Contribution of the N-Terminal Region of Hirudin to Its Interaction with Thrombin

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ABSTRACT: Hirudin is a 65-residue polypeptide that specifically inhibits thrombin by forming a tight, noncovalent complex with the enzyme. The role of the two amino-terminal valine residues and the N-terminal α-amino group of hirudin in the formation of the complex has been investigated by site-directed mutagenesis and chemical modification. Replacement of the two N-terminal valyl residues of recombinant hirudin by polar amino acids resulted in an increase in the inhibition constant (K_I). In contrast, replacement of these residues by hydrophobic amino acids had little effect on the value for K_I. These results demonstrated that the hydrophobic nature of the N-terminal residues of hirudin was important for its interaction with thrombin. Addition of a single amino acid to the N-terminus of hirudin resulted in a marked increase in the value of K_I. A similar effect was observed when the positive charge of the α-amino group was removed by acetylation. In contrast, amidination of this group, which preserves the positive charge, resulted in a less pronounced increase in the value of K_I. Thus, it appears that a positive charge immediately adjacent to the N-terminal hydrophobic residue is required for optimal binding to thrombin.

Hirudin is a 65-residue protein found in the salivary glands of the medicinal leech *Hirudo medicinalis* (Markwardt, 1970; Bagdy et al., 1976; Dodt et al., 1986). It specifically inhibits the blood-clotting protease thrombin (Walsmann & Markwardt, 1981) and is presumably used, in conjunction with other protease inhibitors (Rigbi et al., 1987), to facilitate the leech's feeding on the blood of its prey. Hirudin is the most potent known inhibitor of thrombin and acts as a slow, tight-binding inhibitor of this enzyme (Stone & Hofsteenge, 1986; Dodt et al., 1988). It has been shown previously that the specificity of the interaction of thrombin with hirudin does not arise solely from the binding of hirudin to the catalytic site of thrombin but that secondary sites on both molecules are also involved (Chang, 1983; Stone et al., 1987; Noë et al., 1988). The availability of recombinant hirudin (Fortkamp et al., 1986; Harvey et al., 1986; Meyhack et al., 1987; Bergmann et al., 1986; Loison et al., 1988) offers the possibility of identifying interaction sites on hirudin by site-directed mutagenesis. Two forms of recombinant hirudin have been produced: the form examined in this study corresponds to the amino acid sequence determined by Bagdy et al. (1976) and Dodt et al. (1984) and is referred to here as r-hir;[1] the second recombinant hirudin variant corresponds to the cDNA sequence determined by Harvey et al. (1986) and is referred to as r-hir-V2. Site-directed mutagenesis has been used to show that the binding of a basic residue of hirudin in the primary specificity pocket of thrombin is not of critical importance for its inhibition of thrombin (Dodt et al., 1988; Braun et al., 1988; Degryse et al., 1989) and that a region of acidic residues in the C-terminal area of hirudin is important for effective binding (Braun et al., 1988).

It has been observed that recombinant hirudin (r-hir) with an eight-residue N-terminal extension had no antithrombin activity (Fortkamp et al., 1986) and another recombinant form (r-hir-V2) with an extra N-terminal methionyl residue had a reduced affinity for thrombin (Loison et al., 1988). These experiments indicate that the N-terminal region of hirudin may be important for its interaction with thrombin. The present paper reports the results of experiments that were performed to investigate this hypothesis more fully. N-Terminally modified hirudins were prepared by using both chemical modification and site-directed mutagenesis, and the inhibitory properties of these hirudin variants were determined. The results suggest the following requirements for the optimal binding of hirudin to thrombin: (1) hydrophobic amino acids should occupy the two N-terminal positions of hirudin; (2) a positive charge should be located immediately adjacent to the N-terminal hydrophobic residue.

Experimental Procedures

Materials. The thrombin substrates D-Phe-Pip-Arg-pNA (S-2238) and D-Val-Leu-Arg-pNA (S-2266) were purchased from Kabi (Molndal, Sweden). The peptide Val-Val-Tyr was purchased from Bachem (Bubendorf, Switzerland). Human thrombin was prepared as described previously (Stone & Hofsteenge, 1986) and was fully active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). Amino acid sequence analysis (Hewick et al., 1981) indicated that thrombin was greater than 99% pure.

Amidolytic Assay of Thrombin. Assays were performed as described previously (Stone & Hofsteenge, 1986) at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% poly(ethylene glycol) M_r 6000.

