Fenton, J. W., II, & Bing, D. H. (1986) *Semin. Thromb. Hemostasis 12*, 200-208.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, Freeman, New York.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301-304.
Fromm, H. J. (1975) *Initial Rate Enzyme Kinetics*, Springer, Berlin.
Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J. 9*, 2361-2365.
Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 91-117.
Lottenberg, R., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 558-564.
Lottenberg, R., Christensen, U., Jackson, C. M., & Coleman, P. L. (1981) *Methods Enzymol. 80*, 341-361.
Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539-557.
Polgár, L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) pp 159-200, Elsevier, Amsterdam.
Rydel, T. J., Ravichrandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 245*, 277-280.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Tipton, K. F., & Dixon, H. B. F. (1979) *Methods Enzymol. 63*, 183-234.

Role of Interactions Involving C-Terminal Nonpolar Residues of Hirudin in the Formation of the Thrombin-Hirudin Complex

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ABSTRACT: The role of interactions involving C-terminal nonpolar residues of hirudin in the formation of the thrombin-hirudin complex has been investigated by site-directed mutagenesis. The residues Phe56, Pro60, and Tyr63 of hirudin were replaced by a number of different amino acids, and the kinetics of the inhibition of thrombin by the mutant proteins were determined. Phe56 could be replaced by aromatic amino acids without significant loss in binding energy. While substitution of Phe56 by alanine decreased the binding energy (ΔG_b° by only 1.9 kJ mol^{-1}, replacement of this residue by amino acids with branched side chains caused larger decreases in ΔG_b°. For example, the mutant Phe56→Val displayed a decrease in ΔG_b° of 10.5 kJ mol^{-1}. Substitution of Pro60 by alanine or glycine resulted in a decrease in ΔG_b° of about 6 kJ mol^{-1}. Tyr63 could be replaced by phenylalanine without any loss in binding energy, and replacement of this residue by alanine caused a decrease of 2.2 kJ mol^{-1} in ΔG_b°. Substitution of Tyr63 by residues with branched side chains resulted in smaller decreases in ΔG_b° than those seen with the corresponding substitutions of Phe56; for example, the mutant Tyr63→Val showed a decrease in binding energy of 5.1 kJ mol^{-1}. The effects of the mutations are discussed in terms of the crystal structure of the thrombin-hirudin complex.

Hirudin is a specific tight-binding inhibitor of thrombin. Its tertiary structure in solution as determined by two-dimensional NMR spectroscopy indicates that it is composed of a relatively compact N-terminal core domain (residues 3-49) and a disordered C-terminal tail comprising residues 50-65 (Clore et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989). In the crystal structure of the complex, the C-terminal tail of hirudin is bound in a long groove on the surface of thrombin that is flanked by positively charged groups (Rydel et al., 1990; Grütter et al., 1990) and has been termed the anion-binding exosite (Fenton, 1989). Electrostatic interactions between the anion-binding exosite and acidic residues in the C-terminal region of hirudin appear to be important for the formation of the thrombin-hirudin complex (Braun et al., 1988; Stone et al., 1989; Betz et al., 1991). In addition, 5 of the 11 C-terminal residues of hirudin are involved in nonpolar interactions with this exosite (Rydel et al., 1990). Phe56′[1] is buried in a hydrophobic pocket as shown in Figure 1 and makes 14 intermolecular contacts less than 4 Å (Rydel et al., 1990). Moreover, the aromatic rings of Phe56′ and Phe34 of thrombin are perpendicular to each other, and this arrangement is considered to be optimal for a favorable interaction between aromatic rings (Burley & Petsko, 1985). Synthetic peptides based on the sequence of the C-terminal tail have been shown to inhibit the cleavage of fibrinogen by thrombin (Krstenansky et al., 1990), and the aromatic nature of Phe56′ appears to be important for the binding of these peptides to thrombin (Krstenansky et al., 1987). After Phe56′, the most important nonpolar residues in the C-terminal region of hirudin appear to be Pro60′ and Tyr63′ (see Figure 1). Both these residues make numerous nonpolar contacts with thrombin; Pro60′ makes 7 intermolecular contacts less than 4 Å while Tyr63′ makes 10 such contacts (Rydel et al., 1990).

Although the crystallographic studies suggest that nonpolar interactions with the C-terminal region of hirudin are important in the stabilization of the complex, quantitative data on the strength of these interactions for individual residues

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[1] Residues in rhir are distinguished from those in thrombin by the use of primed numbers; e.g., Phe56′ represents the phenylalanine at position 56 in rhir. The numbering of residues in thrombin is that of Bode et al. (1989) which is based on chymotrypsin numbering.

