

Hirudin inhibits thrombin by forming a tight equimolar complex ($K_d = 10^{-14}$ M). Results obtained from a range of biochemical studies have suggested that the thrombin–hirudin complex differs from the previously studied complexes of other serine proteases and their inhibitors (Read and James, 1986; Stone *et al.*, 1987; Braun *et al.*, 1988; Wallace *et al.*, 1989; Dodt *et al.*, 1990). It was thought that the unique specificity of hirudin for thrombin and the tightness of binding resulted from interactions both at the active site of the enzyme and at secondary binding sites distant from the active site (see Stone *et al.*, 1990; Chang 1989). Also for hirudin, evidence has been obtained to indicate that the interacting surface is not restricted to a single loop as in the case of other inhibitors of serine proteases (Read and James, 1986; Stone *et al.*, 1990). Thrombin has a clear specificity for arginyl residues in the P1-position (nomenclature of Schechter and Berger, 1967); therefore it was proposed that one of the basic amino acid residues in hirudin would be the reactive site residue (Fenton, 1989; Johnson *et al.*, 1989). However, protein engineering studies have shown that a basic amino acid residue does not play an essential role in complex formation (Braun *et al.*, 1988; Dodt *et al.*, 1988; Degryse *et al.*, 1989).

The unique biochemical properties of the thrombin–hirudin interaction are evident from the structure of the complex between human α-thrombin and recombinant desulphato-hirudin (variant 1) reported here. This structure reveals a mode of binding that has not been observed previously for a protease inhibitor. The unique features are: (i) extended areas of hirudin and thrombin are in close contact; (ii) the primary specificity pocket of the enzyme is not occupied by hirudin; and (iii) the three N-terminal amino acids of hirudin bind in the active site and run in a direction that is exactly opposite to that of the substrate-like inhibitor D-Phe-Pro-ArgCH$_2$Cl (Bode *et al.*, 1989).

## Results

### *Structure solution*

Four different implementations of the fast rotation function (Crowther, 1972) were used to find the relative orientations of the model of thrombin (Bode *et al.*, 1989) to the thrombin–hirudin complex in our crystal unit cell. All gave similar solutions, although with different degrees of clarity. The clearest result gave a solution that was 4.8 σ above the mean. Two implementations of the Crowther and Blow translation function (1967) were then used to position the

thrombin molecule in the crystallographic unit cell of the complex. Both systems gave extremely clear answers and were able to distinguish between the two possible enantiomorphic space groups $P4_12_12$ and $P4_32_12$ (95% above the highest noise peak for $P4_32_12$ versus 4% for $P4_12_12$). The highest peak in the packing function was near the special position $x = y = 0.5$, $z = 0$. The correct solution, as suggested by the translation function, while high, was not the highest, non-special peak. Examination of the packing of the thrombin molecule in the $P4_32_12$ unit cell on a graphic display unit showed only one region where symmetry related neighbours were closer than their van der Waals radii should allow. More importantly, there was also room for the hirudin molecule near the active site of thrombin.

Using phases calculated from the thrombin molecule properly oriented and positioned in our unit cell, a 2Fo-Fc Sim-weighted (Sim, 1959) difference electron density map showed strong features in the 'solvent' region near the active site of thrombin where hirudin presumably binds. Initially, it was not possible to correlate the electron density with the sequence, although the folding of the core was known from two-dimensional NMR (Clore *et al.*, 1987; Haruyama and Wüthrich, 1989). Solvent flattening (Wang, 1985), flattening out the thrombin density as well as solvent and using only the volume of hirudin was tried, to add some information about the hirudin into the calculated phases. Various maps using different coefficients and ways of combining the solvent flattened phases with the thrombin derived phases were tried. The map with coefficients Fo-0.8Fc and the solvent flattened phases alone had features which were recognizable as $\beta$-structure. These features allowed us to locate the hirudin core and to place the disulphide bonds in a region where the highest electron density peaks were located. Using these as guides, the whole core of hirudin could be built reasonably well into the electron density. Partial refinement of this still incomplete model and examination of 2Fo-Fc difference maps led to the location of the amino terminal residues (1–4) and the long flexible carboxyl tail of hirudin (residues 46–65).

