Interaction of Hirudin with the Dysthrombins Quick I and II†

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Received January 22, 1991; Revised Manuscript Received April 2, 1991

ABSTRACT: The interaction of hirudin with the dysfunctional enzymes thrombin Quick I and II has been investigated. Natural and recombinant hirudin caused nonlinear competitive inhibition of thrombin Quick I. The results were consistent with thrombin Quick I existing in two forms that have different affinities for hirudin. The affinities of these forms for natural hirudin were respectively 10^4- and 10^6-fold lower than that of α-thrombin. In contrast, truncated hirudin molecules lacking the C-terminal tail of the molecule caused linear inhibition of thrombin Quick I. These results indicate that different modes of interaction of the two forms of thrombin Quick I with the C-terminal tail of hirudin were the cause of the nonlinear inhibition. Comparison of the dissociation constants of thrombin Quick I with the truncated and full-length forms of hirudin suggested that the interactions that normally occur between the C-terminal tail of hirudin and thrombin were completely disrupted with the low-affinity form of thrombin Quick I. Thrombin Quick II displayed an affinity for natural hirudin that was 10^3-fold lower than that observed with α-thrombin. In contrast, it bound a mutant hirudin with altered N-terminal amino acids only 16-fold less tightly. These results are discussed in terms of structural alterations in the active-site cleft in thrombin Quick II.

Prothrombin Quick is a congenital dysfunctional form of the coagulation factor prothrombin (Quick et al., 1955; Owen et al., 1978). Upon activation of prothrombin Quick, two dysthrombins (Quick I and II) are isolated (Henriksen et al., 1980; Henriksen & Owen, 1987). Thrombin Quick I has nearly normal activity with thrombin-specific low molecular weight substrates, but its activity with fibrinogen is about 100 times lower than that observed with α-thrombin (Henriksen & Owen, 1987). The primary structural defect in thrombin Quick I has been identified as the substitution of a cysteine for Arg67[1] (Henriksen & Mann, 1988). This arginine is found in the anion-binding exosite of thrombin (Bode et al., 1989) and is one of the sites that is cleaved during the conversion of α- to γ-thrombin (Boissel et al., 1984). This degraded form of the enzyme also has a much reduced activity with fibrinogen (Lewis et al., 1987).

Thrombin Quick II has negligible activity both with thrombin-specific low molecular weight substrates and with fibrinogen (Henriksen & Owen, 1987). This reduced activity is the result of substitution of a valine for Gly226 (Henriksen & Mann, 1989). In human α-thrombin, Gly226 forms part of the primary specificity pocket (Bode et al., 1989) and it has been argued that the substitution of a valyl residue for Gly226 will result in the closing of this pocket with the result that neither low molecular substrates nor fibrinogen would be able to bind to thrombin Quick II (Henriksen & Mann, 1989).

The salivary gland of the leech *Hirudo medicinalis* contains a number of inhibitors of proteases including hirudin, a specific inhibitor of thrombin (Sawyer, 1986). The major form of hirudin is a polypeptide of 65 amino acids that contains three disulfide bonds (Bagdy et al., 1976; Dodt et al., 1985). The solution structures of natural and recombinant hirudin have been determined by two-dimensional nuclear magnetic resonance techniques (Clore et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989). In these studies, it was found that hirudin consists of a compact N-terminal core (residues 1–49) held together by the three disulfide bridges and a disordered C-terminal tail (residues 50–65). The structure of the thrombin–hirudin complex has also recently been determined by X-ray crystallography (Rydel et al., 1990; Grütter et al., 1990). These structures indicate that hirudin inhibits thrombin by a novel mechanism, as had previously been suggested by the results of solution studies [see, for example, Stone et al. (1987), Dodt et al. (1988), Braun et al. (1988), and Degryse et al. (1989)]. Hirudin binds with its N-terminal three residues in the active-site cleft of thrombin with its polypeptide chain running in the direction opposite to that expected for a substrate. The primary specificity pocket of thrombin is not, however, occupied by hirudin (Rydel et al., 1990; Grütter et al., 1990). Hirudin uses other binding sites to achieve a specific interaction with thrombin. The two most important binding sites for hirudin are hydrophobic regions in the active site and a surface groove on thrombin that has been termed the anion-binding exosite (Fenton, 1981). The C-terminal tail of hirudin lies in this long surface groove, and numerous electrostatic and hydrophobic interactions are made between the two molecules in this region (Rydel et al., 1990).

