Contribution of interactions with the core domain of hirudin to the stability of its complex with thrombin

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The importance of interactions with residues 15–21 in the core domain of hirudin for the formation of the complex with thrombin has been investigated by site-directed mutagenesis. Contacts made by Leu-15 were found to be particularly important; replacement of this residue by alanine led to a decrease in the binding energy ($\Delta\Delta G_b^\circ$) of $15 \text{ kJ} \cdot \text{mol}^{-1}$. Comparison with effects obtained in previous mutagenesis studies indicate that interactions with the side chain of Leu-15 contribute more to the stability of the complex than those of any other hirudin residues. Interactions with the side chains of Glu-17, Asn-20 and Val-21

also contributed significantly to binding energy; the $\Delta\Delta G_b^\circ$ value for these mutations was between 3 and $6 \text{ kJ} \cdot \text{mol}^{-1}$. Examination of the crystal structure of the thrombin–hirudin complex suggested the possibility that ionic interactions that would increase binding energy could be engineered by mutating Ser-19, Asn-20 and Glu-49 to acidic residues. The stability of the thrombin–hirudin complex was not, however, increased by these substitutions. The results obtained are discussed in terms of the crystal structure of the thrombin–hirudin complex.

INTRODUCTION

The trypsin-like serine protease thrombin plays a central role in blood coagulation. Cleavage of fibrinogen by thrombin leads to the formation of a fibrin clot. Thrombin further stimulates clot formation by activating coagulation factors V, VIII and XIII. The activity of thrombin is also important for the control of clot formation. In complex with thrombomodulin, it activates Protein C which subsequently shuts down the coagulation cascade by inactivating factors Va and VIIIa (Davie et al., 1991). The pivotal role of thrombin in blood coagulation requires the restriction of its specificity. This restriction is achieved, in part, by surface loops that partially occlude the active site and by the unique anion-binding exosite distant from the active-site cleft (Bode et al., 1989, 1992).

Thrombin is inhibited with absolute specificity by hirudin, a small protein of 65 residues that was originally isolated from the leech *Hirudo medicinalis* (Walsmann and Markwardt, 1981). The thrombin–hirudin complex is non-covalent and extremely stable; its dissociation constant is 10^{-14} M (Stone and Hofsteenge, 1986). The solution structure of hirudin as determined by n.m.r. spectroscopy reveals the segmentation of the protein into two domains (Haruyama and Wüthrich, 1989; Folkers et al., 1989). The compact N-terminal core domain consists of β -strands and well-defined turns held together by three disulphide bonds. This domain is connected to the floppy C-terminal tail by a polyproline-II helix. Kinetic studies and X-ray structures of the hirudin–thrombin complex have revealed an unusual mechanism of inhibition (Stone and Hofsteenge, 1986, 1991; Rydel et al., 1990, 1991; Grütter et al., 1990). The rate-limiting step of complex formation is the binding of the negatively charged C-terminal tail to the anion-binding exosite of thrombin, while the active site is occluded by the N-terminal domain in a subsequent fast step (Stone and Hofsteenge, 1986; Stone et al., 1989; Jackman et al., 1992). The orientation of the first three residues

