Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T., & Iwanaga, S. (1990) *J. Biol. Chem. 265*, 16068–16073.
Teng, C.-M., & Seegers, W. H. (1981) *Thromb. Res. 23*, 255–263.
Willis, T. W., & Tu, A. T. (1988) *Biochemistry 27*, 4769–4777.
Willis, T. W., Tu, A. T., & Miller, C. W. (1989) *Thromb. Res. 53*, 19–29.

Interaction of the N-Terminal Region of Hirudin with the Active-Site Cleft of Thrombin

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ABSTRACT: Site-specific substitutions of the first five amino acids of the thrombin inhibitor hirudin have been made and the effects of these substitutions on the kinetics of formation of the thrombin–hirudin complex evaluated. The effects of different substitutions of Val1 indicate that nonpolar interactions play a major role in the binding of this residue. In the second position (Val2), polar amino acids were better accommodated than in the first. The mutant with arginine in the second position bound particularly well to thrombin; its dissociation constant was 9-fold lower than that of wild-type recombinant hirudin. Comparison of the effects of single and double mutations involving Val1 and Val2 indicates that there was no cooperativity in the binding of these two residues. Elimination of the hydrophobic interactions made by the aromatic ring of Tyr3 of hirudin resulted in a large loss of binding energy (12.7 kJ mol^{-1}). Replacement of Thr4 of hirudin by serine and alanine suggested that both the γ-methyl and the hydroxyl group of the threonine were important in the stabilization of the thrombin–hirudin complex. Replacement of Asp5 of hirudin by alanine and glutamate caused about the same loss in binding energy (5 kJ mol^{-1}). The effects of site-specific substitutions are discussed in terms of the crystal structure of the thrombin–hirudin complex. Molecular modeling provided plausible explanations for many of the observed effects. For instance, such studies suggested that the improved binding of the mutant with arginine in the second position could be due to an interaction of the arginine with the primary specificity pocket.

The crystal structures of thrombin–hirudin complexes indicate that hirudin inhibits thrombin by a previously unobserved mechanism for proteinase inhibition (Rydel et al., 1990, 1991; Grütter et al., 1990). The three N-terminal amino acids of hirudin are bound in the active-site cleft of thrombin while the C-terminal region of hirudin is bound to a positively charged surface groove on thrombin. The first three residues of hirudin form a parallel β-sheet with residues Ser214–Gly219[1] of thrombin. The orientation of the hirudin polypeptide chain within the active site is opposite of that observed with other inhibitors which form an antiparallel β-sheet with residues Ser214–Gly219. The α-amino group of hirudin forms hydrogen bonds with the carbonyl of Ser214 and the hydroxyl of Ser195 (Rydel et al., 1990, 1991). As shown in Figure 1, the side chain of the first amino acid [Val1′ in recombinant hirudin variant 1 (rhir)[2] and Ile1′ in recombinant hirudin variant 2, Lys47] makes numerous nonpolar interactions with thrombin residues including His57, Tyr60A, Trp60D, and Leu99 (Rydel et al., 1990, 1991; Grütter et al., 1990). The second residue of hirudin is located at the edge of the primary specificity pocket but, unlike other inhibitors of serine proteinases, does not penetrate this pocket. Tyr3′ is buried in a hydrophobic pocket constituted by residues Tyr60A, Ile174, Trp60D, and Leu99 of thrombin (Figure 1). This binding site is occupied by the D-phenylalanine of the inhibitor D-Phe-Pro-ArgCHCl$_2$ and has been called the aryl-binding pocket (Bode et al., 1989, 1990). The active site of thrombin is closed off by the N-terminal core domain of hirudin, and Thr4′ and Asp5′ seem to be important for the positioning of the core domain. A hydrogen bond between O^{γ} of Thr4′ and the ϵ-amino group of Lys47′ is thought to stabilize and position the core domain (Rydel et al., 1990, 1991). The orientation of the core domain is also aided by a salt bridge made between Asp5′ and Arg221A of thrombin (Figure 1; Rydel et al., 1990, 1991). This salt bridge should also make a direct contribution to the binding energy.

Previous investigations established a role for hydrophobic interaction with the three N-terminal amino acids of hirudin in the formation of the thrombin–hirudin complex (Wallace et al., 1989; Winant et al., 1991; Lazar et al., 1991). In the present study, site-directed mutagenesis has been used to study cooperative effects in the binding of the first two amino acids. In addition, the importance of the interactions made by Tyr3′, Thr4′, and Asp5′ has been examined. The changes in the binding energies (ΔG°_{b}) for the mutant hirudins were interpreted in terms of the crystal structures of the complexes and allowed the quantitative assessment of the importance of contacts revealed by these structures.

