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Impact of Protein–Protein Contacts on the Conformation of Thrombin-bound Hirudin Studied by Comparison with the Nuclear Magnetic Resonance Solution Structure of Hirudin(1-51)

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The impact of protein–protein interactions on the conformation of the N-terminal hirudin domain consisting of residues 1 to 51 in the X-ray crystal structure of a hirudin–thrombin complex was investigated through comparisons with the nuclear magnetic resonance solution structure of hirudin(1-51). The close overall similarity observed between these two structures contrasts with the behavior of the C-terminal 17-residue polypeptide segment of hirudin, which is flexibly disordered in solution but exhibits a defined conformation in the complex with thrombin. Localized structural differences in the N-terminal domain include that residues 1 to 3 of hirudin in the crystalline complex form a hydrogen-bonding network with thrombin that is reminiscent of a parallel β-sheet. Moreover, the backbone conformation of residues 17 to 20 in the complex does not contain the characteristic hydrogen bond observed for the type II′ reverse turn in the solution structure, and the side-chains of Ser19 and Val21 have significantly different orientations in the two structures. Most of these structural changes can be related directly to thrombin–hirudin contacts, which may also be an important factor in the mechanism of hirudin action. In this context, it is of special interest that other residues that also make numerous contacts with thrombin, e.g. Thr4, Asp5 and Asn20, have identical conformations in free hirudin and in the complex.

Keywords: hirudin; thrombin; NMR; X-ray crystallography; protein–protein interactions

1. Introduction

Motivated by the biomedical interest in the regulation of thrombin activity by hirudin (Lent, 1986; Märki & Wallis, 1990), considerable effort has been focused on structure determinations of the individual components as well as hirudin–thrombin complexes. As a result, X-ray crystal structures are available for thrombin (Bode *et al.*, 1989) and two 1 : 1 hirudin–thrombin complexes (Grütter *et al.*, 1990; Rydel *et al.*, 1990, 1991). Nuclear magnetic resonance (NMR†) solution structures have been published for natural hirudin (Clore *et al.*, 1987), which contains a sulfated tyrosine residue in position 63, recombinant desulfatohirudin (Folkers *et al.*, 1989; Haruyama & Wüthrich, 1989), the mutant Lys47 → Glu of recombinant desulfatohirudin (Folkers *et al.*, 1989), and the N-terminal 51-residue domain, hirudin(1-51) (Szyperski *et al.*, 1992, accompanying paper). Comparative studies of these

† Abbreviations used: NMR, nuclear magnetic resonance; HV1, hirudin variant 1; HV2-K47, hirudin variant 2 containing Lys in position 47; hirudin(1-51), N-terminal 51-residue domain of hirudin variant HV1; r.m.s.d., root-mean-square deviation.

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different structures should be a promising avenue toward new insights into the basis of the novel inhibitor–protease interactions implicated for the hirudin–thrombin system (Dodt *et al.*, 1985). Previously, using as a reference the NMR solution structures of desulfatohirudin (Folkers *et al.*, 1989; Haruyama & Wüthrich, 1989), major differences between the structures of the polypeptide segment 49–65 in free hirudin and in the complex with thrombin were described (Grütter *et al.*, 1990; Rydel *et al.*, 1990, 1991). With a high-quality NMR structure of hirudin(1-51) (Szyperski *et al.*, 1992) as a reference, these comparative investigations have been extended to the N-terminal domain of residues 1 to 51.

