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Ionic interactions in the formation of the thrombin–hirudin complex

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Site-directed mutagenesis has been used to examine the importance of each of the acidic *C*-terminal residues of hirudin in the formation of its complex with α-thrombin. The contribution to binding energy of acidic residues in the 11 *C*-terminal amino acids varied from 2.3 to 5.9 kJ·mol^{-1}. The differences between the contributions of individual residues were smaller than would be expected from the crystal structures of the thrombin–hirudin complex. In particular, the small effect (2.4 kJ·mol^{-1}) for the replacement of Asp-55 was surprising in view of the two salt bridges made by this residue. The results of studies involving multiple mutations indicated that the additivity of the effects varied with the position of the mutation. Whereas the effect of mutations involving the glutamic acid residues at positions 61 and 62 were additive, non-additivity was observed with the glutamic acid residues at positions 57 and 58.

INTRODUCTION

Hirudin is a leech protein that inhibits thrombin by forming a tight complex involving multiple sites of interaction on the two molecules (Rydel *et al.*, 1990; Grütter *et al.*, 1990). Ionic interactions have been found to play an important role in the formation of this complex (Stone *et al.*, 1989), and a number of these interactions occur between negatively charged residues in the *C*-terminal region of hirudin and a positively charged surface groove on thrombin (Braun *et al.*, 1988; Stone *et al.*, 1989; Grütter *et al.*, 1990; Rydel *et al.*, 1990). Although crystallographic studies indicate that only two of the four glutamic acid residues in the *C*-terminal region of the hirudin would be expected to make a significant contribution to binding energy (Rydel *et al.*, 1990; Grütter *et al.*, 1990), protein-engineering studies based on a limited number of mutants suggested that each of these residues makes about the same contribution to binding energy (Braun *et al.*, 1988; Stone *et al.*, 1989). In an attempt to resolve this inconsistency, we have made additional hirudin mutants and determined kinetic constants for their interaction with thrombin. The results demonstrated that the contribution to the binding energy of each of the acidic residues between positions 55 and 65 varied only from 2.3 to 5.9 kJ·mol^{-1}. In addition, a non-additivity of mutational effects was observed. The relative contributions of the *C*-terminal acidic residues, as well as the non-additivity of mutational effects, are discussed in relation to the crystal structures.

EXPERIMENTAL

Materials

The substrates D-Phe-Pip-Arg *p*-nitroanilide and D-Val-Leu-Arg *p*-nitroanilide were purchased from Kabi (Molndal, Sweden). Human thrombin was prepared and characterized as previously described (Stone & Hofsteenge, 1986). Recombinant hirudin (variant 1) was a gift from Ciba–Geigy (Basel, Switzerland).

Methods

Amidolytic assays of thrombin were performed at 37 °C as previously described (Stone & Hofsteenge, 1986).

Site-directed mutagenesis of the hirudin gene, expression of the mutant hirudins in *Escherichia coli*, purification of mutant proteins and characterization of these mutant proteins by amino acid analysis and peptide mapping were performed as previously described (Braun *et al.*, 1988).

Data analysis

The concentration of active hirudin molecules was determined by titration of 2.90 nM-thrombin in the presence of 200 μM-D-Val-Leu-Arg *p*-nitroanilide (Wallace *et al.*, 1989). Kinetic parameters were determined by analysing progress-curve data for the inhibition of thrombin (20–50 pM) in the presence of D-Phe-Pip-Arg *p*-nitroanilide (~ 100 μM). Each progress-curve experiment consisted of one reaction without hirudin and five others with different concentrations of hirudin. Data analysis was performed as previously described (Braun *et al.*, 1988) to yield estimates for the association rate constant (k_1), dissociation rate constant (k_2) and dissociation constant (K_1). At least two progress-curve experiments were performed for each form of hirudin, and the weighted mean values of the estimates of the parameters are given.