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[1] The numbering of the thrombin is that of Bode et al. (1989) and is based on chymotrypsin numbering. Residues in recombinant hirudin are distinguished from those in thrombin by the use of primed numbers.
[2] Abbreviation: rhir, recombinant hirudin variant 1.

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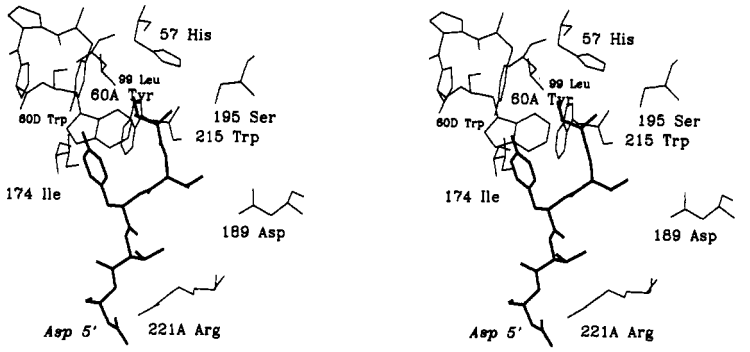

FIGURE 1: Stereodiagram plot of the binding of the first five amino acids of rhir to the active-site cleft of thrombin. rhir is shown in thick lines. In general, only thrombin residues directly involved in interactions with rhir are shown. The structure shown is that of the complex of thrombin with recombinant hirudin variant 2 Lys47 (Rydel et al., 1990, 1991); valyl residues were substituted for Ile1′ and Thr2′ of recombinant hirudin variant 2 Lys47 by molecular modeling.

EXPERIMENTAL PROCEDURES

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

Amidolytic 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 0.2% (w/v) poly(ethylene glycol) M_r 6000.

Site-Directed Mutagenesis, Purification, and Characterization of Mutated Forms of Hirudin. Mutations were introduced using standard methods of site-directed mutagenesis in M13 (Kunkel, 1985). The mutated proteins were expressed in *Escherichia coli* and isolated as described by Braun et al. (1988). Mutant hirudins were characterized by N-terminal sequencing and amino acid composition (Knecht & Chang, 1987). The concentration of the mutant Y3A was determined by amino acid analysis. The concentrations of all other hirudin variants were determined by titration of thrombin in the presence of D-Val-Leu-Arg-*p*-nitroanilide as described by Wallace et al. (1989).

DATA ANALYSIS

Determination of Kinetic Parameters. The inhibition of thrombin by hirudin can be represented by the mechanism shown in Scheme I (Stone & Hofsteenge, 1986). An equation that relates the association rate constant (k_1), the dissociation rate constant (k_2), and the dissociation constant (K_i) is also given in Scheme I.

Scheme I

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad K_i = k_2/k_1$$

Most of the forms of hirudin were tight, slow-binding inhibitors of thrombin under the conditions of the assay. For these forms, estimates of k_1, k_2, and K_i were determined from progress curve experiments as described by Braun et al. (1988). Each progress curve experiment consisted of six assays in the presence of 100 μM D-Phe-pipecolyl-Arg-*p*-nitroanilide and 10–50 pM thrombin; one assay did not contain hirudin, and the five others had different concentrations of hirudin. The progress curves of the thrombin-catalyzed formation of *p*-nitroaniline were analyzed by nonlinear regression and the estimates of the parameters calculated as described previously (Braun et al., 1988).

For the hirudin forms V1K and V2G, slow-binding inhibition was not observed with the concentrations necessary to achieve inhibition. Assays for these forms were performed in the presence of 50 μM D-Phe-pipecolyl-Arg-*p*-nitroanilide and 50 pM thrombin. Steady-state velocity data were fitted to the equation for tight-binding inhibition to yield estimates for the dissociation constant as described previously (Stone & Hofsteenge, 1986; Wallace et al., 1989).

The hirudin form Y3A behaved as a classical competitive inhibitor of thrombin. The conditions for the assays to determine K_i were as described above for tight-binding inhibition. Initial velocity data were fitted to the Dixon equation by using weighted, robust linear regression to yield estimates of the apparent dissociation constant (Cornish-Bowden & Endrenyi, 1981), and the values of K_i were calculated as described by Segel (1975).

The kinetic parameters for each form of hirudin were determined at least twice, and the values given represent the weighted means of these determinations.