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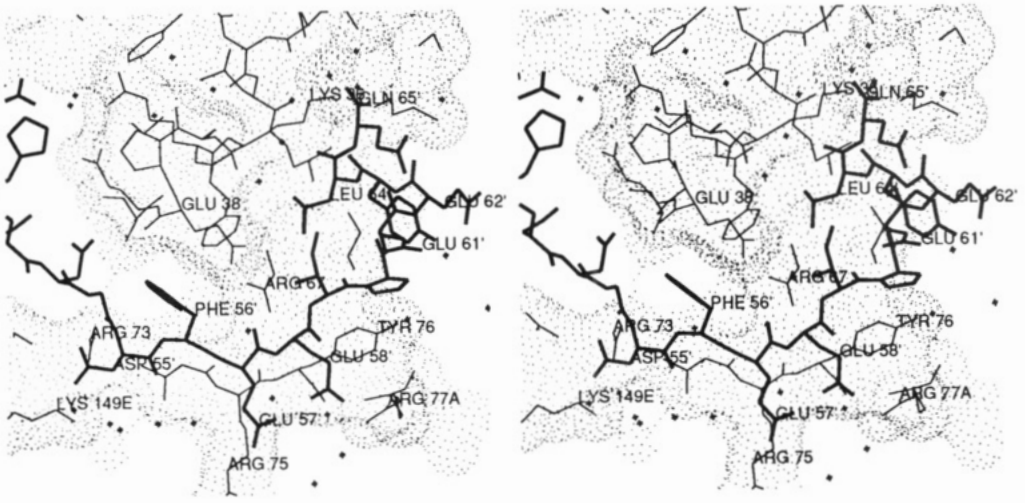

FIGURE 1: Stereo plot of the binding of the C-terminal region of hirudin to the anion-binding exosite of thrombin. Residues of the anion-binding exosite of thrombin are displayed in thin connections together with the Connolly surface of this region of thrombin. Thick connections are used for the C-terminal region of hirudin. The structure displayed is that determined by Rydel et al. (1990).

are not available. In order to address this problem, Phe56′, Pro60′, and Tyr63′ of hirudin have been replaced by site-directed mutagenesis, and the effects of these changes on the inhibitory properties of hirudin have been investigated.

Materials and Methods

Materials. The substrates D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238) and D-Val-Leu-Arg-*p*-nitroanilide (S-2266) were obtained from Kabi-Vitrum (Molndal, Sweden). Human α-thrombin was prepared from plasma and characterized as described previously (Stone & Hofsteenge, 1986); the sample used was fully active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). The hirudin mutants F56L and Y63E were kindly provided by Dr. J. Dodt (Technische Hochschule, Darmstadt, Germany) and Drs. H. Grossenbacher, B. Meyhack, and M. Ingueno (Ciba-Geigy, Basel, Switzerland), respectively.

Expression and Characterization of Mutant Hirudins. Standard site-directed mutagenesis methods were used to make site-specific substitutions in recombinant hirudin (rhir)[2] (Kunkel, 1985). The mutant rhir polypeptides were expressed in *Escherichia coli* and purified using ion-exchange and reverse-phase chromatography as described by Braun et al. (1988). The purity and identity of the proteins were verified by amino acid analysis (Knecht & Chang, 1987) and peptide mapping (Braun et al., 1988).

Amidolytic Assay of Thrombin. Assays were performed as previously described (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. Concentrations of active hirudin molecules were determined by titration of 2.0 nM thrombin in the presence of 200 μM D-Val-Leu-Arg-*p*-nitroanilide as described by Wallace et al. (1989). For the determination of kinetic parameters for the inhibition by hirudin, assays were performed as described by Stone and Hofsteenge (1986) using 100 μM D-Phe-pipecolyl-Arg-*p*-nitroanilide (K_m = 3.6 μM) and 20 or 50 pM thrombin.