2. Methods

The NMR solution structure of hirudin(1-51) is represented in the usual way (Wüthrich, 1986, 1989) by a group of 20 conformers. These were calculated from the NMR data with the program DIANA (Güntert *et al.*, 1991) and refined by energy minimization with the program AMBER (Singh *et al.*, 1986), as described in the accompanying paper (Szyperski *et al.*, 1992). For quantitative comparisons of these conformers among themselves and with other structures, we used r.m.s.d. values (McLachlan, 1979). The mean solution conformation was obtained by first superimposing the 20 energy-minimized DIANA conformers so as to minimize the r.m.s.d. for the backbone atoms N, C^{α} and C′ of residues 3 to 30 and 37 to 48, and then averaging the Cartesian co-ordinates of corresponding atoms in the 20 globally superimposed conformers. Displacements, D (Billeter *et al.*, 1989), are used to quantify the local precision of the solution structure and local differences between crystal and solution structure. Displacements are a generalization of the usual r.m.s.d. in that the set of atoms used for the superposition of the conformers, M_{sup}, differs from the set of atoms for which the r.m.s.d. of the positions is actually calculated, $M_{r.m.s.d.}$. For a given residue i, we calculated the *displacements after global superposition*, D_{glob}, where M_{sup} consists of the backbone atoms N, C^{α} and C′ of residues 3 to 30 and 37 to 48, and $M_{r.m.s.d.}$ consists either of the backbone atoms N, C^{α} and C′ of residue i, or of the side-chain heavy atoms of residue i.

Hirudin(1-51) is derived from the hirudin variant HV1 (Scharf *et al.*, 1989) and differs in the 7 positions 1, 2, 24, 33, 35, 36 and 49 from the hirudin variant HV2-K47 (Harvey *et al.*, 1986) used in the X-ray crystal structure determination of the hirudin–thrombin complex by Rydel *et al.* (1990, 1991). For the calculation of the r.m.s.d. values that involve side-chain atoms of these residues, we identified corresponding heavy atoms. For example, $O^{\gamma 1}$ of Thr was taken to correspond to $C^{\gamma 2}$ of Val. Furthermore, following the definitions of χ^1 angles (IUPAC-IUB Commission of Biochemical Nomenclature, 1970) the relation:

$$\chi^1(\text{Val}) = \chi^1(\text{Ile, Thr}) - 120^\circ \qquad (1)$$

was used when comparing the χ^1 angles of Val with those of corresponding Ile or Thr residues.

3. Results

(a) *Hirudin free in solution and bound to thrombin*

Hirudin is a small protein of 65 amino acid residues. Natural hirudin contains a sulfato-tyrosyl residue in position 63 (Markwardt, 1970). The X-ray and NMR structures of prime interest were obtained with recombinant desulfatohirudin. For the NMR studies reported by Folkers *et al.* (1989). Haruyama & Wüthrich (1989) and Szyperski *et al.* (1992), the form HV1 was used, and for the X-ray studies reported by Rydel *et al.* (1990, 1991) the form HV2-K47, which differs from segment 1–51 of HV1 by the amino acid substitutions Val1 → Ile, Val2 → Thr, Gln24 → Lys, Asp33 → Asn, Glu35 → Lys, Lys36 → Gly and Gln49 → Glu (Scharf *et al.*, 1989; Harvey *et al.*, 1986). Hirudin in solution contains a globular N-terminal domain of residues 1 to 48, which includes the three disulfide bonds 6–14, 16–28 and 22–39, and a negatively charged, flexibly disordered C-terminal tail of residues 49 to 65. To obtain a high-quality NMR structure of the globular domain of hirudin, the flexible tail was cleaved off in hirudin(1-51). The dominant conformational change in hirudin upon binding to thrombin is that the aforementioned flexibly disordered tail of residues 49 to 65 in the solution structure is well structured due to numerous specific contacts with the protease (Grütter *et al.*, 1990; Rydel *et al.*, 1990). In contrast, the global appearance of the N-terminal domain is closely similar in free and complexed hirudin (Fig. 1), warranting a detailed analysis of local conformational differences.