RESULTS AND DISCUSSION

Previous kinetic studies with mutants of hirudin involving single and multiple mutations of the four *C*-terminal glutamic acid residues (57, 58, 61 and 62) suggested that each of these residues makes about the same contribution to binding energy (Braun *et al.*, 1988; Stone *et al.*, 1989). In contrast, examination of the X-ray crystal structures of the thrombin–hirudin complexes reveals that, whereas hirudin residue (h-)Glu-57 and h-Glu-58 are involved in ionic interactions with Arg-77A (residues without the h- are in thrombin) and possibly Arg-75, the h-Glu-61 and h-Glu-62 do not make any ionic interactions, as shown in Fig. 1. The side chain of h-Asp-55 also makes salt bridges with Arg-73 and Lys-149E (Fig. 1), whereas h-Asp-53, which is an asparagine residue in the hirudin variant in the structure described by Rydel *et al.* (1990), does not participate in any ionic interactions. In order to try to resolve these inconsistencies between the previous

Abbreviations used: Pip, piperidyl; the sequence numbering of thrombin is that given by Bode *et al.* (1989); 'h-' preceding the three-letter code indicates a residue in hirudin.

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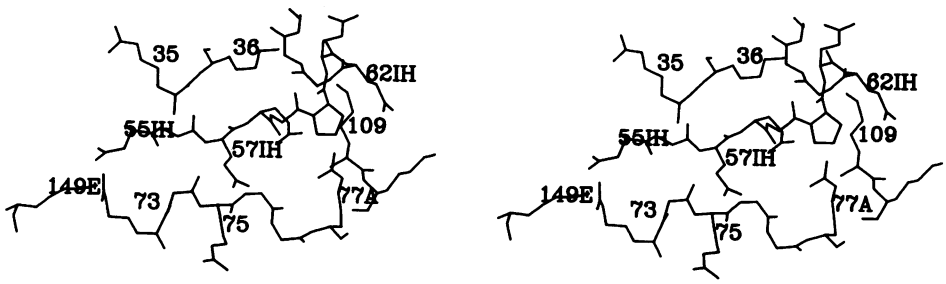

Fig. 1. Stereo pair of the binding of hirudin residues 55–65 to thrombin

The Figure is drawn from the co-ordinates of the structure determined by Rydel *et al.* (1990). Hirudin residues are numbered with the suffix 'IH'. Only the side chains of the acidic residues and h-Pro-60 of hirudin are shown. In addition, only thrombin residues directly interacting with these hirudin residues and some residues that may be responsible for longer-range ionic interactions are shown.

Table 1. Kinetic parameters for the inhibition of thrombin by mutants of recombinant hirudin

Estimates of the association and dissociation rate constants (k_1 and k_2 respectively) together with the dissociation constant (K_I) were determined as described under 'Data analysis'. Binding energy (ΔG_b) was calculated using the relationship:

$$\Delta G_b = RT\ln(K_I)$$

In the forms the prefix and suffix letters are amino acids in one-letter code (D, aspartic acid; E, glutamic acid; A, alanine; N, asparagine; Q, glutamine) and the number(s) are the position(s) in the sequence.

Form of hirudin	$10^{-8}\times k_1$ ($M^{-1}\cdot s^{-1}$)	$10^{5}\times k_2$ (s^{-1})	K_I (pM)	ΔG_b ($-kJ\cdot mol^{-1}$)
rhir*	1.37±0.03	3.17±0.11	0.237±0.006	75.0
D53A	1.23±0.01	2.61±0.10	0.210±0.005	75.2
D55N	0.755±0.018	3.12±0.27	0.595±0.021	72.6
E57Q	0.219±0.002	5.16±0.09	2.30±0.02	69.1
E58Q	1.09±0.07	19.9±1.7	1.83±0.04	69.7
E61Q*	0.957±0.020	3.56±0.14	0.372±0.012	73.7
E62Q*	0.671±0.023	3.70±0.21	0.552±0.025	72.2
E57,58Q*	0.218±0.006	5.15±0.18	2.36±0.05	69.0
E61,62Q	0.454±0.014	3.94±0.11	0.87±0.04	71.5
E57,58,62Q*	0.074±0.001	6.38±0.20	8.60±0.16	65.6
E57,58,61,62Q*	0.045±0.001	6.30±0.17	14.1±0.2	64.4

* From Braun *et al.* (1988)

protein-engineering studies and the X-ray crystal structures, single mutations have been made for all the acidic residues in recombinant hirudin (variant 1) between residues 53 and 65 and the kinetic parameters for the inhibition of thrombin by these mutants determined (Table 1).

