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Quantitative Evaluation of the Contribution of Ionic Interactions to the Formation of the Thrombin–Hirudin Complex

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ABSTRACT: The effect of ionic strength on the kinetics of inhibition of human α-thrombin has been examined by using genetically engineered forms of hirudin that differed only in the number of negatively charged residues in the carboxyl-terminal region of the molecule. Analysis of the data obtained allowed the binding energy for the thrombin–hirudin complex to be divided into contributions from ionic and nonionic interactions. The contribution of nonionic interactions to the binding energy was the same for each of the forms whereas the ionic contribution varied with the charge of the molecule. Each of the negatively charged residues made an approximately equal contribution of −4 kJ mol^{-1} to the binding energy. For native hirudin, ionic interactions accounted for 32% of the binding energy at an ionic strength of zero.

Hirudin is a polypeptide inhibitor of thrombin. It has been isolated from the medicinal leech in 2 forms consisting of 65 amino acids and containing a sulfated tyrosyl residue (Bagdy et al., 1976; Dodt et al., 1984, 1986). Hirudin reacts rapidly with thrombin to form a tight complex with a dissociation constant in the femtomolar range (Stone & Hofsteenge, 1986; Braun et al., 1988a; Dodt et al., 1988). The formation of this complex involves interactions with the active site of thrombin and with additional secondary binding sites (Stone et al., 1987). In fact, the contribution to the tightness of the complex made by the binding of hirudin to the primary specificity pocket of thrombin appears to be small (Dodt et al., 1988; Braun et al., 1988a). The acidic C-terminal region of hirudin has been implicated in interactions with a secondary binding site on thrombin (Chang, 1983; Braun et al., 1988a). This negatively charged region of hirudin is thought to interact with an op-

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positely charged secondary binding site on thrombin (Fenton et al., 1988; Fenton & Bing, 1986; Noë et al., 1988; Stone et al., 1987). The proposed secondary binding site includes residues 62–73 of the B chain of human α-thrombin, and these residues correspond to a surface loop in α-chymotrypsin (Birktoft & Blow, 1972; Furie et al., 1982). Cleavage within this region with trypsin produces human β_T-thrombin (Braun et al., 1988b) which has a much reduced affinity for hirudin (Stone et al., 1987). Moreover, antibodies that bind to residues 62–73 block the interaction of hirudin with thrombin (Noë et al., 1988). Evidence that the C-terminal region of hirudin binds to this secondary binding site is provided by studies with fragments of hirudin. A C-terminal peptide of hirudin (residues 53–65) protected human α-thrombin from cleavage by trypsin and was able to compete with the above antibodies for binding to residues 62–73 of thrombin. In contrast, hirudin with the C-terminal peptide removed (residues 1–52) did not protect human thrombin against cleavage with trypsin and did not compete with the antibodies for binding to thrombin (S. Dennis, J. Hofsteenge, and S. R. Stone, unpublished results). C-Terminal peptides of hirudin have also been shown to block the cleavage of fibrinogen (Krstenansky & Mao, 1987; Krstenansky et al., 1987; Mao et al., 1988) as do the antibodies that bind to residues 62–73 of human α-thrombin (Noë et al., 1988).

Thus, there appears to be good evidence that an ionic interaction occurs between the C-terminal region of hirudin and a region of human α-thrombin that includes residues 62–73 of the B chain. The quantitative importance of this ionic interaction for the formation of the thrombin–hirudin complex is, however, not known, and the present study seeks to address this problem. The effect of ionic strength on the kinetic properties of native hirudin and a number of genetically engineered hirudins has been examined. Analysis of the effect of ionic strength permitted the binding energy for each of the hirudins to be divided into contributions made by ionic and nonionic interactions. The variation of the ionic contribution with the charge of the engineered hirudins allowed the average free energy contribution of a single negative charge to be evaluated.

Experimental Procedures

Materials. The substrate D-Phe-Pip-Arg-pNA[1] was from Kabi Vitrum, Molndal, Sweden, and all other chemicals were of the highest purity available commercially. Thrombin was prepared as previously described (Stone & Hofsteenge, 1986) and was free from proteolytically degraded forms and other proteins as judged by gel electrophoresis and Edman degradation. The preparation of thrombin used was 97% active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). Native hirudin (hir-SO_3) was a gift from Plantorgan Werk, Bad Zwischenahn, West Germany, and was homogeneous as judged by Edman degradation with an N-terminal sequence corresponding to that determined by Bagdy et al. (1976) and Dodt et al. (1984). Recombinant hirudin (r-hir) and the mutant recombinant forms (E62Q, $E_2$57,58Q_2, $E_3$57,58,62Q_3, and $E_4$57,58,61,62Q_4) were produced and characterized as previously described (Braun et al., 1988a).