### *Interactions between thrombin and hirudin*

The structure of hirudin between residues 5 and 45 is very similar to the structure of this part of hirudin determined by two-dimensional NMR (Clore *et al.*, 1987; Haruyama

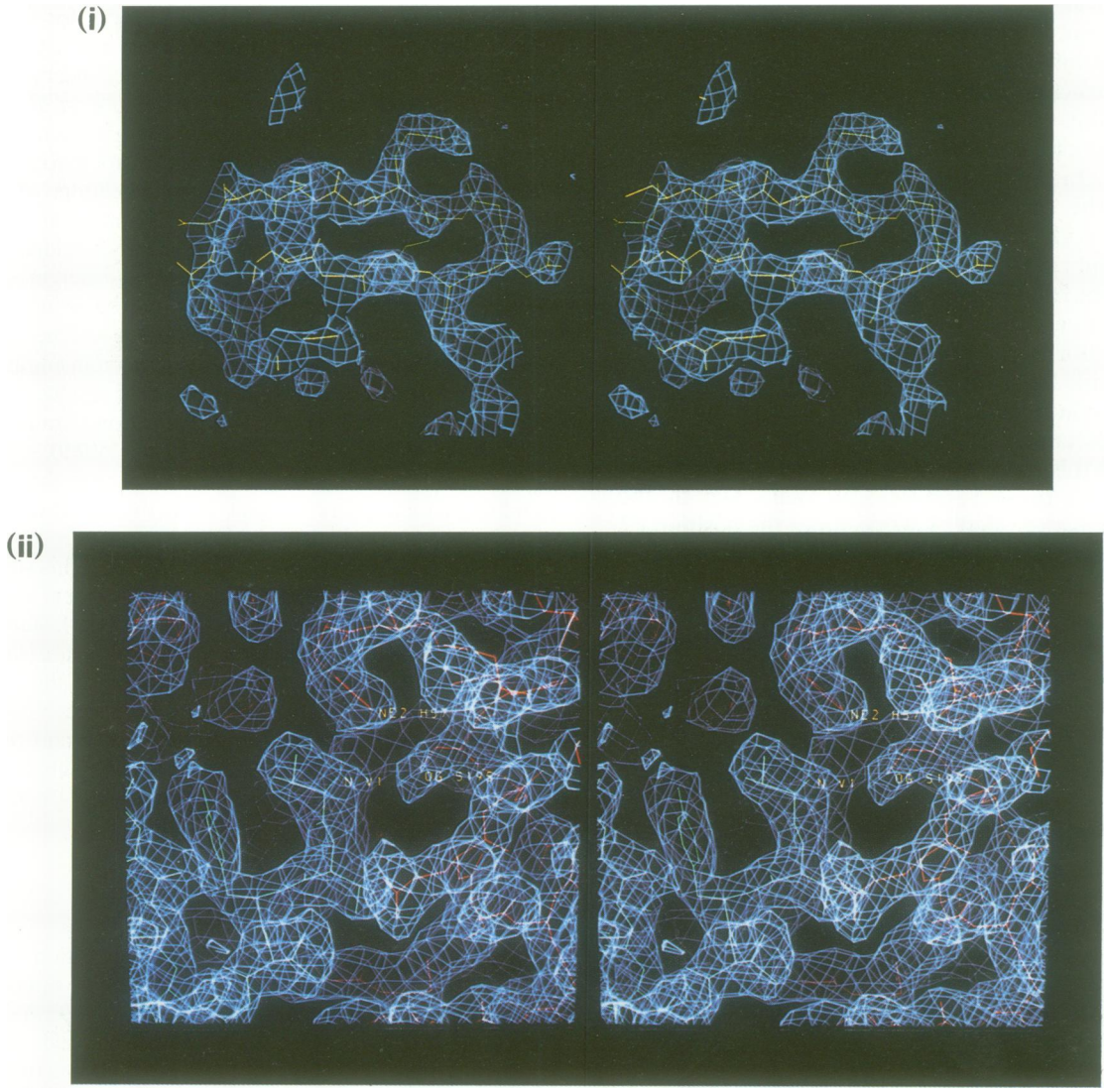


**Fig. 1.** Stereo views of difference electron density maps for (i) two antiparallel $\beta$-strands of hirudin (residues 28–39, 2Fo-Fc coefficients) and (ii) N-terminal residues of hirudin (light green) in the active site of thrombin (red, 3Fo-2Fc coefficients). The catalytic residues Ser195 and His57 of thrombin and the amino terminus of hirudin Val1 are labelled.