Theory and Data Analysis. Under the assay conditions used, hirudin is a slow, tight-binding inhibitor of thrombin (Stone & Hofsteenge, 1986), and a kinetic mechanism that describes the interaction of hirudin (I) with thrombin (E) is given in Scheme I. An expression that relates the dissociation constant (K_i) for the complex to the association (k_1) and dissociation (k_2) rate constants is also given in Scheme I. Estimates for K_i, k_1, and k_2 were determined for each mutant hirudin by analyzing progress curves for thrombin-catalyzed formation of *p*-nitroaniline from D-Phe-pipecolyl-Arg-*p*-nitroanilide in the presence of different concentrations of hirudin. Each progress curve experiment consisted of six assays, one without hirudin and five others with different hirudin concentrations. The apparent kinetic parameters (k_1, k_2, and K_i) were obtained by nonlinear regression analysis of the progress curves as described previously (Stone & Hofsteenge, 1986). The true values of the parameters were calculated by correcting for the concentration of substrate as described by Braun et al. (1988). The kinetic parameters for each mutant were determined at least twice, and the values given represent the weighted means of these individual determinations. By use of the relationship given in eq 1, the

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

Gibbs standard free energy for the formation of the thrombin–hirudin complex, denoted as binding energy (ΔG°_b), was calculated from K_i values where R is the gas constant and T is the absolute temperature.

Scheme I

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

ΔG°_b can be divided into the contributions made by nonionic (ΔG°_{nio}) and ionic interactions (ΔG°_{io0}). Estimates of ΔG°_{nio} and ΔG°_{io0} can be theoretically obtained by examining the dependence of the value ΔG°_b on ionic strength, and eq 2 has been

$$\Delta G^\circ_b = \Delta G^\circ_{nio} + \Delta G^\circ_{io0} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \qquad (2)$$

shown to describe this dependence for the thrombin–hirudin interaction (Stone et al., 1989) where C_1 is an empirical parameter related to the Debye–Hückel screening parameter. For the mutant F56V, ΔG°_b was determined at 13 values of ionic strength between 0.05 and 0.625 M. The values of ΔG°_b obtained were weighted according to the inverse of their variances and fitted to eq 2 by nonlinear regression to yield estimates of ΔG°_{nio} and ΔG°_{io0}. This treatment of ionic strength effects assumes that changes in ionic strength have specifically affected the ionic interactions that occur between thrombin

[2] Abbreviation: rhir, recombinant hirudin variant 1.

Table I: Kinetic Parameters for the Interaction of Mutants of Hirudin with Thrombin[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	0.231 ± 0.006	75.0	
F56Y	1.67 ± 0.04	3.46 ± 0.02	0.207 ± 0.009	75.3	−0.3
F56W	1.21 ± 0.05	4.92 ± 0.46	0.407 ± 0.022	73.6	1.4
F56A	0.961 ± 0.030	4.68 ± 0.02	0.487 ± 0.010	73.1	1.9
F56L	0.107 ± 0.015	7.59 ± 0.13	7.10 ± 0.07	66.2	8.8
F56I	0.157 ± 0.008	10.9 ± 0.7	6.95 ± 0.13	66.3	8.7
F56V	0.070 ± 0.003	9.5 ± 0.5	13.6 ± 0.4	64.5	10.5
F56T	0.046 ± 0.003	12.1 ± 0.7	26.3 ± 0.3	62.8	12.2
P60A	0.156 ± 0.003	4.73 ± 0.12	3.03 ± 0.05	68.4	6.6
P60G	0.213 ± 0.028	4.54 ± 0.58	2.13 ± 0.04	69.3	5.7
Y63F	1.25 ± 0.03	3.26 ± 0.14	0.261 ± 0.009	74.7	0.3
Y63A	0.855 ± 0.025	4.53 ± 0.22	0.526 ± 0.023	72.9	2.2
Y63E	0.662 ± 0.018	3.22 ± 0.25	0.486 ± 0.026	73.1	1.9
Y63L	0.654 ± 0.010	5.13 ± 0.17	0.785 ± 0.015	71.9	3.1
Y63V	0.307 ± 0.013	5.13 ± 0.24	1.67 ± 0.03	69.9	5.1

[a] Assays were performed and data analyzed as described under Materials and Methods to yield the estimates of the parameters. The standard errors of the estimates are also given. The binding energy (ΔG°_b) was calculated by using the relationship given in eq 1. The value of $\Delta\Delta G^\circ_b$ is the decrease in binding energy caused by a particular mutation. [b] The values for rhir were determined previously (Braun et al., 1988).