Amidolytic Assay of Thrombin. Assays were performed as previously described at 37 °C in Tris-HCl buffer containing 0.1% (w/v) poly(ethylene glycol) and NaCl (Stone & Hofsteenge, 1986). The concentrations of Tris and NaCl were chosen such that the desired ionic strength was obtained and are given in Table I. The concentrations of Tris and NaCl necessary to achieve the desired ionic strength were calculated by assuming a pK_a of 7.8 for Tris at 37 °C (Ellis & Morrison, 1982), and the ionic strength was checked by measuring the conductance of each of the solutions. In all cases, the measured values were within 5% of the calculated values. In previous studies, it was shown that the substrate D-Phe-Pip-Arg-pNA could compete with hirudin by binding at the active site and at a second lower affinity site which had a dissociation constant of 0.6–0.9 mM (Stone & Hofsteenge, 1986; Stone et al., 1987). In the present study, the concentration of substrate was maintained at a precisely known concentration in the region of 100 μM so that its binding at the lower affinity site would not be significant.

Table I: Composition of Assay Solutions at Different Ionic Strengths and Kinetic Constants for α-Thrombin with D-Phe-Pip-Arg-pNA at These Ionic Strengths[a]

	salt composition of assay mixture		kinetic constants	
ionic strength	[Tris] (M)	[NaCl] (M)	K_m (μM)	k_{cat} (s^{-1})
0.05	0.02	0.04	3.3 ± 0.1	210 ± 2
0.075	0.03	0.06	3.8 ± 0.1	215 ± 2
0.10	0.04	0.08	3.5 ± 0.1	217 ± 4
0.125	0.05	0.10	3.6 ± 0.2	228 ± 9
0.15	0.05	0.125	3.3 ± 0.1	226 ± 3
0.175	0.05	0.15	3.4 ± 0.3	233 ± 7
0.20	0.05	0.175	3.1 ± 0.2	231 ± 6
0.25	0.05	0.225	3.0 ± 0.1	227 ± 3
0.30	0.05	0.275	2.8 ± 0.2	222 ± 6
0.35	0.05	0.325	2.9 ± 0.1	221 ± 4
0.40	0.05	0.375	3.1 ± 0.1	227 ± 4
0.50	0.05	0.475	3.1 ± 0.1	228 ± 4
0.625	0.05	0.60	3.0 ± 0.4	243 ± 4

[a] Assays to determine the kinetic constants were performed as described under Experimental Procedures. The concentration of α-thrombin was 50 pM, the concentration of D-Phe-Pip-Arg-pNA was varied from 2 to 21 μM (six points), and the initial velocities obtained were fitted to the Michaelis–Menten equation using weighted linear regression (Hofsteenge et al., 1986). These analyses yielded the values for the kinetic constants which are given in the table together with their standard errors.

Theory and Data Analysis

Hirudin is a slow-binding inhibitor of thrombin, and the formation of the thrombin–hirudin complex can be represented by the scheme:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

where E, I, and EI represent thrombin, hirudin, and the thrombin–hirudin complex, respectively. The dissociation constant of the complex (K_I) can be related to the association (k_1) and dissociation (k_2) rate constants by the expression $K_I = k_2/k_1$. The standard Gibbs free energy change for the formation of the complex, referred to in this paper as binding energy (ΔG°_b), is related to K_I by eq 1 where R is the gas

$$\Delta G^\circ_b = RT \ln K_I \quad (1)$$

constant and T is the absolute temperature. ΔG°_b can be divided into components due to ionic (ΔG°_{io}) and nonionic interactions (ΔG°_{nio}):

$$\Delta G^\circ_b = \Delta G^\circ_{nio} + \Delta G^\circ_{io} \quad (2)$$

The value of ΔG°_{io} will be dependent on ionic strength (I), and according to Debye–Hückel theory, this dependence is described by eq 3a where ΔG°_{io0} is the ionic interaction energy

[1] Abbreviation: D-Phe-Pip-Arg-pNA, D-Phe-pipecolyl-Arg-*p*-nitroanilide.