Site-Directed Mutagenesis, Expression, and Characterization of Mutant Hirudins. Site-directed mutagenesis of the hirudin gene and expression of the mutant hirudins in *Escherichia coli* were performed as described previously (Braun et al., 1988). Hirudins were purified by ion-exchange and reversed-phase chromatography and their structure and purity

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[1] Abbreviations: pNA, *p*-nitroanilide; Pip, pipecolyl; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; r-hir, recombinant hirudin; r-hir-V2, recombinant hirudin variant 2; hir-QQR, recombinant hirudin with the mutations K27Q, K36Q, and K47R; S-DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid.

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verified by amino acid composition analysis (Heinrikson & Meredith, 1984), peptide mapping, and peptide sequencing (Braun et al., 1988). In all cases, the protein chemical data agreed with the DNA sequence.

Chemical Modification of the α-Amino Group of Hirudin. In order to achieve specific modification of the α-amino group, a mutant hirudin was prepared in which the lysine residues at positions 27, 36, and 47 were replaced by Gln-27, Gln-36, and Arg-47, respectively. The resulting mutant is referred to as hir-QQR.

(A) Preparation of Acetylated hir-QQR. hir-QQR (10 nmol, 74 μg) was dissolved in 50 μL of 300 mM sodium phosphate buffer, pH 7.5, and 50 μL of acetic anhydride was added. The mixture was vortexed vigorously and cooled on ice for 1 h with additional vortexing every 10 min. The mixture was then diluted with 500 μL of ice-cold distilled water, frozen in dry ice, and lyophilized. Since this procedure may also acetylate the side chains of tyrosyl residues, the product was treated with 50 μL of 1 M hydroxylamine hydrochloride in 300 mM sodium phosphate, pH 7.5, for 10 min at room temperature. The mixture was diluted with 1 mL of water, frozen in dry ice, and lyophilized. The product was redissolved in 0.1% (v/v) TFA and separated from the unreacted material by reversed-phase HPLC on an Aquapore C_8 column using a gradient of 10–40% acetonitrile in 0.1% (v/v) TFA. The acetylated derivative was digested with thermolysin as described previously (Braun et al., 1988). Three peptides were obtained by reversed-phase HPLC; only one of the peptides had an altered retention time compared with the peptide map of unmodified hir-QQR. Amino acid analysis of this peptide indicated that it corresponded to residues 1–29 of hir-QQR, and sequence analysis showed that the N-terminus of this peptide was blocked.

(B) Amidination of hir-QQR with Ethyl Acetimidate. Ten nanomoles (74 μg) of hir-QQR was dissolved in 10 μL of 8% sodium bicarbonate. Five microliters of 1 M ethyl acetimidate in ethanol was added and the mixture incubated at room temperature for 30 min. A further 5 μL of the ethyl acetimidate solution was then added; at the same time, 10 μL of 8% sodium bicarbonate was added to maintain the pH of the reaction mixture at approximately 8.0. This step was repeated every 30 min for a total of 6 times, after which the mixture was allowed to stand overnight at room temperature. The ethanol was removed by evaporation in a Speedvac concentrator (Savant, Hicksville, NY) and the remaining solution diluted with 100 μL of distilled water. The product was isolated by reversed-phase HPLC.

Determination of pK_a Values for α-Amino Groups of Native and Denatured hir-QQR and Val-Val-Tyr. The pH dependence of the initial rate of reaction between the α-amino group and 2,4,6-trinitrobenzenesulfonic acid (TNBS) was determined essentially as described in procedure 2 of Fields (1971). Briefly, Val-Val-Tyr, native hir-QQR, or reduced and carboxamidomethylated hir-QQR [prepared by the method of Crestfield et al. (1963)], at a concentration of 1 μM, was reacted with TNBS (10 mM) in the presence of 1 mM sodium sulfite. The reactions were performed over the pH range from 7.5 to 10.0 in sodium phosphate–tetraborate buffers with a constant ionic strength of 0.125 M (Ellis & Morrison, 1982). The formation of (2,4,6-trinitrophenyl)amino–sulfite complex was detected by its absorbance at 420 nm.