As discussed in the accompanying paper (Szyperski *et al.*, 1992), the high-quality NMR structure of hirudin(1-51) is a faithful representation of the N-terminal domain of the complete hirudin polypeptide chain 1–65 in solution. We shall make frequent reference to the precision of the NMR structure for hirudin(1-51) (Szyperski *et al.*, 1992) and the crystal structure of the thrombin–hirudin complex (Rydel *et al.*, 1991) to properly assess the significance of local structural differences between the N-terminal globular domain in the crystal and in solution. Figure 2A shows that the backbone conformation in the NMR structure of hirudin(1-51) is well defined for residues 3 to 30, 37 to 41 and 45 to 47, with backbone atom displacements after global best fit, D_{glob}, smaller than 0·5 Å (1 Å = 0·1 nm). Residues 3 and 42 to 44 have only slightly increased values of D_{glob}, but the N-terminal dipeptide, a loop formed by residues 31 to 36 (Fig. 1) and the C-terminal tripeptide are only poorly constrained by the NMR data. The Figure shows further that there are wide variations in the precision of the structure determination for the side-chains of individual residues, with 20 "best-defined" residues having values smaller than 0·75 Å for D_{loc} of all heavy atoms. The X-ray crystal structure of the hirudin–thrombin complex reported by Rydel *et al.* (1991) was solved at 2·3 Å resolution and refined to an R-factor of 17·3%. The average of the B-values for hirudin heavy atoms is about 50% greater than the corresponding quantity for thrombin, and they increase from the N toward the C terminus (Fig. 2B). These observations suggest that the B-factors may reflect primarily a distribution of multiple global positionings of the hirudin molecule

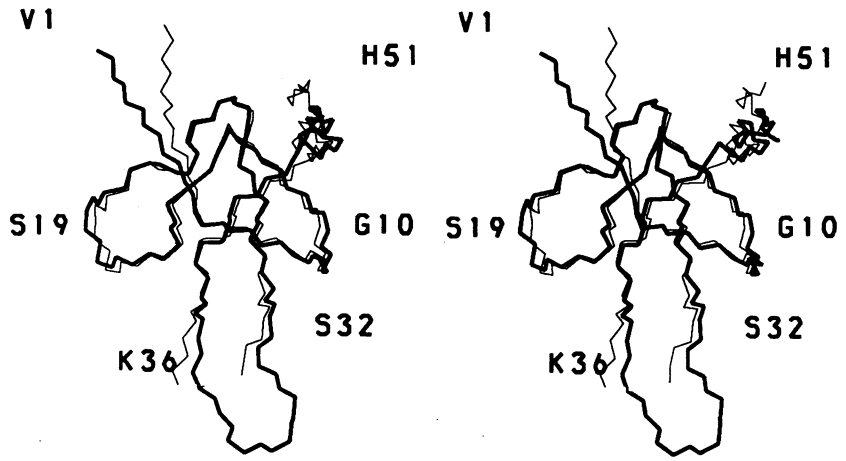
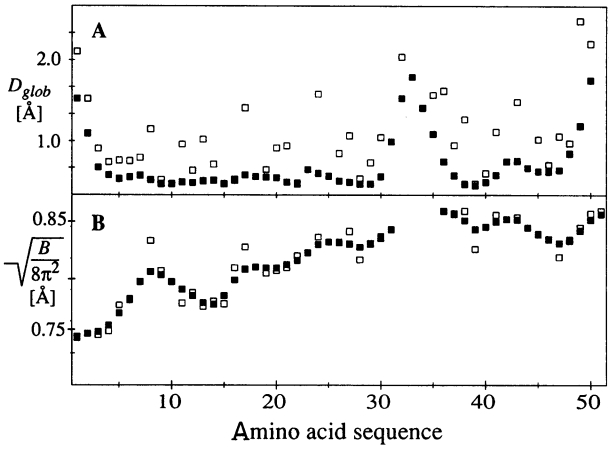


Figure 1. Stereo view of the polypeptide backbone of the mean NMR solution structure of hirudin(1-51) (bold line) and of the corresponding 51-residue segment of recombinant desulfatohirudin in the X-ray crystal structure of the thrombin complex reported by Rydel *et al.* (1990, 1991) (thin line). The 2 structures were superimposed for minimal r.m.s.d. of the backbone atoms N, C^{α} and C′ of residues 3 to 30 and 37 to 48. No co-ordinates for residues 32 to 35 of hirudin are reported in the crystal structure.

relative to thrombin in the complex, rather than local segmental disorder. The low temperature factors of residues 1 to 5 of hirudin (Fig. 2B) are due to very intimate contact with thrombin, resulting in almost complete burial of the N-terminal pentapeptide segment (Tables 5 and 7 in Rydel *et al.*, 1991). This segment might represent a pivot for global motions of hirudin relative to thrombin in the complex.