$$\Delta G^\circ_{io} = \Delta G^\circ_{io0} \exp(-C_1\sqrt{I}) \quad (3a)$$

at an ionic strength of zero and C_1 is a constant that is related to those terms in the Debye–Hückel screening parameter that are independent of ionic strengths, and the distance between the two charges (Adamson, 1979). Debye–Hückel theory is only strictly applicable at low ionic strength, and additional terms may be introduced in eq 3a to account for deviations from Debye–Hückel theory in a semiempirical way (Adamson, 1979). A relationship of the form of eq 3b has been shown

$$\Delta G^\circ_{io} = \Delta G^\circ_{io0} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (3b)$$

to apply to the interactions of electron-transfer proteins over a wide range of ionic strengths (Meyer et al., 1984; Tollin et al., 1984; Przysiecki et al., 1985) and fitted the data well in the present study. At low ionic strength, the denominator of eq 3b approaches unity, and eq 3b reduces to the Debye–Hückel equation (eq 3a). Substitution of eq 3a and 3b into eq 2 leads to the following expressions for the dependence of binding energy on ionic strength:

$$\Delta G^\circ_b = \Delta G^\circ_{nio} + \Delta G^\circ_{io0} \exp(-C_1\sqrt{I}) \quad (4a)$$

$$\Delta G^\circ_b = \Delta G^\circ_{nio} + \Delta G^\circ_{io0} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (4b)$$

For ionic interactions, the association rate constant (k_1) will also be dependent on ionic strength (Laidler, 1987), and the following expressions can be derived from eq 3a and 3b to describe this dependence (Laidler, 1987; Meyer et al., 1984; Tollin et al., 1984; Przysiecki et al., 1985):

$$\ln k_1 = \ln k_{1\infty} - \frac{\Delta G^\circ_{io0}}{RT} \exp(-C_1\sqrt{I}) \quad (5a)$$

$$\ln k_1 = \ln k_{1\infty} - \frac{\Delta G^\circ_{io0}}{RT} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (5b)$$

where $k_{1\infty}$ is the association rate constant in a medium of infinite ionic strength (i.e., with an infinite dielectric constant). For these derivations, $k_{1\infty}$ is equal to $k_{2\infty} \exp(-\Delta G^\circ_{nio}/RT)$ where $k_{2\infty}$ is the dissociation rate of the complex at infinite ionic strength.

Values for k_1 and K_I were determined as described previously (Stone & Hofsteenge, 1986; Stone et al., 1987) by examining the progress curve for the inhibition of thrombin in the presence of D-Phe-Pip-Arg-pNA. Under conditions where slow, tight-binding inhibition was observed, the data obtained with at least six different concentrations of hirudin were fitted to eq 4 of Stone and Hofsteenge (1986). This analysis yielded apparent values for k_1 and K_I (k_1' and K_I'). Previous studies (Stone & Hofsteenge, 1986; Stone et al., 1987) have shown that, under the assay conditions used, k_1' approximates k_1 and that K_I equals $K_I'/(1 + S/K_m)$ where S is the substrate concentration and K_m is its Michaelis constant. For each ionic strength used, values for the K_m of the substrate D-Phe-Pip-Arg-pNA were determined as previously described (Hofsteenge et al., 1986), and the determined values are given in Table I.

Under certain assay conditions, the tight-binding assumption was not essential (i.e., the hirudin concentration was greater than 10 times that of thrombin), and the data obtained from at least three curves at one hirudin concentration were fitted to the equation for slow-binding inhibition [eq 1 of Morrison (1982)]. These analyses yielded values for v_i and v_s, the initial and steady-state velocities, respectively, and k', the apparent first-order rate constant for the development of the inhibition. For the inhibition of thrombin by hirudin, the values for K_I' and k_1' can be calculated by using the expressions given in eq 6 and 7 (Stone et al., 1987). Values for K_I were calculated from those of K_I' as described above.

$$K_I' = v_s I/(v_i - v_s) \quad (6)$$

$$k_1' = (k'/I)(1 - v_s/v_i) \quad (7)$$

For a particular hirudin variant, values of k_1 were weighted according to the squared inverse of their standard error and fitted to either eq 5a or eq 5b by weighted nonlinear regression to yield estimates of $k_{1\infty}$ and ΔG°_{io0}. In a similar way, values for ΔG°_b were calculated from K_I by using eq 1 and fitted to either eq 4a or eq 4b to yield estimates for ΔG°_{nio} and a second estimate for ΔG°_{io0}.