Data Analysis

Determination of Concentrations of Hirudin Variants. The concentration of active hirudin molecules that formed a tight complex with thrombin was determined by titration of 1.0 nM thrombin in the presence of 200 μM D-Val-Leu-Arg-pNA. The dependence of the steady-state velocity on the amount of hirudin present in the assay could be described by eq 1 (Stone & Hofsteenge, 1986)

$$v_s = (v_0/2E_t) \times \{[(K_{I'} + \alpha I_\alpha - E_t)^2 + 4K_{I'}E_t]^{1/2} - (K_{I'} + \alpha I_\alpha - E_t)\} \quad (1)$$

where v_s is the observed steady-state velocity, v_0 is the steady-state velocity in the absence of hirudin, E_t is the total molar enzyme concentration, $K_{I'}$ is the apparent inhibition constant, I_α is the microliters per milliliter hirudin added, and α is the molar concentration of 1.0 μL/mL hirudin. Analysis of the data yielded values for α that could be used to calculate the concentrations of hirudin in the stock solutions. In this manner, the molar concentrations of the mutant hirudins V1,2I, V1,2F, V1,2L, V1L, V2L, hir-QQR, and N^α-acetimidylated hir-QQR were determined. For the mutants V1,2S, V1,2K, V1,2G, V1,2E, V1E, V2E, M0-hir, G0-hir, G0-hir-QQR, and the acetylated hir-QQR, the binding to thrombin was not tight enough to permit the determination of the molar concentration by using the above procedure. Concentrations for these mutants were obtained by amino acid analysis, and these preparations were assumed to contain only active molecules.

Determination of Inhibition Constants. Three types of inhibition were observed, and these corresponded to classical, tight-binding, and slow, tight-binding inhibition in the nomenclature of Morrison (1982). It should be noted that the distinction between the three types of inhibition is somewhat arbitrary and will depend on the reaction conditions [see Morrison and Walsh (1987)]. Classical inhibition was observed with mutants that exhibited a low affinity for thrombin under the chosen reaction conditions and, concentrations of hirudin at least 10 times the concentration of thrombin were used in order to obtain inhibition. Data for such mutants were obtained in the presence of 200 μM D-Val-Leu-Arg-pNA or 50 μM D-Phe-Pip-Arg-pNA and were fitted to the Dixon equation (Segel, 1975) by weighted linear regression. These analyses yielded values for the apparent inhibition constant ($K_{I'}$). The value for the true inhibition constant (K_I) can be calculated from the value for $K_{I'}$ by using the expression (Stone & Hofsteenge, 1986):

$$K_{I'} = K_I(1 + S/K_m) \quad (2)$$

where S is the substrate concentration and K_m is the Michaelis constant for the substrate used. A value of 298 μM was determined for the K_m of D-Val-Leu-Arg-pNA, and this value was used in the calculations for experiments using this substrate. In experiments using D-Phe-Pip-Arg-pNA, the previously determined values for K_m (Stone & Hofsteenge, 1986; Stone et al., 1989) were used in the calculations.

Tight-binding and slow, tight-binding inhibition data were obtained in the presence of 100 μM D-Phe-Pip-Arg-pNA. Data conforming to tight-binding inhibition were fitted to eq 1 of Stone and Hofsteenge (1986) by nonlinear regression. These analyses yielded values for $K_{I'}$, and values for K_I were calculated as described above.

The slow, tight-binding inhibition of thrombin by hirudin can be represented by the scheme (Stone & Hofsteenge, 1986):

$$\text{thrombin} + \text{hirudin} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{thrombin–hirudin}$$

Slow, tight-binding inhibition data were fitted to eq 4 of Stone and Hofsteenge (1986) by nonlinear regression. These analyses yielded apparent values for K_I, the association rate constant (k_1), and the dissociation rate constant (k_2). The true values were calculated as previously described (Braun et al., 1988).

Table I: Inhibition Constants of Hirudins with Modifications to the α-Amino Group[a]

form of hirudin	type of inhibition	K_I (pM)	$-\Delta G^\circ_b$ (kJ mol^{-1})	N-terminal structure[b]
		Modifications to r-hir		
r-hir[c]	slow, tight	0.231 (0.006)	75.0	$^+NH_3$—
M0-hir	tight	5430 (330)	49.0	$^+NH_3CH[(CH_2)_2SCH_3]C(=O)NH$—
G0-hir	tight	488 (52)	55.3	$^+NH_3CH_2C(=O)NH$—
		Modifications to hir-QQR		
hir-QQR[d]	slow, tight	1.39 (0.02)	70.4	$^+NH_3$—
acetylated hir-QQR	classical	9340 (1220)	47.7	$CH_3C(=O)NH$—
N^α-acetimidyl-hir-QQR	tight	24.3 (3.5)	63.0	$CH_3C(=^+NH_2)NH$—
G0-hir-QQR	tight	4120 (540)	49.8	$^+NH_3CH_2C(=O)NH$—

