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pH Dependence of the Interaction of Hirudin with Thrombin

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ABSTRACT: The kinetics of the inhibition of human α-thrombin by recombinant hirudin have been studied over the pH range from 6 to 10. The association rate constant for hirudin did not vary significantly over this pH range. The dissociation constant of hirudin depended on the ionization state of groups with pK_a values of about 7.1, 8.4, and 9.2. Optimal binding of hirudin to thrombin occurred when the groups with pK_a values of 8.4 and 9.0 were protonated and the other group with a pK_a of 7.1 was deprotonated. The pH kinetics of genetically engineered forms of hirudin were examined in an attempt to assign these pK_a values to particular groups. By using this approach, it was possible to show that protonation of His51 and ionization of acidic residues in the C-terminal region of hirudin were not responsible for the observed pK_a values. In contrast, the pK_a value of 8.4 was not observed when a form of hirudin with an acetylated α-amino group was examined, and, thus, this pK_a value was assigned to the α-amino group of hirudin. The requirement for this group to be protonated for optimal binding to thrombin is discussed in terms of the crystal structure of the thrombin-hirudin complex. Examination of this structure allowed the other pK_a values of 7.1 and 9.2 to be tentatively attributed to His57 and the α-amino group of Ile16 of thrombin.

Hirudin was originally isolated from the leech as a specific inhibitor of thrombin (Markwardt, 1970). The major form of hirudin is 65 amino acids in length (Bagdy et al., 1976; Dodt et al., 1984) and is composed of an N-terminal core region held together by 3 disulfide bonds and a flexible C-terminal tail (Folkers et al., 1989; Haruyama & Wüthrich, 1989). Hirudin inhibits thrombin by a unique mechanism. The three N-terminal residues of hirudin are bound in the active-site cleft of thrombin, but the orientation of the polypeptide chain of hirudin within the active site is opposite to that observed with other proteinase inhibitors. Moreover, hirudin does not bind to the primary specificity pocket of thrombin. Hirudin uses binding sites removed from the catalytic center in order to achieve a specific interaction with thrombin. In particular, the C-terminal region of thrombin is bound to a positively charged surface groove on thrombin that has been called the anion-binding exosite (Rydel et al., 1990; Grütter et al., 1990).

The kinetic mechanism for the inhibition of thrombin by hirudin involves at least two steps. The first step, which is rate-determining with picomolar concentrations of hirudin, does not involve the binding of hirudin to the active site of thrombin. In a subsequent step, hirudin binds at the active site to form a tighter complex (Stone & Hofsteenge, 1986; Stone et al., 1987). In the present study, the kinetic mechanism of the interaction of hirudin with thrombin has been

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further investigated by examining the pH dependence of the interaction. The ionization states of three groups that ionize between pH 6 and 10 were found to be important for the formation of the complex. By using modified hirudins, it was possible to assign one of the pK_a values to the α-amino group of hirudin. The possible identity of the other two groups is discussed with reference to the tertiary structure of the thrombin–hirudin complex (Rydel et al., 1990; Grütter et al., 1990).

Experimental Procedures

Materials. The substrates D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238) and D-Val-Leu-Arg-*p*-nitroanilide were obtained from Kabi-Pharmacia (Molndal, Sweden). Human α-thrombin was prepared and characterized as described previously (Stone & Hofsteenge, 1986); the preparation used was fully active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). Recombinant hirudin variant 1 (rhir)[1] was a gift from Ciba-Geigy (Basel, Switzerland).

Preparation and Characterization of Different Hirudin Forms. The mutant hirudins H51Q, rhirQQR (rhir with the mutations K27Q, K36Q, and K47R), and N52M were prepared and characterized as described previously (Braun et al., 1988; Wallace et al., 1989; Dennis et al., 1990). The truncated form of hirudin, rhir(1–52), was produced by CNBr cleavage of the mutant N52M and isolation of the N-terminal fragment by HPLC (Dennis et al., 1990).