of hirudin in the active site of thrombin is opposite to that observed with other proteinase inhibitors; residues 1–3 of hirudin form a parallel β -sheet with the residues 214–216 of thrombin in contrast with the usual antiparallel β -sheet. [The numbering of residues in thrombin is that of Bode et al. (1989) and is based on that of chymotrypsinogen. Residues in thrombin that correspond to insertions in the chymotrypsin sequence are denoted by a letter (A, B and so on) following the residue number. Residues in hirudin are distinguished from those in thrombin by the use of primed numbers.] Moreover, in contrast with other inhibitors of serine proteinases, hirudin does not occupy the primary specificity pocket of its target proteinase (Rydel et al., 1990, 1991; Grütter et al., 1990). The tightness of the thrombin–hirudin complex is the result of numerous electrostatic and hydrophobic interactions between the two proteins. Site-directed mutagenesis experiments have illustrated the importance of hydrophobic contacts with both the N-terminal three amino acids of hirudin and with residues in the C-terminal tail (Betz et al., 1991a,b, 1992). In particular, it was found that contacts with the three N-terminal residues account for about 40 % of the binding energy of the complex (Betz et al., 1992). The X-ray structure of the hirudin–thrombin complex shows an additional patch of close contacts with a segment of hirudin comprising residues Leu-15'–Val-21'; about 20 % of the close contacts between the proteins are made by these residues (Rydel et al., 1991). The side chains of Leu-15', Ser-19', Asn-20' and Val-21' of hirudin are buried in hydrophobic pockets of thrombin, which are mainly formed by the aliphatic portions of side chains of charged residues (Lys-173, Glu-217 and Arg-221A, see Figure 1). Glu-17' also forms a salt bridge with Arg-173 (Rydel et al., 1991). The significance of some of these contacts is further suggested by the fact that similar contacts are made by the fibrinopeptide A portion of the fibrinogen A α chain (Stubbs et al., 1992). In order to assess the strength of the hydrophobic interactions at this site, each residue of the section Leu-15'–Val-21' has been replaced by alanine. The

Abbreviation used: rhir, recombinant hirudin-variant 1.

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results indicated that the majority of residues in this segment, particularly Leu-15', make interactions that contribute to the stability of the complex.

Examination of the structures of thrombin–hirudin complexes (Rydel et al., 1991; Grütter et al., 1990) suggested that it may be possible to engineer salt bridges by mutating several residues in the core domain of hirudin and thereby increase the binding energy. In a previous study, the successful engineering of an ionic interaction with arginine in the second position of hirudin was reported (Betz et al., 1991b). Aspartic acid was substituted for Ser-19' and Asn-20' in an attempt to engineer ion pairs with the positively charged residues Arg-173, Arg-221A and Lys-224 in thrombin. A third possible ion pair with core domain residues was identified between Gln-49' and Lys-60F of thrombin. Such a salt bridge actually occurs in the complex between thrombin and recombinant hirudin-variant 2 (Rydel et al., 1991); in hirudin-variant 2, a glutamate residue occurs at position 49. Although molecular modelling indicated that each of the ionic interactions with acidic residues at positions 19, 20 and 49 could be reasonably formed, no increase in binding energy was observed for any of the substitutions.

MATERIALS AND METHODS

Materials

The substrates D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238) and D-Val-Leu-Arg-*p*-nitroanilide (S-2266) were obtained from Chromogenix (Molndal, Sweden). Human α -thrombin was prepared and characterized as described previously (Stone and Hofsteenge, 1986).

Site-directed mutagenesis, purification and characterization of mutated forms of hirudin

The wild-type DNA of hirudin-variant 1 was mutated by using the uracil-containing template method in M13mp19 (Kunkel, 1985). The mutated proteins were expressed in *Escherichia coli* JM101 and isolated as described previously (Braun et al., 1988). Mutant proteins were characterized by N-terminal sequencing and amino acid analysis (Betz et al., 1991b). The concentrations of the mutant proteins were determined by titration against thrombin in the presence of D-Val-Leu-Arg-*p*-nitroanilide as described by Wallace et al. (1989).

Amidolytic assays

These were performed at 37 °C in 0.05 M Tris/HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.2% (w/v) poly(ethylene glycol) M_r 6000 as previously described (Betz et al., 1991a).

Determination of kinetic parameters

The kinetic parameters of the interactions of hirudin (I) and thrombin (E) are defined in Scheme 1:

Scheme 1

Most forms of hirudin were slow, tight-binding inhibitors of thrombin. The kinetic parameters of these hirudin variants were determined from progress-curve experiments as described previously (Braun et al., 1988). For each determination, the form-

ation of *p*-nitroaniline from D-Phe-pipecolyl-Arg-*p*-nitroanilide was monitored in six assays, one in the absence of hirudin and the others with different concentrations of hirudin. The kinetic parameters were obtained from a non-linear fit of the data to the equation describing the mechanism shown in the above scheme (Braun et al., 1988). The substrate concentration was 100 μ M and the enzyme concentration was between 10 and 50 pM. Mutants are identified in the following text by using the single-letter code for the residue mutated followed by its number and the single letter code for the new amino acid. Thus, L15A represents a mutant hirudin in which Leu-15' has been replaced by an alanine. For the hirudin variant L15A, the concentrations required for inhibition were too great to observe slow-binding inhibition and steady-state data were fitted to the equation for tight-binding inhibition using non-linear regression (Stone and Hofsteenge, 1986; Williams and Morrison, 1979). The kinetic parameters for each mutant were determined at least twice and values given in Tables 1 and 2 represent the weighted means of the estimates.