Table II: Kinetic Parameters for Forms of Hirudin Containing Single and Double Mutations[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	0.231 ± 0.006	75.0	
F56V	0.070 ± 0.003	9.5 ± 0.4	13.6 ± 0.4	64.5	10.5
D55N[c]	0.755 ± 0.002	4.49 ± 0.16	0.595 ± 0.021	72.6	2.4
E57Q[c]	0.219 ± 0.002	5.04 ± 0.06	2.30 ± 0.02	69.1	5.9
F56V/D55N	0.219 ± 0.015	7.88 ± 0.55	36.0 ± 0.5	62.0	13.0
F56V/E57Q	0.0181 ± 0.0044	18.1 ± 0.4	101 ± 1	59.3	15.7

[a] Estimates of the kinetic parameters were obtained as described in Table I. [b] The values for rhir were determined previously (Braun et al., 1988). [c] These values are from Betz et al. (1991).

and hirudin. It seems possible, nevertheless, that nonionic interactions might also be affected by higher salt concentrations. The problem of possible salt effects on nonionic interactions has been previously discussed (Stone et al., 1989; Stone & Hofsteenge, 1991). Given the possibility that the ionic strength has affected both ionic and nonionic interactions, the quantitative significance of the values obtained for ΔG°_{nio} and ΔG°_{io0} is not clear. In previous studies, however, changes in these parameters with different mutations have been consistent with the nature of the mutation. For mutations that altered ionic interactions, only ΔG°_{io0} was affected while ΔG°_{nio} remained constant (Stone et al., 1989), whereas mutations that modified hydrophobic interactions resulted in a change in ΔG°_{nio} without affecting ΔG°_{io0} (Wallace et al., 1989).

Results

Substitutions Involving Phe56′. Replacement of Phe56′ by the aromatic amino acids tryptophan and tyrosine resulted in only a slight change in the binding energy of hirudin (Table I). The mutant with tyrosine substituted for Phe56′ (F56Y) exhibited essentially the same inhibitory properties as rhir while the tryptophan-substituted mutant (F56W) displayed an increase in the dissociation constant (K_i) of about 2-fold. The removal of the aromatic side chain of Phe56′ in the mutant F56A also led to a 2-fold increase in the K_i value which corresponds to a decrease of 1.9 kJ mol^{-1} in binding energy (Table I). Marked increases in the value of K_i were, however, observed when Phe56′ was replaced by amino acids containing branched side chains (Table I). Substitution of leucine or isoleucine for Phe56′ caused an increase of about 30-fold in the K_i value; ΔG°_b decreased by 8.8 kJ mol^{-1} for both mutants. Replacement of Phe56′ by valine or threonine resulted in even larger increases in the value of K_i; the mutants F56V and F56T exhibited K_i values that were respectively 60- and 110-fold greater than that determined for rhir (Table I). The decreases in ΔG°_b for F56V and F56T were 10.5 and 12.2 kJ mol^{-1}, respectively. For all mutants, the decrease in affinity was predominantly due to a decrease in the value of the association rate constant (k_1, Table I).

The large decrease in binding energy observed for mutants in which Phe56′ had been replaced by amino acids with branched side chains (F56V, F56L, F56I, and F56T) was unexpected in view of the small decrease observed when alanine was substituted for Phe56′ (F56A). In order to test whether these replacements had affected the interactions made by adjacent amino acids (Asp55′ and Glu57′), the double mutants F56V/D55N and F56V/E57Q were made. If the mutation Phe56′→Val affects the interactions of Asp55′ or Glu57′, smaller decreases in binding energy would be expected for mutations Asp55′→Asn and Glu57′→Gln in the mutant F56V compared with the same mutations in rhir. There would be a nonadditivity of mutational effects because the mutation at one position would influence the strength of the interaction at the second position (Wells, 1990). The data presented in Table II indicate that this was not the case. The mutations Asp55′→Asn and Glu57′→Gln caused similar decreases in binding energy in both rhir and F56V. Thus, it can be concluded that the Phe56′→Val substitution has not disrupted interactions made by its neighboring residues.