Acetylation of the α-amino group of rhirQQR to yield Ac-rhirQQR was performed as described by Wallace et al. (1989). In addition, after isolation of the product by HPLC, the sample was digested with 1% (w/w) leucine aminopeptidase in 40 mM Tris-HCl buffer, pH 7.4, for 24 h at 37 °C. This additional step ensured that the preparation of Ac-rhirQQR was not contaminated with any residual full-length rhirQQR. After the digestion, Ac-rhirQQR was repurified by HPLC as described by Wallace et al. (1989). FAB-MS analysis of the product yielded the mass expected for Ac-rhirQQR.

The concentrations of rhir, rhirQQR, and H51Q 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). Amino acid analysis (Knecht & Chang, 1986) was used to estimate the concentrations of rhir(1–52) and Ac-rhirQQR.

Amidolytic Assays of Thrombin. These assays were performed at 37 °C in a three-buffer system containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.05 M Tris, 0.05 M ethanolamine, 0.2 M NaCl, 0.2% (w/v) poly(ethylene glycol) M_r 6000, and the substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide as described previously (Stone et al., 1991). The pH of the assay was measured at 37 °C with a Radiometer PHM83 pH meter. The three-buffer system was chosen because it provided adequate buffering capacity over the range of pH 6–10 that was used. Moreover, using this buffer system, the ionic strength of the assay deviated only slightly from 0.3 M over the pH range 6–10 (Ellis & Morrison, 1982). This point was important because the kinetic parameters for the inhibition of thrombin by hirudin have been shown to be dependent on ionic strength (Stone & Hofsteenge, 1986; Stone et al., 1989). At the ionic strength of 0.3 M, thrombin was stable over the entire pH range for at least 30 min. At a lower ionic strength of 0.15 M, a slight loss of thrombin activity was detected over time periods greater than 20 min at the extremes of the pH range. The pH dependence of the binding of rhir and rhirQQR was, however, essentially the same at this lower ionic strength.

Slow, tight-binding inhibition of thrombin was observed with rhir, H51Q, and rhirQQR. In assays to determine the kinetic parameters for the inhibition of thrombin by these forms of hirudin, progress curves of thrombin-catalyzed *p*-nitroaniline formation were followed over a period of 20 min. Thrombin and the substrate D-Phe-pipecolyl-Arg-*p*-nitroaniline were present in these assays at concentrations of 0.1 nM and 100 μM, respectively. Inhibition of thrombin by rhir(1–52) and Ac-rhirQQR was studied in assays containing 50 pM thrombin and 50 μM D-Phe-pipecolyl-Arg-*p*-nitroanilide. Slow-binding behavior was not observed with these forms of hirudin, and the initial velocity of the *p*-nitroaniline formation was measured over the first 10 min of the assay.

Data Analysis

Estimation of the Kinetic Parameters for the Inhibition of Thrombin by Different Forms of Hirudin. Estimates of the dissociation constant (K_I) and association rate constant (k_1) for rhir, rhirQQR, and H51Q were determined at each pH by analyzing progress curve data for the thrombin-catalyzed formation of *p*-nitroaniline 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 concentrations of hirudin used varied over a 5-fold range that was adjusted such that accurate estimates of the K_I values could be obtained. The apparent values of the kinetic parameters ($k_{1'}$ and $K_{I'}$) were obtained by nonlinear regression analysis of the progress curve data (Stone & Hofsteenge, 1986). Under the conditions of the assay, k_1 is approximately equal to $k_{1'}$, and the value of K_I can be calculated by using the relationship given in eq 1 (Braun et al., 1988; Stone & Hofsteenge, 1986) where S is

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

the concentration of substrate in the assay and K_m is the Michaelis constant for the substrate at the pH of the assay. Values of K_I were calculated by using the above relationship together with the known substrate concentration and the K_m value at the pH of the assay. The K_m was calculated by interpolation from data previously obtained for the pH dependence of this parameter (Stone et al., 1991).

AcHrhirQQR and rhir(1–52) were relatively poor inhibitors of thrombin and did not exhibit slow-binding inhibition. For these forms of hirudin, six assays were performed at each pH value: one without inhibitor and five others with different inhibitor concentrations. The concentrations of Ac-rhirQQR and rhir(1–52) were varied over a 5-fold range that was chosen such that at least one concentration was below and one above the value of $K_{I'}$. Fitting initial velocity data to the Dixon equation by using weighted linear regression yielded estimates for $K_{I'}$ (Cornish-Bowden & Endrenyi, 1981). Values of K_I were calculated by using eq 1 as described above.