[a] Assays were performed and data were analyzed according to the appropriate equation for the type of inhibition observed as described under Experimental Procedures. The estimates of K_I are given together with their standard errors in parentheses after the determined value. [b] The structure given represents the N-terminal structure of each hirudin; the right-hand N is that of the amino group of Val-1. [c] This value was determined previously (Braun et al., 1988). [d] The values of k_1 and k_2 for hir-QQR were $(1.43 \pm 0.08) \times 10^8$ M^{-1} s^{-1} and $(1.99 \pm 0.11) \times 10^{-4}$ s^{-1}, respectively.

Determination of the pK_a of α-Amino Groups. The reaction of TNBS with the α-amino group was followed by the increase in absorbance at 420 nm. Values obtained for the initial rate of reaction (v_i) were used to determine the pK_a of the reacting amino group. The variation of v_i with pH was described by eq 3 where v_i is the initial rate of reaction at a particular pH

$$v_i = \frac{V_{max}}{1 + H/K_a} \quad (3)$$

value, V_{max} is the maximum rate of reaction, H is the hydrogen ion concentration, and K_a is the dissociation constant of the α-amino group. Values of v_i were weighted according to the inverse of their standard errors and fitted to eq 3 by weighted linear regression to yield an estimate of K_a.

Effect of Ionic Strength on Formation of the Thrombin–Hirudin Complex. The standard Gibbs free energy change for the formation of the thrombin–hirudin complex, referred to in this paper as binding energy (ΔG°_b), can be related to K_I by eq 4 where R is the gas constant and T is the absolute

$$\Delta G^\circ_b = RT \ln K_I \quad (4)$$

temperature. ΔG°_b can be divided into components due to ionic and nonionic interactions. The contribution due to ionic interactions will be dependent on the ionic strength (I), and eq 5 has been shown to describe the effect of ionic strength on the value of ΔG°_b for the interaction between thrombin and hirudin (Stone et al., 1989):

$$\Delta G^\circ_b = \Delta G^\circ_{nio} + \Delta G^\circ_{io0} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (5)$$

where ΔG°_{nio} is the contribution of nonionic interactions to the complex formation, ΔG°_{io0} is the contribution of ionic interactions at an ionic strength of zero, and C_1 is a constant related to the Debye–Hückel screening parameter. For two mutants (V1,2G and V2E), the dependence of ΔG°_b on ionic strength was determined. Values of ΔG°_b were weighted according to the inverse of their standard errors and fitted to eq 5 by weighted nonlinear regression to yield estimates for ΔG°_{nio} and ΔG°_{io0} for these mutants.

Results

Effect of Addition of Amino Acid Residues to the N-Terminus of Hirudin. The importance of the N-terminal region of hirudin for its inhibitory properties was suggested by the observation that an additional methionyl residue at the N-terminus of r-hir-V2 decreased its affinity for thrombin (Loison et al., 1988). In order to test whether the effect of the additional methionine was peculiar to this variant (r-hir-V2), the mutant M0-hir that contains an additional methionyl residue on the N-terminus of r-hir was constructed. Kinetic analysis of this hirudin variant showed that its inhibition constant (K_I) had increased 20 000-fold compared to r-hir, which corresponds to a 26 kJ mol^{-1} decrease in binding energy (Table I). Treatment of this mutant with CNBr resulted in removal of the N-terminal methionine as determined by peptide sequence analysis and yielded a molecule with an affinity for thrombin that was indistinguishable from that of r-hir (data not shown). Methionine has a large hydrophobic side chain, and the effect of the additional methionyl residue could be due either to this side chain or to the displacement of the α-amino group. In order to test these possibilities, an additional glycine was added to r-hir instead of the methionine. This mutant (G0-hir) also displayed a reduced affinity for thrombin; its binding energy was reduced by 19.7 kJ mol^{-1}. Thus, the effect observed seems to be largely due to the displacement of the α-amino group.