Results

Values of the association rate constant (k_1) and the dissociation constant (K_I) were determined for six hirudin variants over a range of ionic strengths from 0.05 to 0.625. Interpretation of ionic strength effects is simpler if only low ionic strengths are used such that the departure from ideal behavior is small (Laidler, 1987). For the thrombin–hirudin interaction, however, it was not possible to obtain data at low ionic strengths because significant losses of thrombin activity were observed at ionic strengths below 0.05 [data not shown; see also Fenton et al. (1977)]; even at an ionic strength of 0.05, a loss of about 20% occurred. This loss was a reduction in the concentration of active sites as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate and titration with hirudin. The loss of activity occurred upon dilution into the low ionic strength buffer, and no further loss of activity was observed. In fact, the enzyme was stable for at least 30 min under all assay conditions as determined by the method of Selwyn (1965). This loss of activity was taken into account for the calculation of rate and dissociation constants.

In order to investigate the putative ionic interaction between the C-terminal region of hirudin and thrombin (Chang, 1983; Stone & Hofsteenge, 1986; Stone et al., 1987), a number of mutants were constructed (Braun et al., 1988a) in which one to four of the glutamyl residues in the C-terminal region were mutated to glutamine (E62Q, $E_2$57,58Q_2, $E_3$57,58,62Q_3, and $E_4$57,58,61,62Q_4). Recombinant hirudin (r-hir) possesses one less negative charge than native hirudin (hir-SO_3) because Tyr-63 is not sulfated in the recombinant form. Thus, variants of hirudin were available that possessed one to five less charges in the C-terminal region than hir-SO_3. The effect of ionic strength on the binding energy, ΔG°_b (calculated from K_I by using eq 1), for these variants is shown in Figure 1. Values of k_1 could not be obtained for native hir-SO_3 at ionic strengths below 0.125 because the association rate became too rapid to measure. Therefore, insufficient data were available for a proper evaluation of ionic strength effects on k_1 for this form of hirudin, and the data are not presented. The data obtained for the remaining five variants are shown in Figure 2.

As ionic strength increased, both ΔG°_b (Figure 1) and k_1 (Figure 2) decreased, and the data were fitted to equations (eq 4b and 5b) that have been shown to describe the effect of ionic strength on electrostatic interactions (Meyer et al., 1984; Tollin et al., 1984; Przysiecki et al.; 1985). Analysis of the data of Figure 1 according to eq 4b allowed the binding energy for each of the hirudin variants to be divided into

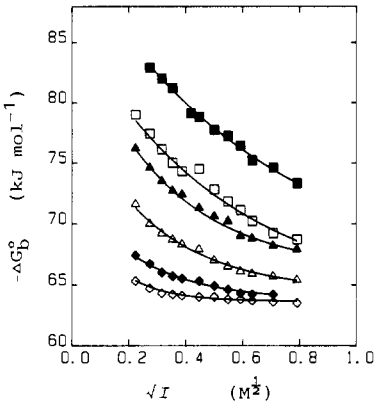

FIGURE 1: Effect of ionic strength on the binding energy of the thrombin–hirudin complex. Binding energies (ΔG°_b) of hir-SO_3 (■), r-hir (□), E62Q (▲), $E_2$57,58Q_2 (△), $E_3$57,58,62Q_3 (◆), and $E_4$57,58,61,62Q_4 (◇) are plotted against the square root of ionic strength (I). Analysis of progress curve data as described under Theory and Data Analysis yielded values for K_I which could be converted to binding energies (ΔG°_b) by using the relationship given in eq 1. The values of ΔG°_b obtained at each ionic strength were then weighted according to the squared inverse of their standard error and fitted to eq 4b by weighted nonlinear regression. The lines drawn represent the fit of the data to this equation.

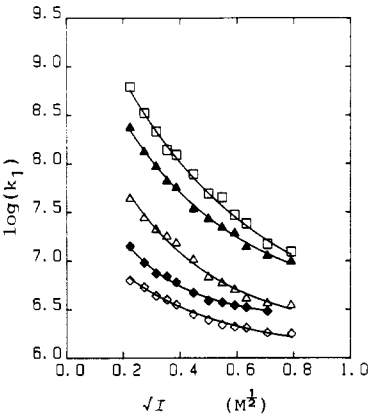

FIGURE 2: Effect of ionic strength on the association rate constant (k_1) for the interaction of hirudin with thrombin. Values of k_1 for r-hir (□), E62Q (▲), $E_2$57,58Q_2 (△), $E_3$57,58,62Q_3 (◆), and $E_4$57,58,61,62Q_4 (◇) are plotted against the square root of ionic strength (I). Analysis of progress curve data as described under Theory and Data Analysis yielded values for k_1 at each ionic strength that were then weighted according to the squared inverse of their standard error and fitted to eq 5b by weighted nonlinear regression. The lines drawn represent the fit of the data to this equation.

contributions due to ionic (ΔG°_{io0}) and nonionic (ΔG°_{nio}) interactions. The value obtained for ΔG°_{nio} was essentially the same for all forms of hirudin, and a weighted mean value of -63.7 ± 0.1 kJ mol^{-1} could be calculated for ΔG°_{nio} from the estimates of this parameter obtained for the six different forms of hirudin (Table II). Thus, mutations that were designed to affect ionic interactions have only affected these interactions, and it can be assumed that the mutations have not caused large alterations in the tertiary structure of other regions of hirudin that interact with thrombin.

