Ionic interactions in the formation of the thrombin–hirudin complex

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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 an important 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 previously described (Stone & Hofsteenge, 1986). Recombinant hirudin (variant 1) was a gift from Clba–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 mM-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 (kₐ), dissociation rate constant (kₜ) and dissociation constant (Kᵣ). 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-61 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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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·mol\(^{-1}\) for hirudin-form E61Q to 5.9 kJ·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 \( < 0.5 \text{ nm (5 Å)} \) 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-Pro-Pro-Arg-CH\(_3\)-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 \( \Delta G_{\text{mut}}(X) \). 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):

\[
\Delta G_{\text{mut}(X,Y)} = \Delta G_{\text{mut}(X)} + \Delta G_{\text{mut}(Y)} + \Delta G_1
\]  

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

<table>
<thead>
<tr>
<th>Form of hirudin</th>
<th>( 10^{-4} \times k_1 ) (s(^{-1}))</th>
<th>( 10^4 \times k_2 ) (s(^{-1}))</th>
<th>( K_1 ) (pm)</th>
<th>( \Delta G_0 ) (kJ·mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhir*</td>
<td>1.37±0.03</td>
<td>3.17±0.11</td>
<td>0.237±0.006</td>
<td>75.0</td>
</tr>
<tr>
<td>D53A</td>
<td>1.23±0.01</td>
<td>2.61±0.10</td>
<td>0.210±0.005</td>
<td>75.2</td>
</tr>
<tr>
<td>D55N</td>
<td>0.755±0.018</td>
<td>3.12±0.27</td>
<td>0.595±0.021</td>
<td>72.6</td>
</tr>
<tr>
<td>E57Q</td>
<td>0.219±0.002</td>
<td>5.16±0.09</td>
<td>2.30±0.02</td>
<td>69.1</td>
</tr>
<tr>
<td>E58Q</td>
<td>1.09±0.07</td>
<td>19.9±1.7</td>
<td>1.83±0.04</td>
<td>69.7</td>
</tr>
<tr>
<td>E61Q*</td>
<td>0.957±0.020</td>
<td>3.56±0.14</td>
<td>0.372±0.012</td>
<td>73.7</td>
</tr>
<tr>
<td>E62Q*</td>
<td>0.671±0.023</td>
<td>3.70±0.21</td>
<td>0.552±0.025</td>
<td>72.2</td>
</tr>
<tr>
<td>E57,58Q*</td>
<td>0.218±0.006</td>
<td>5.15±0.18</td>
<td>2.36±0.05</td>
<td>69.0</td>
</tr>
<tr>
<td>E61,62Q</td>
<td>0.454±0.014</td>
<td>3.94±0.11</td>
<td>0.87±0.04</td>
<td>71.5</td>
</tr>
<tr>
<td>E57,58,62Q*</td>
<td>0.074±0.001</td>
<td>6.38±0.20</td>
<td>8.60±0.16</td>
<td>65.6</td>
</tr>
<tr>
<td>E57,58,61,62Q*</td>
<td>0.045±0.001</td>
<td>6.30±0.17</td>
<td>14.1±0.2</td>
<td>64.4</td>
</tr>
</tbody>
</table>

* From Braun et al. (1988)
Thrombin–hirudin interaction

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-terminal 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:

\[ k_i = k_{n,k_1} \]

with 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 \( k_1 \). 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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REFERENCES


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