and Wüthrich, 1989). The three disulphide bonds of hirudin (6–14, 16–28 and 22–39) are located within this region. The loop region comprising residues 30–37 of hirudin, which is flexible in the NMR structure, has a defined position in the crystal structure owing to crystal contacts (Figure 1i). This loop is not in direct contact with thrombin. A defined structure for the first three amino acids of hirudin together with the C-terminal 18 amino acids could not be determined by two-dimensional NMR studies. In the crystal structure, however, these regions exhibit a well-defined structure owing to the contacts that they make with thrombin (Figure 2).

The structure presented in Figure 2 illustrates the uniqueness of the thrombin–hirudin interaction. Crystallographic and other studies have indicated that other inhibitors of serine proteases make contacts with their target enzymes mainly in the region of the active site (Read and James, 1986). In contrast, hirudin interacts with thrombin over an extended area and in regions that are far removed from the active site. Thus, hirudin makes a larger number of close contacts with thrombin than other protease inhibitors make with their target enzymes; whereas the area of contact of bovine pancreatic trypsin inhibitor with trypsin is 475 Å$^2$, the area for hirudin with thrombin is ~1400 Å$^2$. This large area of contact between hirudin and thrombin provides opportunity for many interactions between the molecules and is probably one of the factors leading to the observed tightness of the complex.

The interaction of hirudin with the active site of thrombin represents a completely novel mode of inhibition. The active site of thrombin is occupied by the N-terminal three residues of hirudin. The polypeptide chain runs in the opposite direction to that expected for a substrate, with the residues Val1 and Tyr3 occupying roughly the binding sites S2 and S3 (compare the orientation of the substrate analogue D-Phe-Pro-Arg$CH_2$Cl and hirudin in Figure 3). The N-terminal peptide chain forms a short parallel $\beta$-pleated sheet with the thrombin segment Gly216 to Gly218 (numbering according to Bode *et al.*, 1989). The side chain of Val2 is located at the position occupied by the side chain Glu192 in the D-Phe-Pro-Arg$CH_2$–thrombin structure; the latter side chain

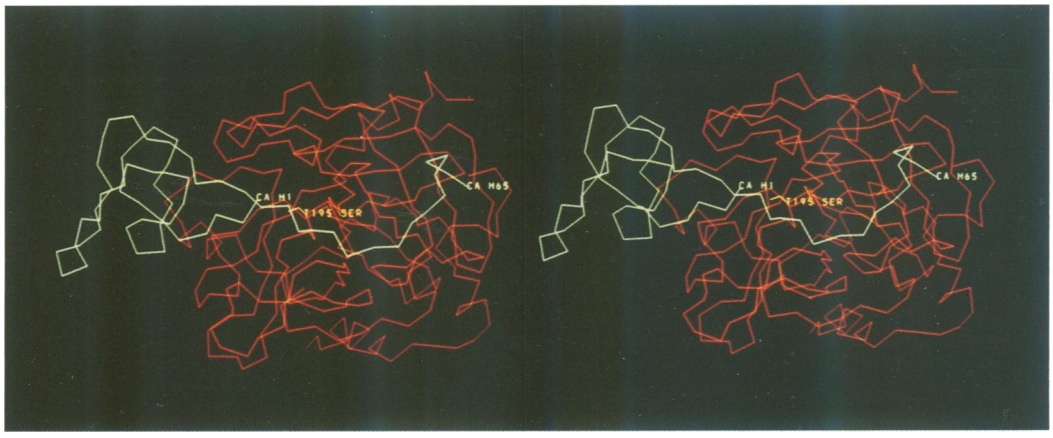


**Fig. 2.** A stereo view of Cα-atoms of the hirudin–thrombin complex viewed with the thrombin (red) active site cleft to the left, harbouring the N-terminal residues of hirudin (green). Spreading across the thrombin molecule from the active site cleft to the right is the secondary binding site, to which the C-terminal tail with residues 55–65 of hirudin binds. The labels H1 and H65 denote the amino and carboxyl-termini of hirudin, respectively. The side chain of the active serine of thrombin is shown in yellow and labelled T195.