Analysis of the pH Dependence of Hirudin Inhibition. The pH dependence of the K_I values for Ac-rhirQQR exhibited a bell-shaped profile that could be described by eq 2 (Cleland, 1977, 1986) where K_I^* is the pH-independent value of K_I and

$$K_I = K_I^*(1 + [H]/K_1 + K_2/[H]) \quad (2)$$

K_1 and K_2 are acid dissociation constants. Estimates of K_I obtained at different pH values were weighted according to

[1] Abbreviations: rhir, recombinant hirudin variant 1; rhirQQR, rhir with mutations K27Q, K36Q, and K47R; Ac-rhirQQR, rhirQQR with an acetylated α-amino group; rhir(1–52), truncated form of rhir consisting of residues 1–51 plus a C-terminal homoserine.

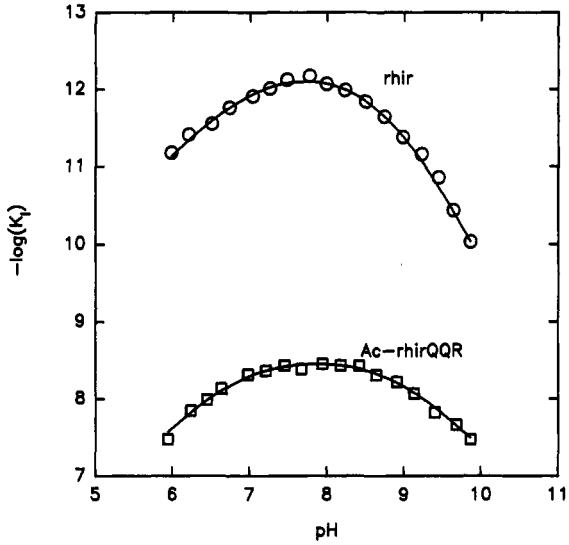

FIGURE 1: Variation with pH of −log K_I for rhir and Ac-rhirQQR. The units of K_I are M, and values are plotted for rhir (○) and Ac-rhirQQR (□). The data points are from single experiments. The curves represent the fit of the data to eq 2 and 3 for Ac-rhirQQR and rhir, respectively, and were drawn using the values of the parameters given in Table I.

their inverse variance and fitted by linear regression to eq 2 to yield estimates of K_I*, pK_1, and pK_2.

The pH dependence for the other forms of hirudin could not be described by eq 2. The plot of −log K_I versus pH exhibited a slope of −2 at higher pH values, and the data were best described by eq 3. Estimates of K_I for these forms of

$$K_I = K_I^*(1 + [H]/K_1 + K_2/[H] + K_2K_3/[H]^2) \quad (3)$$

hirudin obtained at different pH values were fitted to eq 3 by weighted linear regression as described above to yield estimates of K_I*, pK_1, pK_2, and pK_3.

RESULTS

The dependence of the dissociation constant (K_I) for rhir on pH was determined over the pH range from 6 to 10. A pH optimum for the interaction between rhir and thrombin was observed between pH 7.5 and 8.0. At lower pH values, the value of K_I increased, and a plot of −log K_I against pH exhibited a slope 1. The value of K_I also increased at higher pH values, but the plot of −log K_I against pH displayed an asymptotic slope of −2 in the alkaline pH range (Figure 1). These observations suggest that the binding of hirudin requires one group on either thrombin or hirudin to be in its deprotonated form and two other groups to be protonated (Cleland, 1977, 1986). In this case, the pH dependence of the K_I value for rhir would be described empirically by eq 3. The data were fitted to this equation, and the fit illustrated in Figure 1 indicates that this model can adequately explain the data. The pK_a values obtained by fitting the data to this equation were 7.1, 8.4, and 9.2 (Table I). In contrast to the observed pH dependence of K_I, the value of the association rate constant (k_1) for rhir did not vary significantly with pH (Figure 2).