The Gibbs standard free energy change for the formation of the complex between hirudin and thrombin (denoted as binding energy, ΔG°_b) was calculated by using eq 1 where R

$$\Delta G^\circ_b = RT \ln K_i \qquad (1)$$

is the gas constant and T is the absolute temperature of the assay. The change in the binding energy caused by a particular mutation ($\Delta\Delta G^\circ_b$) is defined relative to the binding energy of rhir.

Additivity of Mutational Effects. The change in binding energy for a double mutant at positions x and y can be related to those for the single mutant by eq 2 (Carter et al., 1984; Ackers & Smith, 1985; Wells, 1990) where $\Delta\Delta G^\circ_{b(x,y)}$ is the

$$\Delta\Delta G^\circ_{b(x,y)} = \Delta\Delta G^\circ_{b(x)} + \Delta\Delta G^\circ_{b(y)} + \Delta G^\circ_I \qquad (2)$$

change in binding energy for the double mutation, $\Delta\Delta G^\circ_{b(x)}$ and $\Delta\Delta G^\circ_{b(y)}$ are the changes for the two single mutations, and 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 measures the cooperativity between the two sites and may be positive or negative depending on whether the effect of the mutations on the interaction between the residues has been favorable or unfavorable. If there is no cooperativity between the two sites ($\Delta G^\circ_I = 0$), a plot of the decrease in binding energy for the double mutation against the sum of the decreases in binding energy for the single mutations should have a slope of 1.

RESULTS

Substitution of Val1′ and Val2′. It had previously been shown that replacement of Val1′ by glutamate caused the same

Table I: Kinetic Parameters for the Inhibition of Thrombin by Mutants of Hirudin with Substitutions of the First Two Amino Acids[a]

form of hirudin	$10^{-8} \times k_1$ (M^{-1} s^{-1})	$10^5 \times k_2$ (s^{-1})	K_i (pM)	$-\Delta G^\circ_b$ (kJ mol^{-1})	$\Delta\Delta G^\circ_b$ (kJ mol^{-1})
rhir[b]	1.37 ± 0.03	3.17 ± 0.11	0.237 ± 0.006	75.0	
V1L[c]			0.235 ± 0.030	75.0	0.0
V1G	1.43 ± 0.09	38.2 ± 0.3	2.67 ± 0.03	68.8	6.2
V1E[c]			295 ± 44	56.6	18.4
V1K			10.2 ± 0.4	65.3	9.7
V1R	1.49 ± 0.01	4.79 ± 0.01	0.322 ± 0.007	74.2	0.8
V1S	0.142 ± 0.004	11.2 ± 0.3	7.86 ± 0.07	66.0	9.0
V2L[c]			10.3 ± 0.7	65.3	9.7
V2G			141 ± 15	58.5	16.5
V2E[c]			248 ± 9	57.1	17.9
V2K	1.05 ± 0.17	9.2 ± 1.5	0.877 ± 0.008	71.7	3.3
V2R	1.15 ± 0.04	0.31 ± 0.06	0.027 ± 0.005	80.6	−5.6
V2S	1.39 ± 0.03	7.30 ± 0.12	0.522 ± 0.005	73.9	2.1

[a] Assays were performed and data were analyzed as described under Experimental Procedures to yield the estimates for the kinetic parameters that are given in the table together with the standard errors of the estimated values. [b] The values for rhir have been previously reported (Braun et al., 1988). [c] The values for these mutants were previously determined (Wallace et al., 1989).

Table II: Kinetic Parameters for the Inhibition of Thrombin by Mutants of Hirudin with Substitutions of Positions 3, 4, and 5[a]

form of hirudin	$10^{-8} \times k_1$ (M^{-1} s^{-1})	$10^5 \times k_2$ (s^{-1})	K_i (pM)	$-\Delta G^\circ_b$ (kJ mol^{-1})	$\Delta\Delta G^\circ_b$ (kJ mol^{-1})
rhir[b]	1.37 ± 0.03	3.17 ± 0.11	0.237 ± 0.006	75.0	
Y3A			30.9 ± 2.2	62.4	12.6
T4S	1.54 ± 0.08	20.6 ± 0.1	1.34 ± 0.02	70.5	4.5
T4A	0.94 ± 0.05	47.7 ± 3.0	5.08 ± 0.10	67.0	8.0
D5A	1.39 ± 0.22	19.4 ± 3.0	1.40 ± 0.02	70.4	4.6
D5E	0.92 ± 0.08	16.8 ± 1.5	1.83 ± 0.05	69.7	5.3