Figures 2. Plots *versus* the amino acid sequence of hirudin(1-51) of (A) average of the global displacements, D_{glob}, relative to the mean NMR structure of the 20 energy-minimized DIANA conformers used to represent the NMR solution structure of hirudin(1-51) after superposition of the backbone atoms N, C^{α} and C′ of residues 3 to 30 and 37 to 48 for minimal r.m.s.d. For the backbone atoms N, C^{α} and C′ of each residue, this quantity is indicated by filled squares and for the side-chain heavy atoms by open squares. B, The quantity $\sqrt{(B/8\pi^2)}$, where B is either the average of the crystallographic B-factors of the backbone atoms N, C^{α} and C′ (filled squares) or of the side-chain heavy atoms (open squares) in the hirudin–thrombin complex as reported by Rydel *et al.* (1991). This presentation was chosen because the mean displacements of the atomic positions are proportional to the square-root of the B-factor (Glusker & Trueblood, 1985).

(b) *Correlation of structural differences with protein–protein contacts in the crystals*

The visual impression of a close global fit between residues 3 to 30 and 37 to 48 of hirudin(1-51) in solution and in the crystalline hirudin–thrombin complex (Fig. 1) is confirmed by r.m.s.d. values of 0·61 Å calculated for the polypeptide backbone atoms, and 0·91 Å for all heavy atoms of these residues (Szyperski *et al.*, 1992). The two structures further have the common feature that the segment of residues 31 to 36 is disordered (Fig. 2). Local conformational differences were identified for the polypeptide segments 1–3 and 17–21.

Overall, there are 103 interactions shorter than 4·0 Å between the N-terminal 51 residues of hirudin and thrombin in the crystal structure of the complex determined by Rydel *et al.* (1991). In addition to possible correlations between these contacts and the conformational differences relative to hirudin(1-51) in solution, we shall consider crystal contacts shorter than 4·0 Å that occur between residues 1 to 51 of desulfatohirudin bound to thrombin and hirudin–thrombin complexes in other asymmetric units of the crystal.

For the N-terminal tripeptide segment the small local r.m.s.d. values for the backbone atoms N, C^{α} and C′ (Fig. 2B in Szyperski *et al.*, 1992) demonstrate that in spite of the large global displacements between the crystal structure and the mean solution conformation (Fig. 2A in Szyperski *et al.*, 1992), this tripeptide adopts a local structure in solution that is close to that in the complex. Both large local r.m.s.d. values calculated for the backbone atoms of residues 2 to 4 between the X-ray crystal structure and the mean NMR solution structure as well as the large difference observed for the ψ-angle of Tyr3 (Fig. 3 of Szyperski *et al.*, 1992) indicate that the reorientation of the N terminus upon complex formation may be described by a rotation around this dihedral angle. In the crystal structure of the complex (Fig. 3), a parallel β-sheet is formed by the