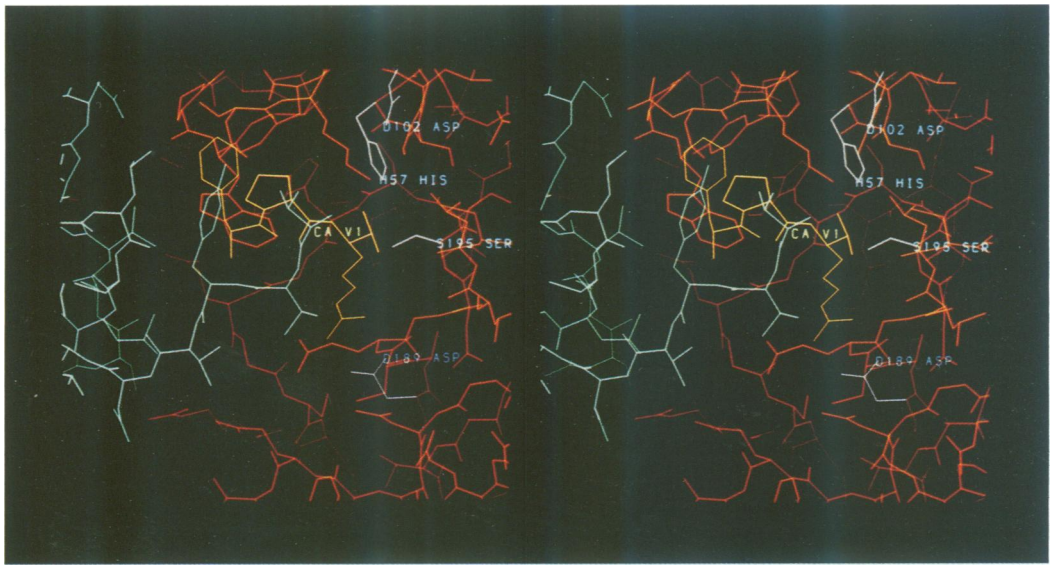


**Fig. 3.** A stereo view of the active site cleft of thrombin (red) when hirudin (light green) is bound. The catalytic triad of thrombin (Ser195, His57 and Asp102) and the specificity pocket Asp189 are labelled, as is the amino-terminal Val1 of hirudin. The tripeptide D-Phe-Pro-Arg$CH_2$Cl (yellow) from the structure of Bode *et al.* (1989) is also included for comparison. The specificity pocket occupied by the arginine side chain of the tripeptide is unoccupied in the hirudin–thrombin complex.

consequently swings away from the active site (Figure 3). The primary specificity pocket (S1) of thrombin is not occupied. The specificity of other inhibitors of serine proteases is determined by interactions with the primary specificity pockets of their target enzymes. The residue of the inhibitor that binds in this pocket has been termed the reactive-site residue (Carrel and Travis, 1985) and changes in this residue, either by site-directed mutagenesis or in natural variants, can completely change the specificity of the inhibitor (Carrel and Travis, 1985; Jallat *et al.*, 1986). The specificity of hirudin is not due to such interactions but to numerous other interactions both within the active site and with surface loops of thrombin away from the active site (Figure 4). This lack of a reactive-site residue in hirudin is presumably why it inhibits no serine proteases other than thrombin (Wallis, 1988).

The N-terminal amino group of hirudin forms hydrogen bonds to the active-site His57 as well as to the main chain carbonyl of Ser214. The importance of these bonds is illustrated by the fact that a decrease of 23 kJ/mol in the binding energy of hirudin is observed upon acetylation of this group (Wallace *et al.*, 1989). The N-terminal three residues of hirudin are bound in a hydrophobic pocket formed by the residue side chains of Trp215, Leu99, His57, Tyr60A and closed partly by Tyr3 of hirudin. Val1 and Tyr3 of hirudin make numerous hydrophobic contacts within this pocket and the considerable contribution of these contacts to the binding energy has been indicated by site-directed mutagenesis. For instance, substitution of the two N-terminal valyl residues of hirudin by more polar seryl residues leads to a 19 kJ/mol decrease in binding energy (Wallace *et al.*, 1989).