A similar conclusion can be drawn from the values of $k_{1\infty}$ obtained from the analyses of the data of Figure 2. The value of $k_{1\infty}$ should represent the rate of association of hirudin with thrombin under conditions where only nonionic forces are involved. It should be constant for each of the hirudins provided no major alterations in tertiary structure have occurred. The results presented in Table III indicate that the estimate of $k_{1\infty}$ obtained was essentially the same for each of the forms. The standard errors of the values of $k_{1\infty}$ estimated for r-hir and E62Q were quite large. The value of $k_{1\infty}$ is determined by extrapolation to infinite ionic strength, and from Figure 2, it is apparent that this extrapolation will be less accurate for r-hir and E62Q than for the other forms. The values obtained for r-hir and E62Q were nevertheless of the same order as those obtained for the other forms.

Two estimates of the contribution of ionic interactions to the binding energy (ΔG°_{io0}) were obtained for each form of hirudin: one from analysis of the effect on ionic strength on ΔG°_b (Table II) and the other from the effect on k_1 (Table III). The estimates of ΔG°_{io0} obtained from the effect of ionic strength on ΔG°_b (Table II) and k_1 (Table III) were generally in good agreement, and as negative charges were removed from the C-terminal region, the value of ΔG°_{io0} decreased. Moreover, the decrease in ΔG°_{io0} was about equal for each charge removed; a plot of the values ΔG°_{io0} versus the charge of hirudin was linear (Figure 3). The linearity of this plot indicates that

Table II: Parameters for the Effect of Ionic Strength on the Binding Energy of the Thrombin–Hirudin Complex[a]

form of hirudin	$-\Delta G^\circ_{nio}$ (kJ mol^{-1})	$-\Delta G^\circ_{io0}$ (kJ mol^{-1})	C_1
hir-SO_3	63.3 ± 4.3	29.6 ± 2.3	0.8 ± 0.3
r-hir	62.2 ± 2.4	25.0 ± 0.7	1.0 ± 0.3
E62Q	65.7 ± 0.6	22.8 ± 2.0	1.9 ± 0.3
$E_2$57,58Q_2	64.1 ± 0.5	17.1 ± 2.0	2.1 ± 0.4
$E_3$57,58,62Q_3	63.6 ± 0.2	10.9 ± 2.0	2.7 ± 0.5
$E_4$57,58,61,62Q_4	63.6 ± 0.1	8.9 ± 1.8	5.0 ± 1.0
weighted mean value	63.7 ± 0.1		

[a] The data of Figure 1 were analyzed according to eq 4b by weighted nonlinear regression as described under Theory and Data Analysis to yield the values of the parameters in the table. The standard errors of the parameters derived from the analysis are also given.

Table III: Parameters for the Effect of Ionic Strength on the Rate of Association of Hirudin and Thrombin[a]

form of hirudin	$10^{-6}k_{1\infty}$ (M^{-1} s^{-1})	$-\Delta G^\circ_{io0}$ (kJ mol^{-1})	C_1
r-hir	1.1 ± 0.7	25.2 ± 0.5	1.1 ± 0.2
E62Q	2.3 ± 1.1	20.5 ± 0.7	1.3 ± 0.3
$E_2$57,58Q_2	1.4 ± 0.4	17.4 ± 1.0	1.7 ± 0.3
$E_3$57,58,62Q_3	2.3 ± 0.2	13.4 ± 1.5	2.7 ± 0.4
$E_4$57,58,61,62Q_4	1.0 ± 0.2	9.2 ± 0.2	1.5 ± 0.3

[a] The data of Figure 2 were analyzed according to eq 5b by weighted nonlinear regression as described under Theory and Data Analysis to yield the values of the parameters in the table. The standard errors of the parameters derived from the analysis are also given.