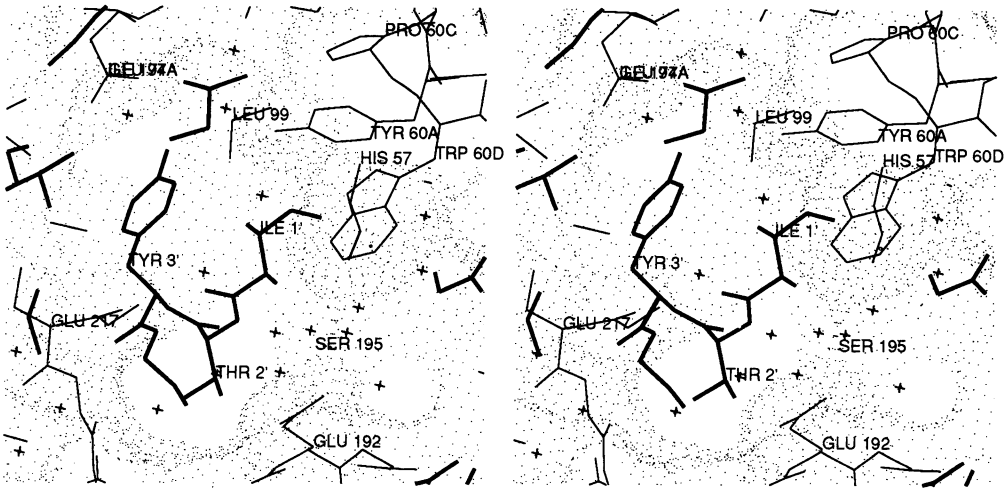


Figure 3. X-ray crystal structure of the HV2-K47 complex with thrombin determined by Rydel *et al.* (1991). The hirudin segment Ile1-Thr2-Tyr3-Thr4-(thick connections) and surrounding residues of thrombin (thin connections) are displayed together with the Connolly surface of the thrombin component and localized solvent molecules (crosses). The surface "hole" marks the entrance to the specificity pocket. The amino-terminal hirudin segment runs parallel to the thrombin segment Ser214 to Gly219; the hirudin residue Ile1 occupies the hydrophobic cavity-like S2-subsite of thrombin; its amino group is in hydrogen bonding distance relative to His57A N^{ε} and Ser195A O^{γ}.

N-terminal tripeptide segment of hirudin and the residues Ser214A to Gly219A of thrombin (Rydel *et al.*, 1991). Out of the total of 103 hirudin–thrombin contacts shorter than 4·0 Å, 41 involve this tripeptide segment (Rydel *et al.*, 1991). When investigating possible conformational changes of the side-chains of this tripeptide segment, one has to take into account that the hirudin variant HV2-K47 in the X-ray crystal structure differs by the substitutions Val1 → Ile and Val2 → Thr from hirudin(1-51). Accordingly, the χ^1 angle of about −60° for Thr2 of HV2-K47 in the complex is conformationally equivalent to the χ^1 angle of about 180° found for Val2 in hirudin(1-51) in solution (Fig. 3 of Szyperski *et al.*, 1992). Thus, the locally well-defined side-chain conformation of Val2 observed in solution seems to be conserved upon binding to thrombin. The space occupied by the *pro-S* methyl carbon atom of Val2 in HV1 can be filled by the γ oxygen atom of Thr2 in HV2-K47 without a need for further structural changes. For Val1, all three rotamers about χ^1 are observed in the NMR solution structure, and this terminal residue is conformationally fixed upon binding to thrombin. The first residue of recombinant hirudin makes a large number of contacts to thrombin (Ile1 of HV2-K47 makes over 20 contacts shorter than 4·0 Å (Rydel *et al.*, 1991)) and the well-defined conformation of residue 1 is a direct consequence of these contacts. Replacement of Val1 of recombinant hirudin by glycine results in a 12-fold increase in the dissociation constant of the complex (i.e. the binding energy decreases by 6·2 kJ·mol^{-1}; Betz *et al.*, 1992), which again demonstrates the important role of residue 1 in the formation of the complex. A large local side-chain displacement of Tyr3 in the complex relative to the mean NMR conformation (Fig. 2C of Szyperski *et al.*, 1992) demonstrates that the aromatic ring of Tyr3 is differentially oriented in hirudin(1-51) and in hirudin bound to thrombin. Since a narrow range of χ^1 angles was found for Tyr3 in hirudin(1-51) in solution, which nonetheless includes the χ^1 value observed for Tyr3 when bound to thrombin, this structural difference seems to come predominantly from a reorientation around χ^2. This reorientation enables favorable contacts between thrombin and both the aromatic ring and the hydroxyl group of Tyr3 (Rydel *et al.*, 1991). These contacts make a substantial contribution to the stability of the complex, as is evidenced by the fact that substitution of Ala for Tyr3 causes a 135-fold increase in K_d (i.e. a decrease in the binding energy of 12·6 kJ·mol^{-1}; Betz *et al.*, 1992).