[a] Estimates of the kinetic parameters were determined as described in the legend to Table I. [b] The values of rhir have been reported previously (Braun et al., 1988).

decrease in binding energy as the replacement of Val2′ by this amino acid (Wallace et al., 1989). In order to test whether both positions are equally sensitive to substitution by other polar or charged residues, Val1′ and Val2′ have been replaced by serine, lysine, and arginine. In contrast to the results obtained upon substitution by glutamate, these amino acids were better accommodated in the binding site for Val2′ than in that for Val1′. Mutation of Val1′ to serine increased the K_i value 33-fold from 0.24 to 7.9 pM ($\Delta\Delta G^\circ_b$ = 9.0 kJ mol^{-1}) whereas replacement of Val2′ by serine resulted in only a 2-fold increase in K_i (0.522 pM) and a decrease in ΔG°_b of 2.2 kJ mol^{-1} (Table I). The differences between the effects of substitutions in positions 1 and 2 were similar when lysine was used for the substitution. In position 1, replacement of valine by lysine caused a 45-fold increase in K_i (9.7 kJ mol^{-1} decrease in ΔG°_b) while the corresponding replacement in position 2 produced only a 4-fold increase in K_i (3.3 kJ mol^{-1} decrease in ΔG°_b). Arginine was better accommodated in both positions than lysine. The mutation Val1′→Arg caused only a 1.4-fold increase in K_i, and arginine in the second position improved the inhibitory properties of rhir; a 9-fold decrease in K_i was observed with the V2R mutant (5.6 kJ mol^{-1} increase in ΔG°_b).

In contrast to the results obtained with the serine, lysine, and arginine substitutions, replacement of Val2′ with glycine was found to have a greater effect than replacement of Val1′. The mutants V1G and V2G had K_i values that were respectively 11- and 590-fold greater than rhir with the corresponding decreases in ΔG°_b being 6.2 and 16.5 kJ mol^{-1} (Table I). These results are qualitatively similar to those previously obtained with leucine substitutions; while the mutant V1L had the same K_i value as rhir, V2L had a value that was 43-fold higher (Table I).

For a number of the mutants, it was possible to measure association (k_1) and dissociation rate (k_2) constants (Table I). For most of the mutants, the altered K_i value was predominantly due to a change in the value of k_2; for example, the 11-fold increase in the K_i value for V1G was due to a 12-fold increase in the value of k_2, and the 9-fold decrease in the value of K_i for V2R was caused by a 10-fold decrease in the k_2 value. The observation that the value of k_1 is not affected by N-terminal mutations is consistent with the proposal that the association of the C-terminal region of hirudin is the rate-limiting step in the formation of the complex (Stone & Hofsteenge, 1986; Stone et al., 1987, 1989). An exception to this correlation between k_2 and K_i was observed with the mutant V1S where the increase in the K_i value was mostly due to a decrease in the value of k_1.

Additivity of Mutational Effects at Positions 1 and 2 of rhir. As outlined under Data Analysis, cooperativity in the binding of the first two residues of rhir can be evaluated by plotting the decrease in binding energy for the double mutation against the sum of the decreases in binding energy for the single mutations. Such a plot should have a slope of 1 if there is no cooperativity in the binding of the two residues (Wells, 1990). Binding energy decreases for a series of double mutations of the first two residues of rhir have been previously reported (Wallace et al., 1989). In addition, the double mutation V1, 2R was made. This mutant exhibited kinetic parameters that were similar to rhir; the K_i value was 0.176 ± 0.007 pM, and the values of k_1 and k_2 were (0.68 ± 0.01) × 10^8 M^{-1} s^{-1} and (1.2 ± 0.1) × 10^{-5} s^{-1}, respectively. The data illustrated in Figure 2 indicate that there was no cooperativity in binding the amino acids in positions 1 and 2 of rhir for most of the substitutions that were made. The majority of the points do not deviate significantly from the line drawn with a slope of 1. The largest deviations were seen with the serine and arginine substitutions.