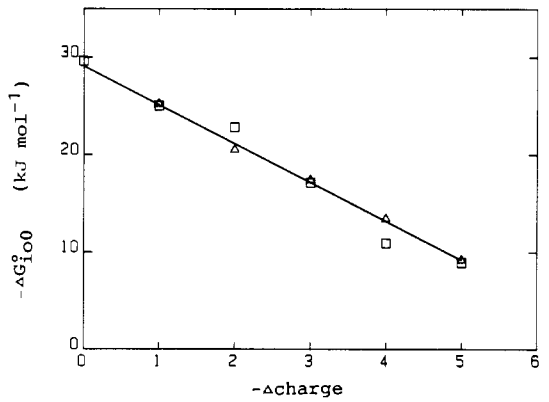

FIGURE 3: Correlation of the ionic contribution to the binding energy of the thrombin–hirudin complex (ΔG°_{io0}) with the charge of hirudin variants. Values for ΔG°_{io0} were determined by analysis of the data of Figure 1 according to eq 4b (□) and by analysis of the data of Figure 2 according to eq 5b (△). The value for Δcharge was calculated relative to the charge of native hirudin. The line drawn represents the best fit of the data obtained by weighted linear regression for the linear dependence of ΔG°_{io0} on Δcharge.

each of the negatively charged residues in the C-terminal region makes an approximately equal contribution to the binding energy, and analysis of the data by weighted linear regression indicated that the average contribution of each residue was -4.0 ± 0.1 kJ mol^{-1}.

The data presented in Figures 1 and 2 were also analyzed according to equations derived from Debye–Hückel theory (eq 4a and 5a). Analysis of the data according to these equations yielded a slightly worse fit to the data in most cases. In general, the standard errors of the estimates of the parameters were slightly higher as were the residual sums of squares (data not shown). The estimates of ΔG°_{nio} and $k_{1\infty}$ obtained by analysis according to Debye–Hückel theory were not significantly different from those presented in Tables II and III. The estimates of ΔG°_{io0} were, however, about 10% lower than those given in Tables II and III. A plot similar to that shown in Figure 3 was obtained for the estimates of ΔG°_{io0} obtained by analysis according to Debye–Hückel theory, but a slightly lower value of -3.7 ± 0.1 kJ mol^{-1} was determined for the average contribution of each negatively charged residue (data not shown). Thus, it seems that the deviations from Debye–Hückel theory at higher ionic strength were not large.

Discussion

In principle, the effect of ionic strength on the interaction between charged molecules can be analyzed in terms of Debye–Hückel theory only when low values of ionic strength are used (Laidler, 1987). Debye–Hückel theory has been shown to adequately describe the effect of ionic strength on a number of chemical reactions (Laidler, 1987) and on the interaction of charged substrates with enzymes [see, for example, Alberty and Hammes (1958), Hammes and Alberty (1958), Nolte et al. (1980), and Argese et al. (1987)]. However, Debye–Hückel theory could not be strictly applied to the thrombin–hirudin interaction because it was only possible to obtain data at higher ionic strengths. Consequently, other treatments of ionic strength effects were examined. The treatment of ionic interactions developed by Watkins (Watkins & Cusanovich, 1983; Meyer et al., 1984; Tollin et al., 1984) has been shown to empirically describe the ionic interactions of electron-transfer proteins at higher ionic strengths (Meyer et al., 1984; Tollin et al., 1984; Przysiecki et al., 1985),[2] and the data obtained in the present study fitted well to equations derived from this treatment (eq 4b and 5b). The primary aim of this investigation was to dissect the binding energy for the thrombin–hirudin interaction into contributions from nonionic and ionic interactions, i.e., to determine values for ΔG°_{nio} and ΔG°_{io0}, and eq 4b and 5b have allowed a relatively accurate estimation of these parameters (Tables II and III). Equations 4b and 5b were not unique in this respect, however, since analysis of the data according to Debye–Hückel theory (eq 4a and 5a) also yielded reasonable estimates of ΔG°_{nio} and ΔG°_{io0}. Thus, although Debye–Hückel theory should not apply at higher values of ionic strength, departures from ideal behavior appear not to have been large.

Using values of ΔG°_{nio} and ΔG°_{io0} obtained for native hirudin (hir-SO_3), it can be calculated that 32% of the binding energy at zero ionic strength is due to ionic interactions. As the ionic strength increases, this contribution will decrease. At a physiological ionic strength of 0.15, the ionic interactions account for 20% (calculated by using eq 4b). For recombinant hirudin (r-hir), the ionic contributions at zero and physiological ionic strengths are 29% and 16%, respectively.