In order to examine whether ionic interactions other than those involving Asp55′ and Glu57′ had been altered by the Phe56′→Val mutation, the contributions of ionic (ΔG°_{io0}) and nonionic interactions (ΔG°_{nio}) to binding energy were evaluated for the mutant F56V by examining the effect of ionic strength on ΔG°_b. The dependence of ΔG°_b for F56V on ionic strength was similar to that previously seen for the thrombin–hirudin interaction (Stone et al., 1989; Stone & Hofsteenge, 1991) and could be described by eq 2 (data not shown). Analysis

Table III: Contribution of Ionic and Nonionic Interactions to the Binding Energy of Two Forms of Hirudin[a]

form of hirudin	ΔG°_{nio} (kJ mol^{-1})	ΔG°_{io0} (kJ mol^{-1})
rhir[b]	62.2 ± 2.4	25.0 ± 0.7
F56V	46.0 ■ 4.4	27.4 ± 3.8

[a] Assays for the mutant F56V were performed at 13 different ionic strengths between 0.05 and 0.625, and the values of ΔG°_b were determined as described under Materials and Methods. The estimates of ΔG°_b at each ionic strength were weighted according to the inverse of their variance and fitted by nonlinear regression to eq 2. This analysis yielded values of ΔG°_{nio} and ΔG°_{io0} which are given together with their standard errors. [b] These values were determined previously (Stone et al., 1989).

of the data yielded the results presented in Table III. The values of ΔG°_{io0} were essentially the same for rhir and F56V whereas the value of ΔG°_{nio} was markedly reduced for F56V. Previous studies have shown that the value of ΔG°_{io0} decreases for mutants in which C-terminal ionic interactions have been disrupted (Stone et al., 1989). Thus, the results obtained with F56V suggest that the mutation Phe56′→Val has not significantly affected ionic interactions between the C-terminal region of hirudin and thrombin.

Substitution of Pro60′ and Tyr63′. Replacement of Pro60′ by alanine or glycine caused about the same decrease in affinity. The value of K_i increased 13- and 9-fold for the mutants P60A and P60G, respectively, and the corresponding decreases in binding energy were 6.6 and 5.7 kJ mol^{-1} (Table I). In both cases, the increase in the magnitude of K_i was mainly caused by a decrease in the value of k_1.

Replacement of Tyr63′ by phenylalanine did not significantly change the inhibitory properties of rhir (Table I). Thus, tyrosine and phenylalanine were able to substitute for each other without loss of binding energy in both position 56 and position 63. The removal of the aromatic ring of Tyr63′ by the substitution alanine for Tyr63′ caused an increase in the value of K_i of about 2-fold and a decrease in ΔG°_b of 2.2 kJ mol^{-1} (Y63A, Table I). This effect is similar to that observed for the replacement of Phe56′ by alanine. The substitution of Tyr63′ by amino acids with branched side chains resulted in smaller increases of the value of K_i than those observed for the same substitutions of Phe56′. The mutants Y63L and Y63V displayed values of K_i that were respectively 3- and 7-fold greater than that of rhir. These increases correspond to decreases in ΔG°_b of 3.1 and 5.1 kJ mol^{-1} for Y63L and Y63V, respectively (Table I). The corresponding decreases in ΔG°_b for the leucine and valine mutations at position 56 were 8.8 and 10.5 kJ mol^{-1}.

Tyr63′ is sulfated in native hirudin, and the absence of this negatively charged sulfate group in rhir results in a 10-fold increase in the K_i value of rhir (Braun et al., 1988; Dodt et al., 1988, 1990). The full activity can be restored by phosphorylation (Hofsteenge et al., 1990) or sulfation of Tyr63′ (Niehrs et al., 1990). A negatively charged glutamate at this position did not decrease the K_i value of rhir to that observed for native hirudin; the K_i value for Y63E was about 2-fold higher than that observed for rhir (Table I).

The observed increases in K_i values for Tyr63′ mutations were mainly due to decreases in the magnitude of k_1 (Table I).

Discussion

The crystal structures of thrombin–hirudin complexes suggest that nonpolar interactions play an important role in stabilizing the complex (Rydel et al., 1990; Grütter et al., 1990). Studies on the ability of peptides based on the C-terminal region of hirudin to inhibit fibrinogen cleavage by thrombin also indicate that nonpolar residues in this region are necessary for optimal inhibitory activity (Krstenansky et al., 1990). In particular, the aromatic nature of Phe56′ was found to be important for the binding of C-terminal peptides to thrombin (Krstenansky et al., 1987). In the crystal structure of the complex, Phe56′ is bound in a hydrophobic pocket where it makes contacts with Phe34, Leu40, Arg73, and Thr74 (Rydel et al., 1990; Figure 1). Molecular modeling based on the structure determined by Rydel et al. (1990) demonstrated that tyrosine could be well accommodated in the hydrophobic pocket in accordance with the observation that the mutant F56Y has the same K_i value as rhir (Table I). In contrast, the aromatic ring of tryptophan cannot be bound in the Phe56′-binding pocket without some unfavorably close contacts, which is consistent with the slightly lower affinity of thrombin for F56W.