To allow the peptide chain of hirudin to leave the active site, the 145–150 loop of thrombin assumes a different conformation to that observed in D-Phe-Pro-Arg$CH_2$–thrombin structure. The main chain of this loop moves to open the active site to allow binding of hirudin (Figure 2). The core region of hirudin closes off the active site of thrombin and the segment comprising residues 45–50 runs along the outside of the Leu59–Phe60H loop. Only weak density is seen for residues 50 and 51 which link the core of hirudin with the C-terminal residues 52–65. The C-terminal segment (residues 55–65) lies in a long groove on the surface of thrombin extending from the active site cleft (Figure 2). The groove is flanked by the thrombin surface loops 70–80 on one side and 35–39 on the other side. A number of possible electrostatic interactions between basic residues in this groove (Arg73, Arg75, Arg77A, Lys81) and the acidic C-terminal residues of hirudin (Asp55, Glu57, Glu61, Glu62) are observed. The side chains of Phe56 and Ile59 make many hydrophobic contacts in this groove. In our current model the proximity of Tyr63 of hirudin to the side chain of Tyr76 of thrombin could explain the greater affinity of thrombin for native hirudin (Braun *et al.*, 1988), which has a negatively charged sulphato-tyrosine at position 63. The importance of negatively charged residues in the C-terminal region of hirudin has been demonstrated by protein engineering studies in which the charge properties of these residues were altered (Braun *et al.*, 1988; Stone *et al.*, 1989; Hofsteenge *et al.*, 1990). Other studies using partially proteolysed forms of thrombin and peptide specific antibodies have indicated that the complementary positively charged region on thrombin is

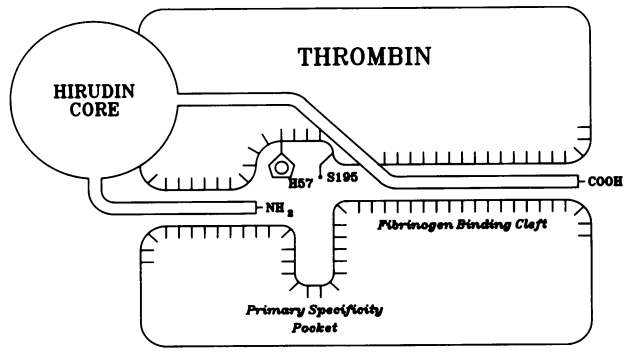


**Fig. 4.** A schematic diagram of the hirudin–thrombin complex. Hirudin binds with its N-terminal three residues to the thrombin active site cleft in a direction opposite to that expected for fibrinogen. The carboxyl tail of hirudin (residues 55–65) binds along the putative fibrinogen binding cleft.

important for a tight interaction with hirudin (Stone *et al.*, 1987; Noé *et al.*, 1988). It also seems likely that the extended groove on the surface of thrombin that binds the C-terminal region of hirudin forms a secondary binding site for fibrinogen (Fenton, 1981; Bode *et al.*, 1989).

We plan to collect high resolution data using synchrotron radiation and we will use the data to refine the structure further.

## Materials and methods

### *Crystallization*

Human α-thrombin was prepared as described previously (Noé *et al.*, 1988) and concentrated in 5–8 mg/ml by the use of Centricon 10 tubes (Amicon) in 0.08 M phosphate buffer containing 0.5 M NaCl and 0.1% PEG 6000. Rec-desulphato-hirudin (variant 1) has been expressed in yeast and purified to homogeneity (Grossenbacher *et al.*, 1987; Meyhack *et al.*, 1989). Thrombin was inhibited by one mole-equivalent of hirudin using a solution of 10 mg/ml in $H_2O$. Crystallization of the complex was achieved by the hanging drop vapour diffusion technique against 3 M phosphate buffer pH 6.2–7.0 or between 20% and 30% PEG 6000 in 0.1 M phosphate buffer pH 6.5–7.5 at 4°C. Initial protein concentration in the drop was between 4 and 5 mg/ml. Often plates or needles were obtained after a few weeks whereas more rarely tetragonal bipyramids up to a size of 0.3 × 0.3 × 0.3 $mm^3$ were grown after ~2 months. The tetragonal crystals have the space group $P4_32_12$ with cell edges of 90.1 Å × 90.1 Å × 132.1 Å, contain one molecule per asymmetric unit, and diffract to a maximum of 2.5 Å resolution on a still picture on a conventional X-ray source.