Availability of the α-Amino Group for Chemical Modification and Determination of Its pK_a Value. The α-amino group could be important for the maintenance of the tertiary structure of hirudin or be involved in a direct interaction with thrombin. Two-dimensional NMR studies indicate that the two N-terminal valyl residues of hirudin do not make any contacts with the rest of the molecule (Clore et al., 1987; Sukumaran et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989) which suggests that, under the conditions of the NMR experiments, the α-amino group is probably not involved in the maintenance of hirudin's tertiary structure. The experiments of Clore, Gronenborn, and co-workers (Clore et al., 1987; Sukumaran et al., 1987; Folkers et al., 1989) were, however, conducted at low pH such that an intramolecular salt bridge between the α-amino group and a carboxyl group would not be favored because the carboxyl groups would be largely protonated. In order to examine whether the α-amino group is involved in an intramolecular salt bridge at neutral pH, the availability of the group for chemical modification and its pK_a have been determined. If the α-amino group is involved in an intramolecular salt bridge, it would not be expected to be readily available for chemical modification and its pK_a would be perturbed.

Hirudin contains four primary amino groups that would react with modifying reagents: Lys-27, Lys-36, Lys-47, and the α-amino group. In order to facilitate specific modification of the α-amino group, a hirudin molecule was constructed that contained the following replacements: K27Q, K36Q, and K47R (referred to as hir-QQR). This mutant had a decreased affinity for thrombin compared with r-hir (Table I); its K_I value is about the same as that previously observed for the mutant in which Lys-47 was replaced by Gln (K47Q; Braun et al., 1988). The α-amino group of hir-QQR was readily

Table II: Kinetic Constants for the Inhibition of Thrombin by Hirudins with Various Substitutions of the Two N-Terminal Valines[a]

form of hirudin	type of inhibition	K_I (pM)	$-\Delta G_b^\circ$ (kJ mol^{-1})	$k_1 \times 10^{-7}$ (M^{-1} s^{-1})	$k_2 \times 10^5$ (s^{-1})
r-hir[b]	slow, tight	0.231 (0.006)	75.0	13.7 (0.3)	3.17 (0.11)
V1,2I	slow, tight	0.099 (0.009)	77.2	17.7 (0.7)	1.75 (0.17)
V1,2F	slow, tight	0.238 (0.008)	74.9	16.2 (0.4)	3.86 (0.16)
V1,2L	slow, tight	9.91 (0.47)	65.3	6.0 (2.0)	59.5 (20.0)
V1,2S	tight	175 (16)	57.9		
V1,2K	tight	152 (16)	58.3		
V1,2G	classical	694 (32)	54.4		
V1,2E	classical	67000 (4600)	42.6		
V1L	tight	0.235 (0.030)	75.0		
V2L	tight	10.3 (0.7)	65.2		
V1E	classical	295 (44)	56.6		
V2E	classical	248 (9)	57.0		

[a] Assays were performed and data were analyzed according to the appropriate equation for the type of inhibition observed as described under Experimental Procedures. The estimates of the constants are given together with their standard errors in parentheses. [b] This value was determined previously (Braun et al., 1988).

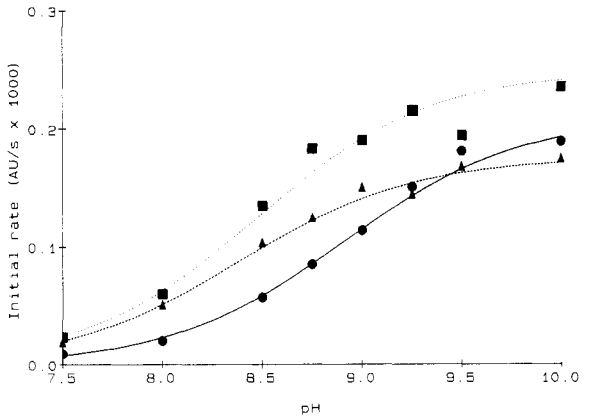

FIGURE 1: Determination of the pK_a's of the α-amino group of the peptide Val-Val-Tyr, denatured hir-QQR, and native hir-QQR. Initial rates of reaction of the α-amino group with TNBS were obtained as described under Experimental Procedures for carboxamidomethylated and undenatured hir-QQR and the peptide. The plot shows the relationship between pH and observed rate for peptide (■···■) and carboxamidomethylated (●—●) and native (▲---▲) hir-QQR. The curves represent a weighted least-squares fit of the data to eq 3.