With the exception of the mutation involving h-Asp-53, the decreases in binding energy caused by the mutations varied only 2.5-fold from 2.3 $kJ\cdot mol^{-1}$ for hirudin-form E61Q to 5.9 $kJ\cdot mol^{-1}$ for form E57Q (Table 1). The side chain of h-Asp-53 does not form any close ionic interactions with residues on thrombin, and thus the observation that the replacement of h-Asp-53 by an uncharged residue does not have any effect on binding energy is consistent with the crystal structures. This aspartic acid residue is not conserved between different hirudin isoforms and is a neutral residue in hirudin variant 2 (Harvey *et al.*, 1986) and hirudin PA (Dodt *et al.*, 1986). The small difference between the effects observed with the other mutations is somewhat surprising. On the basis of the structure presented in Fig. 1, larger differences would be expected between the residues that form intermolecular salt bridges (h-Asp-55, h-Glu-57 and h-Glu-58) and those that do not make any close [< 0.5 nm (5 Å] ionic interactions (h-Glu-61 and h-Glu-62). The small effect that was observed for the mutation of h-Asp-55 is particularly unexpected. The X-ray crystal structure described by Rydel *et al.* (1990) indicates a major role for this residue in the formation of the complex with thrombin. The effect of mutating this residue to an asparagine residue was less than that observed for the mutation of h-Glu-57 or h-Glu-58 and was about the same as that observed with h-Glu-61 and h-Glu-62 (Table 1). The reason for this discrepancy is not clear. The salt bridge between h-Asp-55 and Lys-149E may not make a large contribution to binding energy. Lys-149E is found in a loop in thrombin that assumes a different conformation in the D-Phe-Pro-Arg-CH_2–thrombin complex (Bode *et al.*, 1989; Grütter *et al.*, 1990; Rydel *et al.*, 1990), and the susceptibility of this loop to cleavage by proteinases suggests that it is mobile (Berliner, 1984; Kawabata *et al.*, 1985; Brezniak *et al.*, 1990). Thus the energetic value of the salt bridge formed with Lys-149E may have to be balanced against the resultant loss of entropy that would occur when the loop containing this residue is frozen in one conformation. However, even if the salt bridge between h-Asp-55 and Lys-149E does not contribute greatly to binding energy, the second salt bridge with Arg-73 would be expected to make a considerable contribution. Further studies are required to resolve this inconsistency between crystallographic and protein-engineering studies.

The changes in binding energy caused by a mutation at a particular position X is usually defined relative to that of the wild-type protein as $\Delta\Delta G_{b(X)}$. The binding energy change for a double mutant (X,Y) can be related to those for the single mutant by eqn. (1) (Carter *et al.*, 1984; Ackers & Smith, 1985; Wells, 1990):

$$\Delta\Delta G_{b(X,Y)} = \Delta\Delta G_{b(X)} + \Delta\Delta G_{b(Y)} + \Delta G_I \quad (1)$$

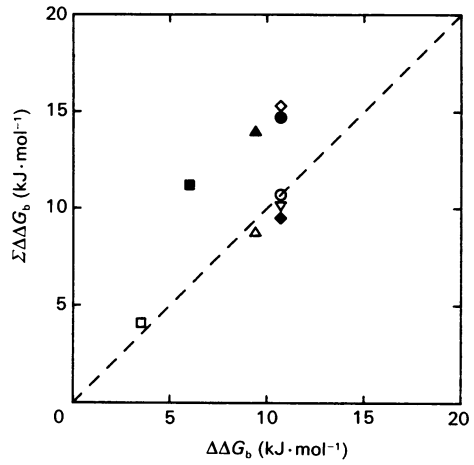

Fig. 2. Plot of the sum of the change in binding energy ($\Sigma\Delta\Delta G_b$) for single mutations against the change in the binding energy for the corresponding multiple mutation ($\Delta\Delta G_b$)

The data were derived from Table 1, and the values summed for a particular point are as follows: □, E61Q+E62Q; ■, E57Q+E58Q; △, E57,58Q+E62Q; ▲, E57Q+E58Q+E62Q; ◆, E57,58Q+E61,62Q; ◇, E57Q+E58Q+E61Q+E62Q; ○, E57,58,62Q+E61Q; ●, E57Q+E58Q+E61,62Q; ▽, E57,58Q+E61Q+E62Q. The broken line represents the expected behaviour if there is no interaction energy between the residues involved, i.e. if ΔG_I in eqn. (1) is equal to zero.