Deviations observed for the ψ angle of Asp5 and the ϕ angle of Cys6 (Fig. 3 of Szyperski *et al.*, 1992) might be related to the fact that six intermolecular hirudin–thrombin contacts shorter than 4·0 Å are found for Thr4 and Asp5 (Rydel *et al.*, 1991). Replacement of Thr4 or Asp5 by Ala results in a decrease in binding energy of about 5 kJ mol^{-1} (A. Betz, P. Hopkins & S. R. Stone, unpublished results). However, in spite of the close intermolecular contacts, the side-chain conformations of Thr4 and Asp5 coincide almost perfectly in solution and when bound to thrombin. This contrasts with the behavior of the side-chain of Thr7, where χ^1 differs by approximately 160° in the two structures, although there is no close interaction with thrombin in the complex (Rydel *et al.*, 1991). This reorientation is probably due to an interaction of the γ hydroxyl group of Thr7 with the side-chain carboxylate group of Asp1A of thrombin (numbering according to Bode *et al.*, 1989) of another complex in the crystal lattice (Fig. 4). Two contacts of 3·5 Å and 3·6 Å are observed between the γ oxygen atom of Thr7 and the carboxyl oxygen

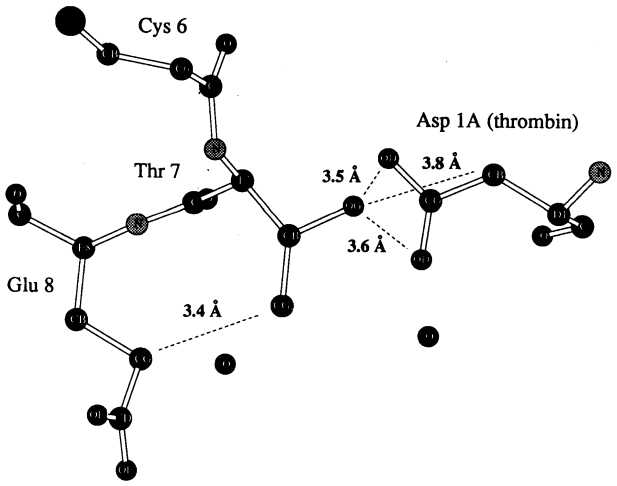


Figure 4. Ball-and-stick representation of the heavy atoms of Cys6, Thr7 and Glu8 from the crystal structure of thrombin-bound hirudin. Thr7 makes close contacts with Asp1A (numbering according to Bode *et al.*, 1989) of a thrombin molecule from a different asymmetric unit in the crystal (Rydel *et al.*, 1991). The oxygen atoms of 2 nearby water molecules observed in the X-ray crystal structure are shown. Carbon atoms are represented as black spheres, nitrogen atoms as light grey spheres and oxygen atoms as dark grey spheres.

atoms of Asp1A, indicating a favorable electrostatic interaction that could not be present with the side-chain conformation found for hirudin(1-51) in solution. In addition, an ensuing intramolecular hydrophobic contact of 3·4 Å between the γ methylene group of Glu8 and the γ methyl group of Thr7 of hirudin (Fig. 4) might stabilize the side-chain conformation of Glu8 found in the complex.