available for reaction with TNBS, acetic anhydride, and ethyl acetimidate (data not shown). The reaction with TNBS was utilized to determine the pK_a of the α-amino group in the native hir-QQR, and in the reduced and carboxamidomethylated protein. From the data shown in Figure 1, pK_a values of 8.39 ± 0.05 and 8.90 ± 0.04 were determined for the α-amino group of the native and denatured hir-QQR, respectively. These values compare well with the determined pK_a of 8.47 ± 0.04 for the N-terminal peptide fragment Val-Val-Tyr. These results indicate that the α-amino group in native hir-QQR is freely accessible to solvent and, given that its pK_a value approximates that of Val-Val-Tyr, it appears not to be involved in intramolecular interactions.

Effect of Modification of the α-Amino Group on the Affinity of Hirudin for Thrombin. Exhaustive reaction with acetic anhydride or ethyl acetimidate resulted in molecules with a blocked α-amino group. It was shown by peptide mapping of the final purified products that no other modification had occurred in the molecules. The structures of the modifying groups, as well as the kinetic properties of the modified hirudins, are shown in Table I. Acetylation of the α-amino group resulted in a considerable loss of binding energy (22.7 kJ mol^{-1}, Table I). This loss was largely due to the removal of the positive charge from the N-terminus, since modification with the similarly sized acetimidyl group resulted in a much smaller loss of binding energy (7.4 kJ mol^{-1}, Table I). The effect on binding energy due to displacement of the positively charged α-amino group of hir-QQR was examined by placing an additional glycine on the N-terminus of hir-QQR. The binding energy of the resultant mutant (G0-hir-QQR) was reduced by 20.6 kJ mol^{-1} (Table I), a similar effect to that observed for G0-hir (Table I). These results illustrate the importance of the position of the α-amino group relative to the N-terminus of r-hir.

Effect of Substitution of the Two N-Terminal Valyl Residues. The importance of the paired hydrophobic residues at positions 1 and 2 of hirudin was examined by site-directed mutagenesis. Replacement of Val-1 and Val-2 by other hydrophobic residues (Ile, Phe, or Leu) resulted in minor changes in binding energy (Table II). Substitution of valine by isoleucine (V1,2I) resulted in a decrease in the value of K_I (0.099 pM compared with 0.231 pM for r-hir). In contrast, substitution by phenylalanine was without effect. The largest effect for the substitution with a hydrophobic residue was observed when the valyl residues were replaced by leucyl residues; a 40-fold increase in the value of K_I corresponding to a decrease of 9.7 kJ mol^{-1} in the binding energy was observed (Table II). Kinetic analysis of the inhibition of thrombin by V1,2L indicated that the increase in K_I was due mainly to an increase in the dissociation rate constant (k_2). By constructing the single mutants V1L and V2L, it could be shown that the decrease in affinity was entirely due to the substitution at position 2; whereas V1L exhibited a K_I value indistinguishable from r-hir, the value for V2L was the same as that observed for V1,2L (Table II).

Replacement of Val-1 and Val-2 by uncharged polar residues resulted in large decreases in affinity. Substitution by glycine yielded a molecule (V1,2G) that exhibited a 3000-fold increase in its K_I value which corresponds to a decrease in binding energy of 20.6 kJ mol^{-1} (Table II). The observed decrease in binding energy for the serine mutant (V1,2S) was somewhat smaller (17.1 kJ mol^{-1}; Table II).

Substitution of Val-1 and Val-2 by charged residues indicated that positively charged residues were much better tolerated in positions 1 and 2 than negatively charged residues. The replacement of Val-1 and Val-2 by lysyl residues resulted in a mutant (V1,2K) with affinity for thrombin similar to that of V1,2S; the binding energy of V1,2K was reduced by 16.7 kJ mol^{-1} compared with that of r-hir (Table II). When both valyl residues were replaced by glutamyl residues in mutant V1,2E, a decrease of 32.4 kJ mol^{-1} in binding energy was observed (K_I = 67 nM; Table II). Two mutants with a single amino acid substitution (V1E and V2E) were constructed to assess the importance of each of the substitutions. Both mutants exhibited about the same affinity for thrombin; the K_I values were 295 and 248 pM for V1E and V2E, respectively