Substitutions of Tyr3′, Thr4′, and Asp5′. Replacement of Tyr3′ by alanine resulted in a 127-fold increase in the K_i value which corresponds to a decrease in binding energy of 12.6 kJ mol^{-1} (Table II). Replacement of Thr4′ by alanine also caused a large decrease in binding energy. The K_i value of 5.1 pM for T4A represented a 21-fold increase relative to rhir while ΔG°_b decreased 8.2 kJ mol^{-1} (Table II). When Thr4′ was replaced by serine, the decrease in binding energy was not as large. For the mutant T4S, the K_i increased 5.7-fold, and ΔG°_b

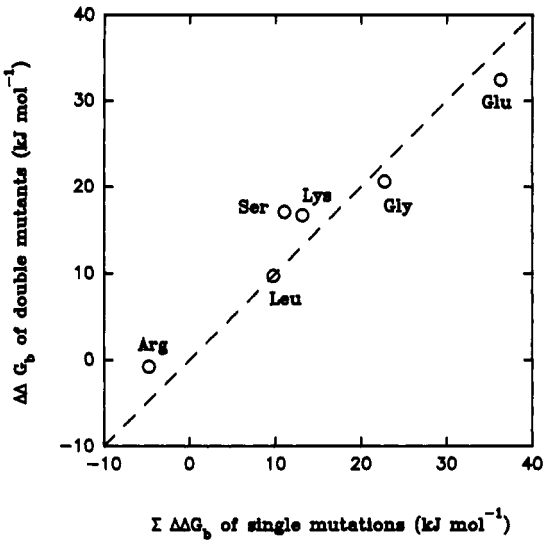

FIGURE 2: Plot of the change in binding energy for a double mutation ($\Delta\Delta G_b$) against the sum of the change in binding energies for corresponding single mutations ($\sum\Delta\Delta G_b$). The data for the single mutations were taken from Table I whereas those for the double mutations were from Wallace et al. (1989) or the value given in the text for V1, 2R. The data points are identified by the amino acids that were mutated. The dashed line represents the expected behavior if there is no cooperativity in the binding of the two residues, if ΔG_I in eq 2 is equal to zero.

decreased by 4.5 kJ mol^{-1} (Table II). The effect of replacing Asp5′ by glutamate or alanine was about the same. The alanine substitution caused a 5.9-fold increase in the value of K_i while the glutamate substitution caused a 7.7-fold increase. The decrease in ΔG_b° was about 5 kJ mol^{-1} for both mutations (Table II). For the mutations involving Thr4′ and Asp5′, it was possible to obtain estimates for the association and dissociation rate constants, and in all cases, the increase in the value of K_i was predominantly due to an increase in the dissociation rate constant (Table II).

Discussion

Effects of Substitution of Val1′. The first amino acid of rhir makes a large number of nonpolar contacts with thrombin (Figure 1; Rydel et al., 1990, 1991; Grütter et al., 1990). Replacement of this residue by leucine should maintain these contacts, and, thus, equivalent binding energies observed for rhir and V1L can be predicted from the crystal structure. Removal of these nonpolar contacts by substituting glycine for Val1′ led to a considerable decrease in binding energy (6.2 kJ mol^{-1}) which indicates an important role for the nonpolar interactions made by Val1′ in the stabilization of the complex. The polar amino acids serine, lysine, arginine, and glutamate were not well accommodated in the first position. These amino acids would not make the same nonpolar contacts as Val1′. The effect observed upon substitution of Val1′ by glutamate was much larger than that observed for other amino acids. Calculation of the electrostatic potential surrounding thrombin indicates that the residues of the active-site cleft of thrombin create a negative potential (Karshikov et al., 1992). Interaction of the negatively charged glutamate with this potential probably contributes to the loss in binding energy observed with the mutant V1E. In addition, the negatively charged glutamyl residue may have disrupted the interactions that the positively charged α-amino group of rhir makes. It has been shown that the α-amino group of rhir must be positively charged for an optimal interaction with thrombin (Wallace et al., 1989; Betz et al., 1992). This group forms hydrogen bonds with Ser195 and the carbonyl of Ser214 (Rydel et al., 1990, 1991), and it seems possible that the close proximity of the negatively charged carboxylate of a glutamate in position 1 destabilizes these interactions. Arginine was better accommodated in position 1 than other polar amino acids (Table I). An arginine in this position could possibly make electrostatic interactions with Glu192 and Asp194. Molecular modeling[3] does not provide a conclusive explanation, however, for why arginine would be bound better in position 1 than lysine.

Effects of Substitution of Val2′. The second amino acid of hirudin is located at the entrance to the primary specificity pocket. This binding site is less hydrophobic than that for the first amino acid (Rydel et al., 1990, 1991; Grütter et al., 1990), and Val2′ makes less nonpolar contacts than Val1′ (Grütter et al., 1990). Consequently, polar amino acids are, in general, better accommodated in position 2 than in position 1. The mutation V2E provides an exception to this generalization. A number of negatively charged residues (e.g., Asp189 and Glu192) are found in the region around the binding site for Val2′, and the poor binding of V2E is presumably a result of repulsive electrostatic interactions with these residues and the negative potential created by other residues in the active site (Karshikov et al., 1992).