The Gibbs standard free energy for the formation of the complexes between hirudin variants and thrombin (also called binding energy) was calculated using eqn. (1):

$$\Delta G_b^\circ = RT \ln K_i \quad (1)$$

where R is the gas constant and T is the absolute temperature at which the assay was performed. The difference in the free standard binding energies of the mutated forms of hirudin and recombinant hirudin-variant 1 (rhir) is referred to as $\Delta\Delta G_b^\circ$.

RESULTS

Hydrophobic interactions with residues Leu-15'–Val-21' in the core domain of hirudin

In order to evaluate the contribution of the interactions with residues between Leu-15' and Val-21' to the stability of the complex, the side chains of each of the residues in this segment were truncated to alanine with the exception of Cys-16' and Gly-18'. While Gly-18' has no side chain to truncate, Cys-16' forms a disulphide bond and its side chain makes no interactions with thrombin. The largest effect was observed with the mutation Leu-15' $\rightarrow$ Ala which caused a 320-fold increase in the inhibition constant (K_i); the corresponding decrease in the binding energy ($\Delta\Delta G_b^\circ$) was 14.9 kJ $\cdot$ mol $^{-1}$ (Table 1). The substitution of Glu-17' caused a 3-fold increase in the K_i value ($\Delta\Delta G_b^\circ = 3.2$ kJ $\cdot$ mol $^{-1}$). Replacement of Ser-19' did not affect the affinity of thrombin for

Table 1 Kinetic parameters for the inhibition of thrombin by mutants of hirudin within the segment Leu-15'–Val-21'

Assays were performed and data analysed as described in the Materials and methods section. The kinetic parameters [association rate constant (k_1) and inhibition constant (K_i)] are defined in Scheme 1. The parameters for each mutant were determined at least twice and the values given represent the weighted mean of these estimates together with the standard error. The values for rhir are from Braun et al. (1988).

Form of hirudin	$10^{-8} \times k_1$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)	$-\Delta G_b^\circ$ (kJ $\cdot$ mol $^{-1}$)	$\Delta\Delta G_b^\circ$ (kJ $\cdot$ mol $^{-1}$)
rhir	1.37 ± 0.03	0.237 ± 0.006	75.0	–
L15A	–	76 ± 5	60.1	14.9
E17A	1.77 ± 0.18	0.815 ± 0.020	71.8	3.2
S19A	1.49 ± 0.04	0.208 ± 0.005	75.3	–0.3
N20A	1.84 ± 0.2	2.58 ± 0.03	68.9	6.1
V21A	1.08 ± 0.02	1.00 ± 0.01	71.2	3.8

Table 2 Kinetic parameters for the inhibition of thrombin by mutants of hirudin with altered electrostatic properties

The kinetic parameters for each mutant and their S.E.M.s were derived as described in the legend of Table 1. The values for rhir are from Braun et al. (1988).

Form of hirudin	$10^{-8} \times k_1$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)	$-\Delta G_b^\circ$ ($kJ \cdot mol^{-1}$)	$\Delta\Delta G_b^\circ$ ($kJ \cdot mol^{-1}$)
rhir	1.37 ± 0.03	0.237 ± 0.006	75.0	–
S19D	0.70 ± 0.02	0.369 ± 0.011	73.8	1.2
N20D	1.43 ± 0.04	0.191 ± 0.011	75.5	–0.5
Q49E	1.24 ± 0.05	0.247 ± 0.010	74.8	0.2

hirudin. The second largest effect was observed on the replacement of Asn-20'; an 11-fold increase in the K_i value was observed ($\Delta\Delta G_b^\circ = 6.1 \text{ kJ} \cdot \text{mol}^{-1}$; Table 1). Exchange of Val-21' led to a 4-fold elevation in the K_i value with a corresponding decrease in binding energy of $3.8 \text{ kJ} \cdot \text{mol}^{-1}$ (Table 1). It was not possible to measure an association rate constant (k_1) for L15A, but the value of this constant for the other mutants was not markedly different from that for rhir (Table 1). These results are consistent with the proposed mechanism for hirudin in which interactions with the C-terminal region are involved in the first and rate-determining step (Stone et al., 1989; Jackman et al., 1992).