In an attempt to assign the observed pK_a values to specific groups, the pH profiles of K_I for mutants of hirudin were examined. The binding of the truncated form rhir(1–52) exhibited the same pH dependence as that seen with rhir (data not shown); pK_a values of 7.1, 8.3, and 9.2 were observed (Table I). Thus, the ionization of groups in the C-terminal region of hirudin (residues 53–65) does not appear to be responsible for any of the observed pK_a values. The C-terminal region of hirudin binds to a surface groove of thrombin called the anion-binding exosite (Rydel et al., 1990; Grütter et al., 1990), and it is also possible to conclude that the observed pK_a values are not due to the ionization of groups in the anion-binding exosite.

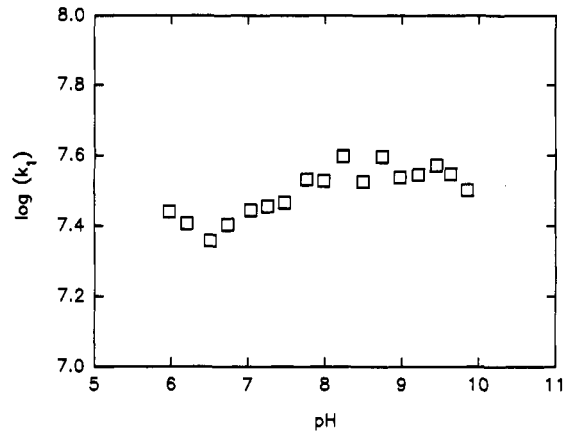

FIGURE 2: Variation with pH of log k_1 for rhir. The units of k_1 are M^{-1} s^{-1}.

Table I: Parameters for the pH Dependence of the Dissociation Constant of Different Forms of Hirudin[a]

form of hirudin	K_I* (pM)	pK_1	pK_2	pK_3
rhir[b]	0.558 ± 0.030	7.06 ± 0.03	8.43 ± 0.05	9.17 ± 0.04
rhirQQR[b]	4.50 ± 0.25	7.11 ± 0.03	8.27 ± 0.06	9.17 ± 0.06
rhir(1–52)[b]	28700 ± 1900	7.06 ± 0.04	8.28 ± 0.08	9.24 ± 0.10
H51Q	0.651 ± 0.050	7.29 ± 0.05	8.60 ± 0.08	9.01 ± 0.06
Ac-rhirQQR	2940 ± 140	6.96 ± 0.05	8.96 ± 0.05	

[a] Estimates for the K_I values for the different forms of hirudin were determined for at least 17 different pH values between 6 and 10 as described under Experimental Procedures. The K_I values were weighted according to the inverse of their variances and fitted by weighted linear regression either to eq 2 for Ac-rhirQQR or to eq 3 for the other forms. These analyses yielded the pK_a values given in the table and the pH-independent value of the dissociation constant (K_I*). The standard errors of the values are also given. [b] For rhir, rhirQQR, and rhir(1–52), data from two pH profiles were analyzed to yield estimates of the parameters. The values given represent the weighted means of these two estimates of the parameters.

It seemed possible that the ionization of the side chain of His51′[2] of hirudin was responsible for the observed pK_a value of about 7. In order to test this hypothesis, the pH dependence of the K_I value for a mutant in which His51′ had been replaced by a glutamine (H51Q) was examined. The pH profile of this mutant was the same as that observed for rhir (data not shown); pK_a values of 7.3, 8.6, and 9.0 were determined from the data (Table I). Thus, the observed pK_a value of about 7 cannot be assigned to His51′. The value of k_1 for H51Q did not vary significantly with pH (data not shown) as was also observed for rhir (Figure 2).

A mutant hirudin in which all three lysyl residues were exchanged (rhirQQR) displayed the same pH dependence as that observed for rhir (data not shown), with pK_a values of 7.1, 8.3, and 9.2 being observed (Table I). Deprotonation of the lysyl side chains of hirudin is, therefore, not responsible for any of the observed pK_a values. As observed with rhir, the value of k_1 for rhirQQR also did not vary significantly with pH (data not shown).

When the α-amino group of rhirQQR was acetylated, the molecule became a much poorer inhibitor of thrombin, and

[2] The numbering of the sequence of thrombin is that of Bode et al. (1989). Residues in rhir are distinguished from those in thrombin by the use of primed numbers.

the pK_a value of about 8.4 was not observed in the pH dependence of binding (Figure 1 and Table I). Ac-rhirQQR showed a simple bell-shaped pH dependence, and analysis of the data yielded pK_a values of 7.0 and 9.0 (Table I). Thus, the pK_a value of 8.4 observed for rhir can be assigned to the α-amino group of the molecule.