Hirudin with serine in the second position (V2S) is a better inhibitor than the molecule with serine in the first position (V1S); the decrease in binding energy for V2S was 2.2 kJ mol^{-1} compared with 9.0 kJ mol^{-1} for V1S (Table I). This can be ascribed to the more polar nature of the binding site for Val2′.

Hirudin with arginine in the second position binds remarkably well to thrombin; the mutant V2R has a K_i value that is 9-fold lower than that of rhir (Table I). The affinity of V2R for thrombin approaches that of natural sulfated hirudin (Stone & Hofsteenge, 1986). Although arginine has not been observed in position 2 in any of the many isoforms of hirudin from the European medicinal leech *Hirudo medicinalis* that have been sequenced (Scharf et al., 1989), arginine was found in this position in a form of hirudin isolated from the southeast Asian buffalo leech *Hirudinaria manillensis* (Maschler et al., 1988). Molecular modeling provides a possible explanation for the increased potency of V2R. It is possible to model the side chain of Arg2′ such that it penetrates the primary specificity pocket of thrombin and makes a salt bridge with Asp189 of thrombin. In this model, the guanidino moiety of the arginine occupies roughly the same position as that of the arginine in the structure of D-Phe-Pro-ArgCH_2–thrombin (Bode et al., 1989). The orientation of the side chain in the modeled structure is, however, different than that observed for the arginine in the D-Phe-Pro-ArgCH_2–thrombin complex. Arg2′ would penetrate the primary specificity pocket from a different angle; the orientation of the side chain would be similar to that modeled by Bode et al. (1990) for the arginine in the thrombin-specific inhibitor MQPA [(2*R*,4*S*)-4-methyl-1-[N^α-(3-methyl-1,2,3,4-tetrahydro-8-quinolinylsulfonyl)-L-arginyl]-2-piperidinecarboxylic acid]. The hirudin V2K did not show an increased affinity for thrombin. The side chain of Lys2′ would not be able to penetrate as deeply into the primary specificity pocket as Arg2′, and the electrostatic interaction with Asp189 would not be as favorable. Electrostatic interactions for Arg2′ and Lys2′ with residues other than Asp189 also seem possible, particularly with Glu192 and Asp194.

Leucine was not bound well in the second position. No conclusive explanation can be given for the decrease in binding

[3] Molecular modeling has been performed using the structure determined by Rydel et al. (1990, 1991).

energy observed with V2L. In some orientations, however, the C^{γ} atoms of Leu2′ would clash with backbone or side chain atoms of Cys191 and Glu192. Replacement of Val2′ by glycine had a larger effect on binding energy than the replacement of Val1′ (Table I), and this result is somewhat surprising since Val1′ makes more nonpolar contacts than Val2′ (Grütter et al., 1990). Replacement of Val2′ by glycine may, however, lead to a loss in binding energy due to an increase in the conformational entropy of the polypeptide backbone of unbound rhir. Consequently, a larger decrease in entropy would be observed when the N-terminal amino acids are immobilized in the active site of thrombin.

Additivity of Mutational Effects at Positions 1 and 2 of rhir. The plot illustrated in Figure 2 indicates that in general there was no cooperativity in the binding of the two N-terminal amino acids of rhir. A plot of the decrease in binding energy for the double mutation against the sum of the decreases in binding energy for the single mutations is expected to have a slope of 1 when there is no cooperativity in the binding of the two residues (Wells, 1990). This lack of cooperativity can be attributed to the wide spatial separation of the side chains of each residue in the complex such that no interaction between the side chains is to be expected. Wells (1990) has analyzed the additivity of mutational effects on catalysis and binding for a number of proteins. In most cases, negligible cooperativity was observed. Significant cooperative effects were only found 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). With hirudin, the largest deviation from the expected behavior for a system lacking cooperativity was observed with the serine substitutions (Figure 2). In most cases for which association (k_1) and dissociation (k_2) rate constants could be measured, the increase in the K_i value was due to an increase in the value of the dissociation rate constant. The mutant V1S was the only form of hirudin for which the increase in the K_i value was caused by a decrease in the value of k_1 (Table I). Thus, the lack of additivity in the case of the serine substitutions may provide another example of where the nonadditivity of mutational effects is due to a change in the mechanism of interaction (Wells, 1990). Similar results were observed with mutations involving Glu58′ of rhir (Betz et al., 1991).