Interactions between hirudin and thrombin in these two regions account for 60% of the intermolecular contacts less than 4 Å (Rydel et al., 1990). Since thrombin Quick I and II represent two dysthrombins that are altered in these two regions, it was of interest to determine to what extent the interaction of hirudin with these two dysthrombins was altered compared with α-thrombin. The results of the studies presented here indicate that thrombin Quick I exists in two forms and that the interaction of hirudin with the anion-binding exosite of one form is completely disrupted. In addition, on

† This study was funded in part by grants from the Deutsche Forschungsgemeinschaft (J.D.), the American Heart Association, Iowa Affiliate (R.A.H.), and the National Institutes of Health (Grant HL-17430) (R.A.H.).

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[1] The sequence numbering for thrombin is that of Bode et al. (1989) and is based on the chymotrypsin numbering.

0006-2960/91/0430-6392$02.50/0

the basis of the affinity of thrombin Quick II for hirudin, it would appear that the alteration of the active-site region in this enzyme is more extensive than a simple closing off of the primary specificity pocket.

Experimental Procedures

Materials. The chromogenic substrates D-Phe-Pip-Arg-pNA,[2] D-Val-Leu-Arg-pNA, and D-Ile-Pro-Arg-pNA were purchased from Kabi Vitrum (Molndal, Sweden), whereas the fluorogenic substrate tos-Gly-Pro-Arg-AMC was from Bachem (Bubendorf, Switzerland). Human α-thrombin was prepared as described previously (Stone & Hofsteenge, 1986) and was fully active as determined by active-site titration (Jameson et al., 1973). Thrombin Quick I and II were prepared as described by Leong et al. (1991). Active-site titration of thrombin Quick I showed it to be 98% active. Native hirudin ($hirSO_3$) was a gift from Plantorgan Werk (Bad Zwischenahn, Germany) and was homogeneous as judged by Edman degradation with an N-terminal sequence corresponding to that determined by Bagdy et al. (1976). Recombinant hirudin (rhir) was provided by Ciba-Geigy (Basel, Switzerland) or produced as described previously (Dodt et al., 1986). The mutant hirudin rhir(V1,2S) and the truncated forms rhir(1–52) and rhir(1–47) were prepared and characterized as described by Wallace et al. (1989), Dennis et al. (1990), and Dodt et al. (1990), respectively. All forms of hirudin displayed a single peak on reversed-phase HPLC, and amino acid and sequence analysis yielded the results expected for a single hirudin species with the proposed primary structure.

Amidolytic Assays for α-Thrombin and Thrombin Quick I. Assays were performed with either chromogenic (Stone & Hofsteenge, 1986) or fluorogenic substrates (Schmitz et al., 1991) at 37 °C in buffers containing 0.1 or 0.5% (w/v) poly(ethylene glycol) (M_r 6000). The chromogenic assays used 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and the substrates D-Val-Leu-Arg-pNA, D-Phe-Pip-Arg-pNA, or D-Ile-Pro-Arg-pNA (Stone & Hofsteenge, 1986), while the fluorogenic assays utilized 10 mM sodium phosphate buffer, pH 7.4, containing 137 mM NaCl, 2.5 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and the substrate tos-Gly-Pro-Arg-AMC.

Under the conditions of the chromogenic assay, there was a progressive increase in the degree of inhibition caused by $hirSO_3$ and rhir over a period of about 5 min. These progress curve data fitted well to the equation for slow-binding inhibition (Morrison, 1982), and the data were fitted to this equation in order to obtain an estimate for the inhibited velocity. Alternatively, the slope of the progress curve was measured after the steady-state velocity had been achieved (after 10–15 min). Under the conditions of the fluorogenic assay, no significant hysteresis was observed.