In the above treatment of ionic strength effects, it has been assumed that the changes in ionic strength have specifically affected the ionic interactions that occur between thrombin and hirudin. It is possible, however, that increasing concentrations of salt have caused changes in the structure of thrombin such that other interactions have also been affected. Such secondary salt effects are difficult to exclude. Indeed, increasing concentrations of salt have been found to cause small changes in the structure of thrombin (Landis et al., 1981; Villanueva & Perret, 1983). Over the range of salt concentrations used in the present study, significant changes in the values of k_{cat} and K_m for the substrate D-Phe-Pip-Arg-pNA were not observed (Table I), and these results suggest that any salt-induced changes in the active-site region of thrombin were minor. Similar conclusions regarding the effect of salt on the active site of thrombin have been drawn from ESR experiments using a spin-labeled derivative of thrombin (Landis et al., 1981). Changes in the binding energy of hirudin caused by salt effects on the structure of thrombin would not necessarily be described by eq 4a or 4b. However, the data fitted well to these equations (Figure 1), and, thus, it can be concluded that any secondary salt effects were minor such that deviations of the data from the above equations were small or that the secondary salt effects could also be described by these equations. It would be expected that any secondary salt effects on thrombin structure would affect the binding of each of the hirudins equally unless the structural changes have affected the areas of thrombin that interact with the C-terminal region of hirudin. However, the value of ΔG°_{io0} showed a linear dependence on the charge of hirudin (Figure 3), and such a dependence would not be expected if the putative salt effect on thrombin structure affected the binding of some hirudins more than others.

A number of theoretical models have been developed to examine electrostatic interactions within and between proteins (Warshel & Russell, 1984; Rogers, 1986), and site-directed mutagenesis is now providing a means of testing these models (Hwang & Warshel, 1987, 1988; Shashidar et al., 1987; Gilson & Honig, 1987; Sternberg et al., 1987). The simplest approach to the evaluation of ionic forces assumes that individual charges interact in a continuous uniform medium. The electrostatic contribution, $\Delta G^\circ_{io}(i)$, of a charged residue at a site, i, that interacts with n other charged residues is given by eq 8

$$\Delta G^\circ_{io}(i) = \sum_{i \neq j}^{n} \frac{z_i z_j e^2}{r_{ij} D_{ij}} \quad (8)$$

(Matthew, 1985) where z_i and z_j are the charge numbers of the residues at i and j, e is the elementary charge, r_{ij} is the separation distance between the two charges, and D_{ij} is the effective dielectric constant between the charges. It should be noted that both favorable and unfavorable interactions (i.e., those between unlike and like charges, respectively) are included in this summation. The data of Figure 3 indicate that each of the negatively charged residues in the C-terminal region of hirudin makes an approximately equal contribution of about -4.0 kJ mol^{-1} to the binding energy. This value is lower than other workers have estimated for charge–charge interactions. In a study investigating the interactions of charged substrates with mutants of subtilisin, Wells et al. (1987) estimated values of -7.5 and -9.6 kJ mol^{-1} for two different ionic interactions. Studies by Fersht and colleagues indicated an even higher value of -13 kJ mol^{-1} for charged

[2] Expressions similar to the equations used in the present study have been derived by Alberty and Hammes (1958) and by Wherland and Gray (1976). Moreover, equations derived from the work of Wherland and Gray (1976) have been shown to apply at higher ionic strengths (Smith et al., 1981; Hall et al., 1987a,b).

hydrogen bonds (Fersht et al., 1985; Fersht, 1987). The difference between previous studies and the thrombin–hirudin interaction may be partly explained by the accessibility of the bonds involved to the bulk solvent. The ionic interactions investigated by Wells et al. (1987) were between a substrate and a side chain in the binding pocket of subtilisin. The binding pocket is removed from the bulk solvent, and dielectric constants of 48 and 61 were estimated for the two interactions investigated (Wells et al., 1987). In contrast, it seems likely that the interaction between the charged regions of thrombin and hirudin involves regions of both molecules that are located on the surface. The C-terminal region of hirudin does not make any contacts with the hydrophobic core of the molecule and may form an irregular extended strand in contact with the bulk solvent (Clore et al., 1987). Evidence from a number of experiments outlined in the introduction suggests that the area of thrombin interacting with the C-terminus of hirudin corresponds to a surface loop. Thus, the applicable dielectric constant for the thrombin–hirudin interaction is probably closer to that of water than that of the binding pocket of subtilisin.

The presence of unfavorable as well as favorable electrostatic interactions for each of the negatively charged residues in the C-terminus of hirudin could lead to a lower value for $\Delta G^{\circ}_{io}(i)$. The clustering of these residues in the primary structure suggests that they may also be close to each other in the thrombin–hirudin complex. Although uncomplexed hirudin would be expected to assume a conformation that would lead to maximum separation of the charges, other structural constraints may lead to them being closer together in the complex. In this case, removal of the negative charge not only would remove favorable interactions between the residue and thrombin but also unfavorable interactions with neighboring residues in hirudin and the net change in $\Delta G^{\circ}_{io}(i)$ would be lower than otherwise expected.