Effects of Substitution of Tyr3′, Thr4′, and Asp5′. In the thrombin–hirudin complex, Tyr3′ is bound in a hydrophobic pocket made by the residues Tyr60A, Ile174, Trp60D, and Leu99 (Figure 1). In addition, an edge-to-face interaction with the aromatic ring of Trp215 is seen (Figure 1; Rydel et al., 1990, 1991; Grütter et al., 1990). D-Phenylalanine of the inhibitor D-Phe-Pro-ArgCH_2Cl is also bound to this site (Bode et al., 1989), and it seems probable that the aryl groups of other inhibitors of thrombin are also bound there (Bode et al., 1990; Turk et al., 1991). The importance of the interaction of Tyr3′ with this site is suggested by the fact that a tyrosine has been found in position 3 in all sequenced forms of hirudin (Scharf et al., 1989; Maschler et al., 1988). Site-directed mutagenesis studies have confirmed this suggested importance. Removal of the aromatic side chain of Tyr3′ in the mutant Y3A resulted in a 12.6 kJ mol^{-1} decrease in binding energy. Similar results were obtained by Lazar et al. (1991).

The hydrogen bond observed between the O^{γ} of Thr4′ and the ϵ-amino group of Lys47′ has been proposed both to stabilize the core domain of rhir and to position this core domain with respect to the active-site cleft of thrombin (Rydel et al., 1990, 1991). If it is assumed that a serine in position 4 would form the same hydrogen bond, the contribution of this hydrogen bond to the stability of the complex can be assessed by comparison of the two mutants T4S and T4A. The difference in the binding energies of these mutants was 3.5 kJ mol^{-1}, and this value corresponds well with those previously observed for the deletion of hydrogen bonds (Fersht, 1987). The reduction in binding energy seen with the mutant T4S suggests that the γ-methyl group of Thr4′ makes a contribution to the stability of the complex. However, a role for this group is not apparent from the crystal structure. The reduced affinity of T4S could be caused by a sum of subtle effects. The conformational entropy of the polypeptide backbone of free rhir would increase upon replacement of Thr4′ by serine because serine does not restrict the movement of the main chain with an γ-methyl group. In addition, it has been suggested that threonine may form a stronger hydrogen bond than serine (Alber et al., 1987).

The structure of the thrombin–hirudin complex suggests that Asp5′ stabilizes the complex by forming a salt bridge to Arg221A of thrombin (Rydel et al., 1990, 1991; Grütter et al., 1990). The importance of this salt bridge was investigated by mutating Asp5′ to alanine and glutamate. Removal of the carboxylate group of Asp5′ in the mutant D5A resulted in a 4.6 kJ mol^{-1} loss in binding energy. Similar values have been obtained upon elimination of salt bridges with the C-terminal region of rhir (Betz et al., 1991) and for the salt bridges located on the surface of other proteins (Horovitz et al., 1990). The results obtained with D5E indicate that the precise geometry of this salt bridge is important for its stabilization of the complex. The additional methylene group in Glu5′ has caused a decrease in binding energy that is about equal to that caused by the deletion of the carboxylate group in D5A (Table II). This observation suggests that the precise orientation of the salt bridge between Asp5′ and Arg221A is important and that this salt bridge may contribute to the positioning of the N-terminal region within the active-site cleft. The slight distortion of the geometry of this salt bridge may have caused a misalignment of the N-terminal region within the active site. The carbonyl of Asp5′ makes a hydrogen bond with the ϵ-amino group of Lys47′, and it is thought that the interactions made with this ϵ-amino group are important for the orientation of the N-terminal domain as discussed above (Rydel et al., 1990, 1991). This interaction may have also been affected by the replacement of Asp5′ by glutamate.

The results of the present study show that contacts made by each of the five N-terminal amino acids of hirudin make a significant contribution to the stabilization of the thrombin–hirudin complex. Some idea of the importance of these interactions can be obtained by summing the values of $\Delta\Delta G_b^{\circ}$ for the mutations V1G, V2S, Y3A, T4A, and D5A (Tables I and II). The result of this calculation suggests that over 40% of the binding energy of rhir may be due to interactions with the first five residues.

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References

Ackers, G. K., & Smith, F. R. (1985) *Annu. Rev. Biochem. 54*, 597–629.

Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41–46.