An exceptionally large local side-chain deviation was found for Leu15, although the range of χ^1 angles in the NMR solution structure deviates by only approximately 40° from the χ^1 angle in the crystal structure. There are eight short contacts to thrombin that could cause a reorientation of Leu15 upon complex formation. However, the isopropyl group is actually in a very similar spatial location in solution and in the crystal, except that due to a rotation around χ^2 the *pro-S* methyl group of the solution structure is superimposed with the *pro-R* methyl group in the crystal, and *vice versa*. By inspection of the electron density map, we conclude that the side-chain conformation observed by NMR in solution is indeed compatible also with the X-ray data. In any case, site-directed mutagenesis studies indicate that the contacts made by Leu15 are important for the formation of the complex. Mutation of Leu15 to Ala causes a 78-fold increase in K_d (i.e. a decrease in binding energy of 11 kJ mol^{-1}; A. Betz, P. Hopkins & S. R. Stone, unpublished results).

Large global displacements of the backbone atoms of residues 18 to 20 (Fig. 2A in Szyperski *et al.*, 1992) in conjunction with large local side-chain displacements of Ser19 and Val21 correlate with the fact that 37 of the 103 hirudin–thrombin contacts shorter than 4·0 Å are observed for residues 18 to 21 (Rydel *et al.*, 1991). The hydrogen bond connecting the amide proton of Asn20 and the carbonyl oxygen atom of Glu17 in the solution structure of hirudin(1-51) is rather long and bifurcated in the crystal structure, with distances Glu17 O′–Asn20 N of 3·3 Å, and Glu17 O′–Asn20 N$^{\delta}$ of 3·2 Å. Inspection of the local r.m.s.d. values of residues 18 to 21 showed that the displacement of the backbone atoms in this region cannot be due to localized hinge motions. The hydrogen bonds between Ser19 O′ of hirudin and Lys224A N$^{\zeta}$ of thrombin, and between Ser19 O$^{\gamma}$ and Arg221A N$^{\eta}$ may be an important factor, while the displacement of Val21 must be due to hydrophobic contacts with thrombin or, indirectly, to the formation of a hydrogen bond connecting the amide proton of Val21, and the carboxylate group of Glu217A of thrombin. Rather unexpectedly, the dissociation constant of the mutant Ser19 → Ala of recombinant hirudin in the complex with thrombin was found to be nearly identical with that of wild-type recombinant hirudin (A. Betz, P. Hopkins & S. R. Stone, unpublished results). In trying to rationalize this observation, one first has to acknowledge that the hydrogen bonds between Ser19 of hirudin and Lys224A of thrombin, and between Ser19 and Arg221A are relatively weak. Moreover, the hydroxyl group of Ser19 of hirudin has to replace a water molecule that is hydrogen-bonded to Lys224A or Arg221A of thrombin, with a free binding enthalpy that is close to that for the hydrogen bonds formed with the hydroxyl group of Ser19. Furthermore, this water molecule should retain its position in the thrombin complex with the Ser19 → Ala mutant, where it occupies the space otherwise occupied by the hydroxyl group of Ser19. The side-chain conformation of Asn20, which alone exhibits 16 contacts shorter than 4·0 Å to thrombin (Rydel *et al.*, 1991) has, quite surprisingly, nearly identical conformations in free hirudin(1-51) and in the complex.

Figure 1 shows that the backbone conformations of residues 29 to 31 and 36 to 37, which flank the loop of residues 32 to 35, clearly differ between the two structures. This observation is manifested also in large global displacements for these residues. These differences cannot be explained in terms of protein–protein interactions. However, the observed conformational differences might be related to three point mutations (Asp33 → Asn, Glu35 → Lys, Lys36 → Gly) in hirudin variant HV2-K47 when compared with hirudin variant HV1.

Overall, the agreement of the backbone conformation of residues 38 to 48 between the mean NMR solution structure of hirudin(1-51) and the crystal structure of the desulfatohirudin–thrombin complex was found to be significantly lower than for the N-terminal segment consisting of residues 3 to 28. This goes along with the fact that the quality of both the NMR solution structure and the X-ray crystal structure decreases towards the C terminus

(Fig. 2). Real differences are likely to occur, however, since the polypeptide segment 47–49 of hirudin makes intimate contacts with the insertion loop around residue 60A of thrombin.

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