Table III: Contribution of Ionic and Nonionic Interactions to the Binding Energy of Two Hirudin Molecules with Substitutions of the N-Terminal Valyl Residues[a]

form of hirudin	$-\Delta G^{\circ}_{nio}$ (kJ mol^{-1})	$-\Delta G^{\circ}_{io0}$ (kJ mol^{-1})
r-hir[b]	62.2 ± 2.4	25.0 ± 0.7
V1,2G	41.5 ± 2.0	27.4 ± 0.8
V2E	47.8 ± 1.3	22.0 ± 0.8

[a] Assays for the mutants V1,2G and V2E were performed at 13 different ionic strengths between 0.05 and 0.625 in the presence of 50 μM D-Phe-Pip-Arg-pNA, and the data were analyzed according to the Dixon equation for classical inhibition. Previously determined K_m values for the substrate (Stone et al., 1989) were used to calculate the values for K_I at each ionic strength. Analysis of these data as described under Experimental Procedures yielded the values for ΔG°_{gio} and ΔG°_{io0} which are given together with their standard errors. ΔG°_{nio} and ΔG°_{io0} represent the nonionic and ionic contributions, respectively, to the binding energy. [b] This value was determined previously (Stone et al., 1989).

(Table II). The decreases in binding energy were 18.4 and 18.0 kJ mol^{-1} for V1E and V2E, respectively. The decreases in binding energy observed for V1E and V2E were about half of that observed for V1,2E which suggests that the effects of the mutations were additive.

Evaluation of the Contributions of Ionic and Nonionic Interactions to the Binding Energy of Two Mutants. It has been shown recently that, by examining the effect of ionic strength on the inhibition of thrombin by hirudin, it is possible to divide the binding energy for hirudin into ionic and nonionic contributions (Stone et al., 1989). The above mutations were designed to affect hydrophobic interactions between the N-terminal region of hirudin and thrombin. For substitutions where large decreases in binding energy have been observed, it would be of interest to determine whether only the hydrophobic interactions with the N-terminal region have been affected or whether the disruption of the interactions with the N-terminal region has resulted in a misalignment of hirudin in the complex such that other interactions have also been perturbed. This question can be partially answered by determining whether the ionic interactions that occur mainly with the C-terminal region of hirudin (Stone et al., 1989) have been altered. The contributions of ionic and nonionic interactions to binding energy were assessed for two mutants (V1E and V1,2G), and the results are given in Table III. In both cases, the decrease in binding energy was predominantly due to a decrease in the nonionic contribution (ΔG°_{nio}). Thus, it can be concluded that any misalignment of hirudin in the complex caused by the replacement of the N-terminal hydrophobic residues has not been large enough to affect the ionic interactions between the C-terminal region of hirudin and thrombin.

Discussion

Previous studies have shown that the tight interaction between hirudin and thrombin involves a number of different areas on both the inhibitor and the protease (Chang, 1983; Stone et al., 1987; Noë et al., 1988; Braun et al., 1988). The C-terminal region of hirudin has been shown to be important for its interactions with thrombin (Chang, 1983; Krstenansky & Mao, 1987; Krstenansky et al., 1987; Braun et al., 1988; Stone et al., 1989). The results presented here indicate that the α-amino group and Val-1 and Val-2 of hirudin also make a significant contribution to the formation of the thrombin–hirudin complex. In fact, the contribution made by the N-terminal hydrophobic residues appears to be greater than that made by the acidic residues in the C-terminal region. Under the assay conditions used in the present study, mutation of Glu-57, -58, -61, and -62 to glutamine resulted in a 11 kJ mol^{-1} decrease in binding energy (Braun et al., 1988) whereas mutation of Val-1 and -2 to polar residues caused a 17–20 kJ mol^{-1} decrease (V1,2S, V1,2G; Table II).