The decrease in binding energy caused by removal of the aromatic side chain in the mutant F56A theoretically permits the assessment of the contribution to binding energy of nonpolar contacts with the aromatic ring and of the edge-to-face interaction between the aromatic rings Phe34 of thrombin and Phe56′. The decrease in binding energy in mutant F56A is smaller than expected. The value of 1.9 kJ mol^{-1} obtained agrees well with the theoretical value for edge-to-face interactions (Burley & Petsko, 1985) as well as with experimentally determined values (Hecht et al., 1984). However, a considerable contribution from the numerous (10) close contacts made by the aromatic ring of Phe56′ was also expected.

No conclusive explanation can be given for the effects observed when Phe56′ was replaced by amino acids with branched side chains. A possible explanation for these results is that the side chains of these residues could not be accommodated in the binding pocket for Phe56′ and that there was a change in the mode of binding of the C-terminal region in order to accommodate these branched-chain residues in position 56. According to this hypothesis, interactions between other hirudin residues and thrombin could also have been affected by these mutations. The possibility that the replacement of Phe56′ by a branched-chain amino acid resulted in a decrease in the contribution of interactions with Asp55′ and Glu57′ appears to be excluded by the results presented in Table II. The mutations Asp55′→Asn and Glu57′→Gln caused the same decreases in binding energy in both rhir and F56V. Moreover, the results presented in Table III suggest that the mutation Phe56→Val has not caused structural changes that disrupt ionic interactions with the C-terminal region of the hirudin.

Molecular modeling indicates that residues with branched side chains would not make the same favorable contacts as Phe56′. Thus, the van der Waals contacts made by Phe56′ appear to be important in stabilizing the complex. Moreover, since Phe56′ could not be replaced by residues of equivalent hydrophobicity without considerable losses in binding energy, it can be concluded that the gain in binding energy obtained from the so-called hydrophobic effect (Creighton, 1991) of burying a hydrophobic residue in the Phe56′ binding pocket appears to be small. The favorable contacts between the side chain of Phe56′ and its binding pocket have been made possible because of a slight change in the conformation of thrombin. The loop 34–41 of thrombin moves upon binding toward Phe56′, and Thr74 moves to close off the binding pocket. The observed movements are not large, with a maximal displacement of about 2 Å in the side chain of Thr74. However, the smaller than expected decrease in binding energy observed when Phe56′ was replaced by alanine suggests that an unfa-

vorable change in free energy may have accompanied the conformational change. The numerous contacts may be Phe56′ could compensate for this unfavorable change in free energy such that the net effect of Phe56′ binding could be favorable but it would not be as large as expected from the observed number of contacts. The conformational change would not be necessary for the binding of alanine. Thus, free energy would not be lost due to the conformational change, and the decrease observed in binding energy caused by the alanine mutation would be smaller than expected if the loss of favorable contacts were considered in isolation. This hypothesis could also explain the effects obtained with amino acids with branched side chains. A conformational change would be necessary to bind these residues, but the fewer contacts made by these residues would not fully compensate for the unfavorable change in free energy and, hence, a decrease in binding energy would be observed.

In contrast to Phe56′, Tyr63′ is not bound in a hydrophobic cleft (Figure 1). However, it also participates in numerous hydrophobic interactions; contacts closer than 4 Å are made with Ile82 and Leu65 of thrombin and to Pro60′ of hirudin (Rydel et al., 1990). The effects on binding energy caused by replacement of Tyr63′ by various amino acids can be readily interpreted in terms of the crystal structure of the complex. The insignificant difference in binding energy between rhir and the mutant Y63F (Table I) indicates that the hydroxyl group of the tyrosine does not contribute to the stability of the complex by hydrogen bonding. In the complex, all potential hydrogen-bonding partners for the hydroxyl of Tyr63′ are located beyond hydrogen-bonding distance.

Tyrosine is necessary in position 63 of the native protein because its hydroxyl group is enzymatically sulfated in the leech and this posttranslational modification causes the 10-fold lower K_i value observed with natural hirudin (Braun et al., 1988; Dodt et al., 1988, 1990). The tyrosine sulfate in native hirudin is presumably involved in a salt bridge or charged hydrogen bond with residues in thrombin. The loss of binding energy observed when Tyr63′ was replaced by glutamate (Table I) indicates, however, that the exact position of the negatively charged group is important. The negatively charged carboxylate of the glutamate is apparently too distant from the groups with which the sulfate moiety interacts to make a hydrogen bond or to form a strong salt bridge. This substitution would also lead to a loss of many of the nonpolar contacts made by Tyr63′.