where ΔG_I (called coupling or interaction energy) is a term that measures the extent to which a mutation at one position affects the contribution of the other position to binding energy. ΔG_I may be positive or negative depending on whether the effect of the mutations on the interaction between the residues has been favourable or unfavourable. Eqn. (1) can be easily expanded to take into account mutations at more than two sites. For a series of mutations, a plot of the sum of the effects of individual mutations ($\Sigma\Delta\Delta G_b$) against the effect of a multiple mutation ($\Delta\Delta G_b$) will yield a straight line with a slope of 1 when ΔG_I is zero. Wells (1990) has applied equations of the same form as eqn. (1) to analyse data obtained for the effects of single and multiple mutations on catalysis and binding for a number of proteins. In most cases, the ΔG_I term was negligible. The ΔG_I term was only significant when the mutated residues were in close contact with each other or when the mutations resulted in a change in the catalytic mechanism (Wells, 1990). Previous studies utilizing the mutants E61Q, E62Q, E57,58Q, E57,58,62Q and E57,58,61,62Q suggested that the ΔG_I term in eqn. (1) was negligible for the ionic interactions involving the *C*-terminal glutamic acid residues of hirudin (Braun *et al.*, 1988; Stone *et al.*, 1989). The results of the present study, however, indicate a lack of additivity for the mutations involving h-Glu-57 and h-Glu-58 (Fig. 2). Four points did not lie on the line expected when ΔG_I is equal to zero (the broken line in Fig. 2). Each of these four deviations were cases in which values of $\Delta\Delta G_b$ for both the mutants E57Q and E58Q were involved in the calculation of $\Sigma\Delta\Delta G_b$. These results indicate that the interaction energy (ΔG_I) between h-Glu-57 and h-Glu-58 is significant. In contrast, the effects of mutating h-Glu-61 and h-Glu-62 were additive (Table 1 and Fig. 2). The results obtained with h-Glu-61 and h-Glu-62 are consistent with the crystal structure (Fig. 1). The side chains of h-Glu-61 and h-Glut-62 are well separated from each other [the two carboxy groups lie about 1 nm (10 Å) apart], and any interaction energy between these two residues would be small. Interpretation of the results obtained with h-Glu-57 and h-Glu-58 is complicated for two reasons: first, the crystal structure in the region of h-Glu-57 and h-Glu-58 is ambiguous and, secondly, there appears to be a change in the mechanism of interaction with the mutant E58Q. The interpretation of the data in terms of the crystal structure is complicated by the fact that h-Glu-57 makes an ion-pair with Arg-75 of a twofold-symmetry-related molecule in the crystal, and it is not known to what extent the conformations of h-Glu-57 and Arg-75 in the crystal structure approximate their conformations in the complex in solution. Moreover, in order to form the salt bridge with h-Glu-58, the N^{η} atoms of Arg-77A have moved by over 0.7 nm (7 Å) (Bode *et al.*, 1989; Rydel *et al.*, 1990), and it has been noted that large conformational changes may result in non-additivity of the mutational effects (Wells, 1990). It follows from the relationship:

$$K_I = k_2/k_1$$

that an increase in K_I could be caused by a decrease in the association rate constant (k_1) and/or an increase in the dissociation rate constant (k_2). In previous studies, increases in the value of K_I for mutants in which the *C*-terminal acidic residues had been replaced were correlated with decreases in the value of k_1 (Braun *et al.*, 1988; Stone *et al.*, 1989). This dependence of the value of k_1 on the charge of the molecule is expected for ionic interactions (Laidler, 1987). For the additional mutants examined in the present study, the same correlation between an increase in K_I and decrease in k_1 was found in all cases, except for E58Q. For this mutant the decrease in affinity was predominantly due to an increase in the value of k_2. This result suggests that there has been a change in the mechanism of interaction with this mutant and that other types of interactions besides ionic ones may have been altered by the replacement of h-Glu-58 by glutamine. Thus the lack of additivity in the case of h-Glu-57 and h-Glu-58 may provide another example of where the non-additivity is due to a change in the mechanism of interaction (Wells, 1990).

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