Discussion

The pH dependence of the K_I value for rhir was bell-shaped, with the slope of the plot of $-\log K_I$ versus pH being 1 on the acidic side and -2 on the alkaline side (Figure 1). Analysis of the data yielded an estimate of 7.1 for the acidic pK_a and estimates of 8.4 and 9.2 for the alkaline pK_a values (Table I). The pK_a values observed in pH profiles of inhibitors such as those shown in Figure 1 are expected to be those of groups in the noncomplexed forms of the inhibitor and enzyme. The data can be interpreted in terms of the binding of rhir being dependent on a group with a pK_a of 7.1 that must be deprotonated and two groups with pK_a values of 8.4 and 9.2 that must be protonated (Cleland, 1977, 1986). The plot of $-\log K_I$ versus pH for rhir did not exhibit a second plateau at low or high pH values (Figure 1). This indicates that the thrombin–hirudin complex only forms to a significant extent when the groups whose pK_a values are seen in the profile are in the appropriate ionization states. Moreover, it can be concluded that the pK_a values of these groups in the complex have been shifted to values outside of the pH range tested (Cleland, 1977, 1986).

Examination of the crystal structures of the thrombin–hirudin complexes helps to establish the identity of the groups responsible for the observed pK_a values. Numerous ionizable groups are found in rhir, and many of these are involved in electrostatic interactions with groups on thrombin (Rydel et al., 1990; Grütter et al., 1990). The expected pK_a values of the majority of these groups are, however, outside of the pH range tested. The carboxylates of Asp5′, Glu17′, Asp55′, Glu57′, Glu58′, and the C-terminal Gln65′ are all involved in ionic interactions (Rydel et al., 1990; Grütter et al., 1990), but their pK_a values are expected to lie below the lowest pH value tested (pH 6). Moreover, the pK_a values of all these groups in rhir as determined from NMR experiments are below 5 (T. Szyperski and K. Wüthrich, personal communication). The only groups in rhir that are involved in electrostatic interactions and whose pK_a values should lie within the pH ranged tested are His51′ and the α-amino group of Val1′. Residues in thrombin involved in electrostatic interactions are Lys36, Glu39, Arg73, Arg77A, Lys149E, Arg173, Arg221A, and Lys224 (Rydel et al., 1990; Grütter et al., 1990), but the expected pK_a values of these groups are also outside the pH range tested. The data obtained with rhir(1–52) confirm that the pK_a values of a number of the above groups do indeed lie outside the pH range tested. Similar pK_a values were seen with rhir(1–52) and rhir (Table I); thus, the pK_a values observed with rhir are not due to the ionization of the acidic groups in its C-terminal region. In addition, it can be concluded that the observed pK_a values are not due to deprotonation of the basic groups in thrombin (Lys36, Arg73, Arg77A, and Lys149E) that interact with C-terminal region of rhir.

Modified hirudins have been used to establish whether the ionization of His51′ or the α-amino group of Val1′ is responsible for one of the observed pK_a values. The replacement of His51′ by a glutamine did not alter the pH dependence of K_I for hirudin (Table I), and it can be concluded that the ionization state of this residue is not important for the binding of hirudin to thrombin. This result is unexpected since His51′ appears to make a salt bridge to Glu39 of thrombin (Rydel et al., 1990). The results of site-directed mutagenesis experiments, however, suggest that the interactions with the side chain of His51′ do not make a large contribution to binding energy. Mutation of His51′ to glutamine, leucine, or aspartate did not significantly reduce the affinity of thrombin for rhir (Dodt et al., 1988; Braun et al., 1988).