The substitution of Ala for Tyr63′ in the mutant Y63A caused a loss of 2.2 kJ mol^{-1} in binding energy which can be rationalized on the basis of the loss of hydrophobic contacts between the aromatic ring and Ile82 of thrombin. The only hydrophobic interaction that would remain is that between the C^{β} atom of alanine and Leu65 of thrombin. The stereochemical properties of leucine and valine would account for the reduction in the affinity of thrombin for Y63L and Y63V (Table I). Molecular modeling indicates that leucine cannot be accommodated in position 63 of hirudin without unfavorably close contacts to Ile82 of thrombin or with atoms of the hirudin backbone. With a valine in this position, repulsive contacts would be encountered either with the hirudin backbone atoms or with the side chain of Ile59′. Thus, the substitution of valine or leucine for Tyr63′ might destabilize the C-terminal 3_{10} helix (Figure 1), and, consequently, contacts made by other residues in the helix could also be affected.

The replacement Pro60′ by alanine or glycine would lead to a loss of all seven of the close contacts (<4 Å) between this residue and Tyr76 of thrombin (Figure 1). This loss would at least partially account for the reduction in binding energy of 6–7 kJ mol^{-1} seen for the mutants P60A and P60G. A prolyl residue in position 60 would also stabilize the complex by reducing the conformational entropy of the polypeptide backbone of the C-terminal region of unbound hirudin which approximates a random-coil in solution (Clore et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989). Replacement of alanine by proline has been shown to increase the stability of T4 lysozyme, and the results were interpreted in terms of the mutation causing a reduction in conformational entropy (Matthews et al., 1987). The reduction in the stability of the complex due to increases in conformational entropy of the hirudin backbone is expected to be greater for the Pro→Gly than for the Pro→Ala mutation. In fact, however, the effect of the latter mutation was greater, which suggests that the contribution to binding energy of the reduction in conformational entropy of free rhir by the prolyl residue is small. Molecular modeling does not indicate any reason for the lower binding energy of P60A compared with P60G.

If the loss of binding energy when alanine is substituted for a particular residue is taken as the contribution of the residue to binding energy, it can be seen that the individual contributions of Phe56′, Pro60′, and Tyr63′ are relatively small (Table I). However, the sum of the contributions of these residues amounts to more than 10 kJ mol^{-1}, which corresponds to about 14% of the overall binding energy. Thus, without considering the contributions of Ile59′ and Leu64′, which have been shown to be necessary for the efficient binding of C-terminal peptides (Krstenansky et al., 1990), the importance of nonpolar interactions between the C-terminal tail of hirudin and the anion-binding exosite of thrombin is apparent.

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References

Betz, A., Hofsteenge, J., & Stone, S. R. (1991) *Biochem. J. 275*, 801–803.

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467–3475.

Braun, P. J., Dennis, S., Hofsteenge, J., & Stone, S. R. (1988) *Biochemistry 27*, 6517–6522.

Burley, S. K., & Petsko, G. A. (1985) *Science 229*, 23–28.

Clore, G. M., Sukumaran, D. K., Nilges, M., Zarbock, J., & Gronenborn, A. M. (1987) *EMBO J. 6*, 529–537.

Creighton, T. E. (1991) *Curr. Opin. Struct. Biol. 1*, 5–16.

Dodt, J., Köhler, S., & Baici, A. (1988) *FEBS Lett. 229*, 87–90.

Dodt, J., Köhler, S., Schmitz, T., & Wilhelm, B. (1990) *J. Biol. Chem. 265*, 713–718.

Fenton, J. W., II (1989) *Semin. Thromb. Hemostasis 15*, 265–268.

Folkers, P. J. M., Clore, G. M., Driscoll, P. C., Dodt, J., Köhler, S., & Gronenborn, A. M. (1989) *Biochemistry 28*, 2601–2617.

Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. *EMBO J. 9*, 2361–2365.

Haruyama, H., & Wüthrich, K. (1989) *Biochemistry 28*, 4301–4312.

Hecht, M. A., Sturtevant, R. T., & Sauer, R. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5685–5689.