The lower value for $\Delta G^{\circ}_{io}(i)$ observed in the present study compared with other studies could also reflect a greater separation of the charges in the case of the thrombin–hirudin complex. For subtilisin, molecular modeling indicated that the charged residues were separated by less than 3.0 Å (Wells et al., 1987). For the thrombin–hirudin complex if it is assumed that the average interaction energy is due to a pair of single charges and that the dielectric constant is 80, the average distance between the charged groups in the thrombin–hirudin complex can be calculated by using eq 8 to be 4.3 Å. If unfavorable interactions do occur, the separation distance will be smaller. In this respect, it is interesting to note that according to Debye–Hückel theory, the constant C_1 of eq 4a and 5a is equal to $0.33r_{ij}$ when r_{ij} is measured in angstroms. The weighted mean value of C_1 was 1.4 which corresponds to a value for r_{ij} of 4.2 Å.

The contribution of ionic interactions to the overall binding energy may be secondary to other functions that they perform. The kinetics of association of hirudin with thrombin proceed via a two-step mechanism (Stone & Hofsteenge, 1986; Stone et al., 1987). The first step appears to be rate determining, and its rate is dependent on ionic strength but independent on the substrate's binding at the active site. The subsequent step is required for the formation of a tighter complex. The ionic interactions involved in the first step could serve primarily to orientate the two molecules as a prelude to the formation of a tighter complex in a subsequent step. Thus, these interactions could be seen as primarily serving a docking function; that is, their function would be to align the two proteins properly and to reduce the number of nonproductive complexes that form before productive complex formation. In this case, the clustering of negatively charged residues in the C-terminal region of hirudin would create a more effective electrical field for long-range orientation. A similar docking function for electrostatic interactions has been proposed for ribonuclease and ribonucleic acid (Matthew, 1985), trypsin and bovine pancreatic trypsin inhibitor (Matthew et al., 1985), and vertebrate cytochrome *c* with bacterial flavodoxin (Matthew et al., 1983).

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β-Lactamase-Catalyzed Aminolysis of Depsipeptides: Amine Specificity and Steady-State Kinetics†

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ABSTRACT: β-Lactamases catalyze not only the hydrolysis but also the aminolysis of certain depsipeptides [Pratt, R. F., & Govardhan, C. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1302–1306]. This paper explores further the specificity of the aminolysis reaction with respect to the structure of the amine and also the steady-state kinetics of the reaction. The amines preferred by the class C β-lactamase of *Enterobacter cloacae* P99 appear to be aromatic D-α-amino acids. The general order of substrate effectiveness at pH 7.5 appears to be aromatic D-α-amino acids > large aliphatic D-α-amino acids > small aliphatic D-α-amino acids ~ small aliphatic L-α-amino acids > large L-α-amino acids. Charges on the aliphatic side chains seem unimportant. Ineffective as acyl acceptors were β-amino acids, α-amino phosphonic acids, and, in general, amines, including amino acid carboxyl derivatives and peptides. There is thus strong evidence for specific interaction between the amine and the enzyme. A detailed kinetics study was made of the P99 β-lactamase-catalyzed aminolysis of *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid by D-phenylalanine. The steady-state kinetics were complex because of the presence of parallel enzyme-catalyzed hydrolysis and aminolysis reactions. An empirical rate equation was obtained for the total reaction. This has important elements in common with that previously found for the aminolysis of specific peptides by the DD-peptidases of various *Streptomyces* strains [e.g., Frere, J.-M., Ghuysen, J.-M., Perkins, H. R., & Nieto, M. (1973) *Biochem. J. 135*, 483–492]. Thus there is further evidence for the close functional similarity between the β-lactamase and DD-peptidase active sites, which supports an evolutionary relationship between the two groups of enzymes. A classical Ping Pong BiBi mechanism does not fit the kinetic data. An ordered BiBi mechanism, with the amino acid binding first, does fit the data, as it did the DD-peptidase results, but is in need of further confirmation.

The major biological role of the β-lactamases is generally believed to be protection of bacteria from β-lactam antibiotics, and indeed, these enzymes do efficiently catalyze the hydrolysis of many such antibiotics. We have shown however that β-lactamases can also catalyze the hydrolysis and aminolysis by D-α-amino acids of certain depsipeptides of general structure **1** (Pratt & Govardhan, 1984). This discovery was significant on two counts. First, it expanded our knowledge of the

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