Engineering of ionic interactions with residues in the core domain of hirudin

Examination of the crystal structure of the thrombin–hirudin complex indicated that it may be possible to increase the tightness of the complex by the engineering of salt bridges through the mutation of Ser-19', Asn-20' and Gln-49' to acidic residues. The hydroxyl of Ser-19' comes within 0.4 nm ($4 \text{ \AA}$) of the guanidinium group of Arg-221A and a similar distance is observed between the O^δ (and/or N^δ) of Asn-20' and the charged groups of Arg-173

and Lys-224 (Rydel et al., 1991). In the complex of recombinant hirudin-variant 2 with thrombin, the residue at position 49 is a glutamate instead of the glutamine in the variant 1 used in the present study and the glutamate at this position forms a close (approx. $3 \text{ \AA}$) ionic interaction with Lys-60F. The replacement of Ser-19' or Asn-20' by aspartate did not have a marked effect on the affinity of thrombin for hirudin; the inhibition constants for S19D and N20D were about equal to that of rhir (Table 2). Similarly, the mutation of Gln-49' to glutamate did not affect the kinetic constants of hirudin (Table 2).

DISCUSSION

The functional importance of residues Leu-15' through to Val-21' is indicated by the high conservation of these residues among hirudin variants (Scharf et al., 1989). With the exception of Ser-19', replacement of residues within this segment by alanine led to significant decreases in binding energy (Table 1). The most important interactions were made by Leu-15'; contacts with the side chain of this residue appear to make a greater contribution to the binding energy than those of any other residue in hirudin apart from Tyr-3' (Betz et al., 1992). Truncation of Leu-15' to alanine would lead to the loss of eight contacts less than 0.4 nm ($4 \text{ \AA}$), four with Glu-217 and four with Arg-221A (Figure 1; Rydel et al., 1991). Glu-17' makes only one contact with thrombin, a salt bridge with a separation of 0.32 nm ($3.2 \text{ \AA}$) to Arg-173 (Figure 1; Rydel et al., 1991). The decrease in binding energy observed for the E17A mutation is within the range observed for the mutation of acidic residues in the C-terminal tail of hirudin (Braun et al., 1988; Betz et al., 1991a). It is interesting to note that in the complex of fibrinopeptide A with thrombin, Arg-173 forms a salt bridge with Glu-11 of the $A\alpha$ chain (Stubbs et al., 1992). The conservation of the Arg-173 ionic interaction with two molecules so different as hirudin and fibrinogen suggests that this ionic interaction may be an important determinant in the specificity of thrombin. The absence of an effect for replacement of Ser-19' by alanine is difficult to rationalize on the basis of the crystal structure (Figure 1). Truncation of Ser-19' to alanine would result in the loss of four contacts with Arg-221A

Figure 1 Interaction of the hirudin segment Leu-15'–Val-21' with thrombin

The van der Waals surface of thrombin is shown and residues 15'–21' of hirudin are displayed in bold without their van der Waals surface. Other residues in hirudin are not shown. Thrombin residues involved in interactions are labelled with their number and the single letter amino acid code; the hirudin residues Leu-15' and Val-21' are labelled with '151H L' and '211H V' respectively. The figure was produced using QUANTA.