Hofsteenge, J., Stone, S. R., Donella-Deana, A., & Pinna, L. A. (1990) *Eur. J. Biochem. 188*, 55–59.
Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 101–117.
Knecht, R., & Chang, J.-Y. (1986) *Anal. Chem. 58*, 2375–2379.
Krstenansky, J. L., Owen, T. J., Yates, M. T., & Mao, S. J. T. (1987) *J. Med. Chem. 30*, 1688–1691.
Krstenansky, J. L., Broersma, R. J., Owen, T. J., Payne, H. P., Yates, M. T., & Mao, S. J. T. (1990) *Thromb. Haemostasis 63*, 208–214.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 81*, 488–492.
Matthews, B. W., Nicholson, H., & Becktel, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6663–6667.
Niehrs, C., Huttner, W. B., Carvallo, D., & Degryse, E. (1990) *J. Biol. Chem. 265*, 9314–9318.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 245*, 277–280.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622–4628.
Stone, S. R., & Hofsteenge, J. (1991) *Biochemistry 30*, 3950–3955.
Stone, S. R., Dennis, S., & Hofsteenge, J. (1989) *Biochemistry 28*, 6857–6863.
Wallace, A., Dennis, S., Hofsteenge, J., & Stone, S. R. (1989) *Biochemistry 28*, 10079–10084.
Wells, J. A. (1990) *Biochemistry 29*, 8509–8517.

Effect of Increased Chain Packing on Gramicidin–Lipid Interactions†

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ABSTRACT: To study the effect of lipid packing on the dynamics of membrane proteins, the changes in the rotational motion of gramicidin tryptophans with increased packing brought about by high hydrostatic pressure through fluorescence spectroscopy were determined. In fluid phase dimyristoylphosphatidylcholine, the rotational motion of the residues decreased slightly with increased packing, but in the gel phase a significant reversible increase was observed. The magnitude of this increase was temperature dependent and much greater at lower temperatures. Quenching studies show that the increase in rotational motion is not due to a change in the location of the peptide in the membrane under pressure. Aromatic ring stacking between residues 9 and 15 appears to be stabilized under pressure, and there is no evidence of pressure-induced changes in peptide aggregation. The increase in rotational motion could be caused by a destabilization of hydrogen bonds between the indole hydrogens and the lipid head group oxygens due to an increase in the thickness of the compressible lipid bilayer with pressure without a concomitant lengthening of the peptide. These results indicate that specific interactions between lipids and proteins may play a major role of regulating the dynamics of membrane proteins.

The role the lipid matrix plays in controlling the structure and dynamics of integral membrane proteins is not fully understood. To a first approximation, membranes stabilize those conformations that will maximally allow for hydrophobic interactions with the lipid chains and more energetic interactions with the polar surface. These interactions are ultimately determined by the relative length of the lipid chain compared to the length of the hydrophobic surface of the protein. The dynamics or fast local motions of peptide residues should be related to the tightness of lipid chain packing. While several studies have been made on the effect of packing on membrane protein activity [e.g., Dornmair and Jahnig (1989) and Dannenberg et al. (1990)], which involves slower larger scale movements of the peptide backbone, little is known about the influence of packing on these smaller scale motions that are ultimately responsible for the larger conformational changes.

In this study, we will investigate the effect of membrane packing on the conformation and dynamics of gramicidin. Gramicidins are small peptides having the general formula (Sarges & Witkop, 1965)

HCO-LVal-Gly-LAla-DLeu-LAla-DVal-LVal-DVal-LTrp-DLeu-LX-DLeu-LTrp-DLeu-LTrp-NHCH$_2$CH$_2$OH

where X is Trp, Phe, or Try at a ratio of ~7:1:2 and where the NH_2-terminal Val is sometimes replaced by Ile. In membranes, these peptides have the ability to form channels selective for monovalent cations. The tertiary structure of gramicidin can vary from an intertwined helical dimer, as found in organic solvents, micelles, and certain membrane conditions to a formyl NH to formyl NH terminal dimer (referred to here as the N to N terminal dimer) of β 6.3 helices as seen in many bilayers [for a recent review, see Wallace (1990)].

The small size of the peptide lends itself well to determine the role each residue plays in ion permeation. One such approach has been to characterize the functional properties of gramicidin analogues with selected amino acid substitutions [e.g., Durkin et al. (1990)]. Recently, Becker and co-workers determined the functional properties of channels formed by GR[1] analogues having one to three Trp → Phe substitutions

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