Betz, A., Hofsteenge, J., & Stone, S. R. (1991) *Biochem. J. 275*, 801-803.
Betz, A., Hofsteenge, J., & Stone, S. R. (1992) *Biochemistry 31*, 1168-1172.
Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467-3475.
Bode, W., Turk, D., & Stürzebecher, J. (1990) *Eur. J. Biochem. 193*, 175-182.
Braun, P. J., Dennis, S., Hofsteenge, J., & Stone, S. R. (1988) *Biochemistry 27*, 6517-6522.
Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835-840.
Cornish-Bowden, A., & Endrenyi, L. (1981) *Biochem. J. 193*, 1005-1008.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301-304.
Grütter, M. G., Priestly, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J. 9*, 2361-2365.
Horovitz, A., Serrano, L., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *J. Mol. Biol. 216*, 1031-1044.
Karshikov, A., Bode, W., Tulinsky, A., & Stone, S. R. *Protein Sci.* (in press).
Knecht, R., & Chang, J.-Y. (1987) *Anal. Chem. 58*, 2375-2379.
Kunkel, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488-492.
Lazar, J. B., Winant, R. C., & Johnson, P. H. (1991) *J. Biol. Chem. 266*, 685-688.
Maschler, R., Steiner, V., Grütter, M. G., & Fritz, R. (1988) Eur. Patent Application 89810414.6, EP-AP0347376.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 249*, 277-280.
Rydel, T. J., Tulinsky, A., Bode, W., & Huber, R. (1991) *J. Mol. Biol. 221*, 583-601.
Scharf, M., Engels, J., & Tripier, D., (1989) *FEBS Lett. 255*, 105-110.
Segel, I. H. (1975) *Enzyme Kinetics*, Chapter 6, Wiley, New York.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Stone, S. R., Braun, P. J., & Hofsteenge, J. (1987) *Biochemistry 26*, 4617-4624.
Stone, S. R., Dennis, S., & Hofsteenge, J. (1989) *Biochemistry 28*, 6857-6863.
Turk, D., Stürzebecher, J., & Bode, W. (1991) *FEBS Lett. 287*, 133-138.
Wallace, A., Dennis, S., Hofsteenge, J., & Stone, S. R. (1989) *Biochemistry 28*, 10079-10084.
Wells, J. A. (1990) *Biochemistry 29*, 8509-8517.
Winant, R. C., Lazar, J. B., & Johnson, P. H. (1991) *Biochemistry 30*, 1271-1277.

Investigation of the Origin of the "S3" EPR Signal from the Oxygen-Evolving Complex of Photosystem 2: The Role of Tyrosine Z†

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ABSTRACT: The origin of the "S3" EPR signal from calcium-depleted photosystem 2 samples has been investigated. This signal is observed after freezing samples under illumination and has been assigned to an interaction between the manganese cluster and an oxidized histidine radical [Boussac et al. (1990) *Nature 347*, 303-306]. In calcium-depleted samples prepared by three different methods, we observed the trapping of the tyrosine radical Y_Z^+ under conditions which also formed the "S3" signal. An "S3"-type signal and Y_Z^+ were also formed in PS2 samples treated with the water analogue ammonia. Following illumination at 277 K, the "S3" and Y_Z^+ signals decayed at the same rate at 273 K in the dark. Both the Y_Z^+ and "S3" signals decayed on storage at 77 K and could be subsequently regenerated by illumination at 8-77 K. No evidence to support histidine oxidation was found. The effects of DCMU, chelators, and alkaline pH on the dark-stable multiline S_2 and the "S3" signals from calcium-depleted samples were determined. Both signals required the presence of EGTA or citrate for maximum yield. The addition of DCMU caused a reduction in the yield of "S3" generated by freezing under illumination. Incubation at pH 7.5 resulted in the loss of both signals. We propose that a variety of treatments which affect calcium and chloride binding cause a stabilization of the S_2 state and slow the reduction of Y_Z^+. This allows the trapping of Y_Z^+, the interaction with the manganese cluster (probably in the S_2 state) resulting in the "S3" signal. The data allow the position of the manganese cluster to be estimated as within 10 Å of tyrosine Z (D1-161).

Electron transfer in photosystem 2 (PS2)[1] follows the absorption of light by the reaction center chlorophyll P680, resulting in the passage of an electron via pheophytin to the primary and secondary plastoquinone acceptors Q_A and Q_B. $P680^+$ is reduced by electrons from the oxygen-evolving com-

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[1] Abbreviations: EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PPBQ, phenyl-1,4-benzoquinone; OEC, oxygen-evolving complex; PS2, photosystem 2; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chl, chlorophyll; H_{pp}, peak to trough line width of EPR spectrum.

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