The results obtained with Ac-rhirQQR suggest that the α-amino group of Val1′ must be protonated for optimal binding and that this group is responsible for the pK_a value of about 8.4. Ac-rhirQQR was made from the mutant rhirQQR in which all the lysines of rhir had been mutated to allow the specific modification of the α-amino group of Val1′. The pK_a value of about 8.4, which was observed in the pH profiles for all other hirudin forms tested, was not observed with Ac-rhirQQR (Table I). The proposal that the pK_a value of about 8.4 is due to the α-amino group of Val1′ is consistent with a number of other results. A value of 8.4 was measured for the pK_a of the α-amino group by titration with 2,4,6-trinitrobenzenesulfonic acid (Wallace et al., 1989), and a pK_a value of 8.1 has been found for this group in NMR experiments (T. Szyperski and K. Wüthrich, personal communication). It was also found that acetylation of the α-amino group resulted in a large decrease in binding energy. In contrast, acetimidation of the α-amino group, which adds a group of similar size to the acetyl group but maintains a positive charge, led to a much smaller decrease in binding energy. The difference between the results obtained with acetylated and acetimidated rhirQQR suggested that a positive charge located at the α-amino group was important for the binding of hirudin to thrombin (Wallace et al., 1989). In the crystal structures of the thrombin–hirudin complexes, the α-amino group of hirudin is well placed to make two hydrogen bonds: one to the carbonyl group of Ser214 and the second to the O^{γ} of Ser195 (Rydel et al., 1990; Grütter et al., 1990). Hydrogen bonds in which one of the partners is charged are expected to be considerably stronger than those in which both partners are uncharged (Fersht, 1987). Thus, deprotonation of the α-amino group of Val1′ would lead to a weakening of the observed hydrogen bonds, and this would explain the observed dependence of the binding of hirudin on the protonation state of this group.

The other two pK_a values of 7.1 and 9.1 are similar to those observed in the pH profile for the substrate analogue N^{α}-dansyl-L-arginine-4-methylpiperidine amide (Stone et al., 1991). These two pK_a values were assigned to His57 and the α-amino group of the thrombin B-chain (Ile16) for the binding of N^{α}-dansyl-L-arginine-4-methylpiperidine amide (Stone et al., 1991), and they can be tentatively assigned to the same groups for hirudin binding. Studies with chymotrypsin have indicated that the α-amino group of Ile16 must be protonated for the enzyme to assume an active conformation (Fersht, 1985). The pH dependence of the binding of hirudin suggests that it only binds to thrombin molecules that have an active conformation, that is, to those molecules with a protonated α-amino group of Ile16. In the crystal structures of the thrombin–hirudin complexes, the $N^{\epsilon 2}$ atom of His57 is close to the nitrogen of the α-amino group of hirudin, but the separation (3.3 Å) of the two atoms is greater than would be expected for a hydrogen bond (Rydel et al., 1990). The results discussed above indicate that the α-amino group of Val1′ must be protonated for optimal binding. Thus, if His57 were protonated, there would be an unfavorable excess of positive charge in the active site and binding of hirudin to thrombin molecules in which His57 was deprotonated would be favored. The results obtained with Ac-rhirQQR are, however, not en-

tirely consistent with this hypothesis. Since the N-terminus of Ac-rhirQQR is neutral, protonation of His57 would not be expected to affect its binding, and the pK_a for His57 should not be observed in the pH profile for this form of hirudin. Examination of the crystal structures of the thrombin–hirudin complexes does not reveal, however, any other groups that could be responsible for the observed pK_a value of 7.1 apart from His51′, and an assignment of the pK_a to this group can be excluded on the basis of the results obtained with H51Q. Protonation of His57 is apparently unfavorable to binding even in the absence of a positively charged N-terminus.

The value of the association rate constant (k_1) for rhir did not vary greatly with pH (Figure 2). The binding of hirudin to thrombin involves at least two steps (Stone & Hofsteenge, 1986). The first and rate-limiting step under the conditions of the assay used in the present study does not involve binding to the active site of thrombin (Stone & Hofsteenge, 1986; Stone et al., 1987). The dependence of the rate of this step on ionic strength and on the charge of the C-terminal region of hirudin has led to the proposal that the rate-limiting step involves ionic interactions between this region of hirudin and the anion-binding exosite of thrombin (Braun et al., 1988; Stone & Hofsteenge, 1986; Stone et al., 1989). The results with rhir(1–52) indicate that the groups involved in these ionic interactions do not ionize within the pH range tested. Thus, if the rate-limiting step in the binding of hirudin to thrombin involves the C-terminal region of hirudin and the anion-binding exosite, the association rate constant would not be expected to depend on pH as was indeed observed (Figure 2).

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