### *Data collection and processing*

X-ray data were collected from one tetragonal crystal using a FAST area detector (Enraf Nonius, Delft, The Netherlands) with a swing-out angle ($\theta$) of 15° over a period of 2 days. Graphite-monochromated CuKα radiation was provided by an FR 571 X-ray generator operated at 40 kV and 70 mA with an apparent focal spot of 0.3 × 0.3 mm. The crystal was rotated about an arbitrary axis for 90°. Frames with a width of 0.1° were taken for 120 s. The crystal to detector distance was 80 mm, allowing data collection to a maximal resolution of 2.8 Å. The data were evaluated on-line using the program system MADNES (Messerschmidt and Pflugrath, 1987). The measured intensity data were processed and merged using the CCP4 program package leading to 10912 or 91% of the possible unique reflections to 2.95 Å. The merging values, defined as $\Sigma(|I-<I>|)/\Sigma I$, were 0.139 at 3.47 Å and 0.205 at 2.95 Å.

### *Structure solution*

The orientation of the thrombin component in the crystal was determined by the fast rotation function (Crowther, 1972) using data to 4.0 Å resolution and the refined model of α-thrombin (Bode *et al.*, 1989). The positioning of the α-thrombin molecule in the unit cell was achieved using the translation function of Crowther and Blow (1967) as implemented in the program TFSGEN (I.Tickle) and TRNSUM in MERLOT (Fitzgerald, 1988). The packing function program PAKFUN (Fitzgerald, 1988) was also used to examine possible translation vectors based solely on packing considerations.

Further rigid-body refinement was performed using the program CORELS (Sussman *et al.*, 1977). A 2Fo-Fc difference Fourier map using phases calculated from the thrombin molecule showed electron density in the area of the active site where hirudin should be bound, but it was not possible to fit hirudin to this electron density. Least squares refinement of the thrombin alone as well as a modified procedure of the solvent flattening method of Wang (1985) were then used to improve the phases. For solvent flattening, all electron density within van der Waals radius of thrombin was set to zero and only the volume of hirudin was considered as protein (calculated to be 6.5% of the unit cell volume, but set conservatively to 10%). Both solvent regions and the thrombin region were set to the same constant level before calculating phases from the modified map. Only a single cycle of this procedure was carried out. Various schemes were tried for combining the phases from the molecular replacement model and those from the solvent flattened map (SIM-weighted combination, straight vector addition, vector addition weighted by molecular weights, etc.) The map with the most easily recognizable features in the hirudin region was one with Fo-0.8Fc (from thrombin), $\alpha_c$ (from solvent and thrombin flattened map). In this map, electron density corresponding to $\beta$-strands in hirudin were recognizable, as well as strong density for the three disulphide bonds. Using these as guides, the core of hirudin, with residues 5–45 (Clore *et al.*, 1987; Haruyama and Wüthrich, 1989), was fitted. The resulting model was partially refined by the restrained least-squares procedure of Hendrickson and Konnert (1980) and in the resulting 2Fo-Fc difference maps the hirudin model was completed. At the hirudin thrombin interface, parts of thrombin had to be refitted. The completed model was further refined using the molecular dynamics refinement package XPLOR (Brünger *et al.*, 1987) to a current R-factor of 0.225 for 10578 reflections between 9 Å and 2.95 Å. Two rounds of refinement were performed, each consisting of Powell minimization to relieve potential bad contacts, fast Fourier transform (FFT) refinement without dynamics, a heat stage of 3000°K for 1 ps in 0.5 fs steps, followed by annealing to 300°K and further FFT refinement.

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