The importance of the positively charged α-amino group was demonstrated by the effect which acetylation of this group had on the binding energy (Table I). Two possible roles of the positive charge might be envisaged: either the N-terminus interacts directly with thrombin, or it is involved in maintenance of the tertiary structure of hirudin in an optimal conformation for thrombin inhibition. The former possibility appears to be more likely for the following reasons. First, two-dimensional NMR studies have shown that the N-terminal region up to Tyr-3 had no structural features, indicating that the residues Val-1 and Val-2 do not interact with the rest of the hirudin molecule (Clore et al., 1987; Sukumaran et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989). Second, the α-amino group in native hir-QQR was readily modified by TNBS, acetic anhydride, and ethyl acetimidate, suggesting that it is freely exposed to the solvent. Similar results have been obtained with 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid (S-DABITC) by Chang (1989). Third, the pK_a value of the α-amino group was found to be similar for both denatured and nondenatured hir-QQR, and was comparable to that of the N-terminal peptide fragment Val-Val-Tyr (Figure 1). These results, when considered together, suggest that the α-amino group is not involved in an intramolecular salt bridge that stabilizes the tertiary structure of hirudin. The recent results of Chang (1989) also favor the proposal that the α-amino group is directly involved in an interaction with thrombin. Although the α-amino group was readily modified by S-DABITC in uncomplexed hirudin, it was protected against modification in the thrombin–hirudin complex. In contrast, the ϵ-amino group of Lys-27 was modified to an equal extent in free and complexed hirudin (Chang, 1989). The positively charged α-amino group must, however, be located precisely adjacent to Val-1. Displacement of the α-amino group by an extra residue as in G0-hir or G0-hir-QQR results in a marked decrease in binding energy. The α-amino group of hirudin presumably makes an ionic interaction with a carboxylate in thrombin, and when the α-amino group is displaced by a residue, this interaction is no longer possible or is much weaker. Additional unfavorable interactions probably also occur in accommodating the extra residue.

All naturally occurring hirudins, whose primary structures have been determined, have an N-terminal hydrophobic amino acid with branched side chain (Bagdy et al., 1976; Dodt et al., 1986; Harvey et al., 1986). The importance of the hydrophobic nature of the N-terminus to the strength of the hirudin–thrombin interaction was demonstrated by the observation that replacement of the two N-terminal valyl residues by polar amino acids caused a marked decrease in the binding energy. Conservative replacements by other hydrophobic amino acids resulted in only moderate changes in the strength of inhibition (Table II). With hydrophobic replacements, only the substitution by leucine in the second position caused a significant decrease in binding energy. It is not clear what particular properties of leucine have caused the decrease in binding energy. The leucyl side chain is branched at C$^{\gamma}$, while the valyl and isoleucyl side chains are branched at C$^{\beta}$. It is possible that the branching of the side chain at C$^{\gamma}$, which results in a greater rotational freedom of the peptide backbone and side chains, causes the reduced affinity with leucine. However, no reduction in affinity is observed with phenylalanine in the second position, and, thus, the exact geometry of the atoms

around the C^{γ} appears to be important also. By far, the largest decrease in binding energy was observed when Val-1 and -2 were replaced by glutamate. In contrast, the effect of substitution by the lysine was much smaller (Table II). The differential effect observed between the mutants V1,2E and V1,2K may be due to the effect of the glutamate replacement on the proposed ionic interaction made by the α-amino group. The presence of a negatively charged residue in the first and second positions would disrupt such an interaction. On the other hand, the positively charged lysyl residue would also be able to participate in this ionic interaction, and the presence of a carboxylate would make it energetically less unfavorable to bind the positively charged lysyl residue in a hydrophobic pocket.

At present, no definitive information is available regarding the site on thrombin that is involved in the hydrophobic interaction with the N-terminus of hirudin. The presence of an apolar binding region on thrombin that is located immediately adjacent to the active site has been proposed on the basis of displacement studies with proflavin dyes (Berliner & Shen, 1977) and indole derivatives (Conery & Berliner, 1983). Other evidence suggests that the hydrophobic D-Phe-Pro portion of the thrombin inactivator D-Phe-Pro-ArgCH_2Cl is also bound to this apolar binding site (Sonder & Fenton, 1984). Thrombin inactivated with D-Phe-Pro-ArgCH_2Cl has a much reduced affinity for hirudin; the binding energy is reduced by about 35 kJ mol^{-1}. Thus, it is apparent that the binding sites for the D-Phe-Pro moiety and for hirudin must overlap, and the apolar binding site adjacent to the active site would appear to be a possible binding site for the N-terminus of hirudin. Any binding site for the N-terminus of hirudin should also contain a carboxylate, either within the hydrophobic pocket or on the surface of the pocket, that would be capable of forming a salt bridge with the α-amino group of hirudin. Further insight into this aspect may be obtained by model building once the tertiary structure of thrombin becomes available (Skrzypczak-Jankun et al., 1989).

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