including a weak hydrogen bond and four other contacts with Lys-224 (Rydel et al., 1991). The hydroxy group of Ser-19' replaces a water molecule that is hydrogen bonded to Arg-221A and/or Lys-224 in the D-Phe-Pro-ArgCH₂-thrombin structure and, consequently, the contribution of the hydrogen bond with Ser-19' may not make a large contribution to binding energy (Fersht, 1987). The loss of the other contacts, however, would be expected to cause a significant loss of binding energy. The truncation of Asn-20' to an alanine would cause the loss of more contacts of less than 0.4 nm (4 Å) than the truncation of any other residue in the segment Leu-15'-Val-21' and therefore the relatively large loss of binding energy (6.1 kJ·mol⁻¹; Table 1) observed upon mutation of this residue to an alanine was not unexpected. The majority of the contacts made by Asn-20' are non-polar ones with Arg-173 (Figure 1) and all 10 of these would be lost upon truncation of the residue to an alanine (Rydel et al., 1991). The side chain of Val-21' lies in a hydrophobic pocket close to Leu-15' where it makes a number of van der Waals contacts with Glu-217 (Figure 1); the four of these that would be lost upon replacement of the residue with alanine would be adequate to explain the modest loss of binding energy observed with V21A (3.8 kJ·mol⁻¹; Table 1).

Comparison of the solution structure of rhir with that of the complexed form indicates that the conformation of a number of residues in the segment Leu-15'-Val-21' has changed significantly upon complex formation. There are relatively large variations in the structure of the side chains of Leu-15', Ser-19' and Val-21' and a global displacement of the peptide backbone between residues 18 and 20 (Szyperki et al., 1992). The numerous close contacts of thrombin with these residues obviously stabilizes their bound conformations. Some idea of the importance of interactions made by residues in the segment Leu-15'-Val-21' can be obtained from the sum of the effects of mutation of individual residues in the segment to alanine; this sum was 28 kJ·mol⁻¹ which represents more than one third of the binding energy for the formation of the complex. Thus, it can be seen that interactions with this region play an important role in the stabilization of the complex. The fact that certain of these interactions (with Arg-173 and Glu-217) are also seen with fibrinopeptide A suggests that this binding region in thrombin may be of general importance in the enzyme's specific interactions with a range of ligands.

Examination of the structure of thrombin-hirudin complexes (Grütter et al., 1990; Rydel et al., 1991) does not indicate any obvious reasons for our failure to engineer ionic interactions that increase the stability of the complex. The failure is most likely due to subtle alterations in the structure of the complexes of thrombin with the mutant proteins. Molecular modelling indicated that an aspartate at position 20 could make ionic interactions with either Arg-173 or Lys-224 (see Figure 1). Arg-173 already makes a salt bridge to Glu-17' and, thus, any gain in interaction energy due to the engineered salt bridge would be at least partially offset by a decrease in the strength of the Glu-17'-Arg-173 interaction. It also seems possible that an interaction of Asp-20' with Lys-224 could disrupt existing contacts with this residue. However, the main contacts made by Lys-224 are with Ser-19' and the results obtained with the S19A mutant suggest that these contacts do not contribute significantly to the stability of the complex (Table 1). An aspartate at position 19 could make

an ionic interaction with Arg-221A (Figure 1). However, this contact would possibly disturb the existing one with Asp-5' which contributes as much as 5 kJ·mol⁻¹ to the binding energy (Betz et al., 1992). In addition, movement of the side chain of Arg-221A could lead to loss of contacts with Leu-15'. The observation that mutation of Gln-49' to glutamate was without effect is particularly puzzling. In the complex of recombinant hirudin-variant 2 with thrombin, the glutamate in position 49 forms a close (approx. 3 Å) ionic interaction with Lys-60F. It is, however, noteworthy that the glutamine in position 49 is conserved in all other (approx. 20) hirudin variants sequenced (Scharf et al., 1989). This conservation suggests some evolutionary advantage for a glutamine over a glutamate in this position. Gln-49' does not participate in the maintenance of the structure of hirudin in solution (Folkers et al., 1989; Haruyama and Wüthrich, 1989; Szyperki et al., 1992), but it may have other functions such as increasing the resistance of hirudin to proteolytic degradation. In conclusion, the results obtained illustrate the difficulties in engineering ionic interactions in proteins. While some studies have successfully engineered such interactions (Wells et al., 1987), difficulties have also been observed by others (Dao-Pin et al., 1991).

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