Fluorescent Titration of Thrombin Quick II. The concentration of thrombin Quick II molecules capable of binding hirudin was determined by fluorescence titration. The binding of hirudin was found to cause an increase in the tryptophan fluorescence of thrombin. This increase was measured with use of a Perkin-Elmer fluorescence spectrometer with excitation and emission wavelengths of 290 and 340 nm, respectively. Titrations were carried out by serial additions of aliquots (1–4 μL) of $hirSO_3$ to a 1.0-cm^2 quartz cuvette that contained about 0.5 μM thrombin Quick II in the buffer used for the chromogenic assays. Equilibrium fluorescence readings were recorded 1 min after the addition of the ligand. If it is assumed that the increase in fluorescence is proportional to the fraction of thrombin Quick II in complex with hirudin, then the equation follows

$$F = F_0 + \Delta F_m \frac{EI}{E_t} \quad (1)$$

where F, F_0, and ΔF_m are the observed fluorescence, the fluorescence in the absence of hirudin, and the increase in fluorescence at an infinite concentration of hirudin, respectively, EI is the concentration of the thrombin Quick II–hirudin complex and E_t is the total concentration of thrombin Quick II molecules that are capable of binding hirudin. By use of the expression for the dissociation constant of the thrombin Quick II–hirudin complex (K_i) given in eq 2, it can be shown that eq 3 describes the dependence of the observed fluorescence on the hirudin concentration

$$K_i = \frac{(E_t - EI)(I_t - EI)}{EI} \quad (2)$$

$$F = F_0 + \frac{\Delta F_m}{2E_t}\{(E_t + I_t + K_i) - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\} \quad (3)$$

where I_t is the total concentration of hirudin and other terms are as previously defined. Fluorescence titration data were fitted to eq 3 by nonlinear regression to obtain an estimate for the concentration of thrombin Quick II (E_t). The concentration of thrombin Quick II estimated by fluorescence titration was 92% of that determined from its absorbance at 280 nm (Henriksen & Mann, 1989). Because of the high concentration of thrombin Quick II used (0.5 μM) relative to the dissociation constant of the complex (Table III), it was not possible to obtain an accurate estimate of this parameter by fluorescence titration.

Determination of the Concentrations of Different Hirudin Forms. The concentrations of $hirSO_3$, rhir, and rhir(V1,2S) were determined by titration with thrombin as previously described (Stone & Hofsteenge, 1986; Wallace et al., 1989). The concentrations of rhir(1–52) and rhir(1–47) were determined by amino acid analysis (Knecht & Chang, 1987; Dodt et al., 1990).

Results

Inhibition of Thrombin Quick I by Hirudin. The inhibition of thrombin Quick I by native ($hirSO_3$) and recombinant hirudin (rhir) was studied over a wide range of hirudin concentrations with use of two assay conditions as described in Experimental Procedures. Both $hirSO_3$ and rhir were found to cause nonlinear competitive inhibition. Figure 1 shows one data set obtained with $hirSO_3$. The data obtained were consistent with the existence of two forms of the enzyme that have different affinities for hirudin. In such a case, the dependence of the velocity on the concentration of inhibitor and substrate will be given by the equation

$$v = \frac{V_{m1}S}{S + K_m(1 + I/K_{i1})} + \frac{V_{m2}S}{S + K_m(1 + I/K_{i2})} \quad (4)$$

where V_{m1} and V_{m2} are the maximum velocities of the two forms of the enzyme, K_{i1} and K_{i2} are the inhibition constants for the two species, and K_m is the Michaelis constant for the

[2] Abbreviations: Pip, pipecolyl; pNA, *p*-nitroanilide; AMC, 7-(4-methyl)coumarinylamide; tos, *p*-toluenesulfonyl; $hirSO_3$, natural hirudin that contains a sulfated tyrosine at position 63; rhir, recombinant hirudin variant 1; rhir(V1,2S), rhir with the valyl residues at positions 1 and 2 mutated to serines; rhir(1–47), rhir residues 1–47; rhir(1–52), rhir residues 1–51 plus a C-terminal homoserine; DIP, diisopropyl phosphoryl.

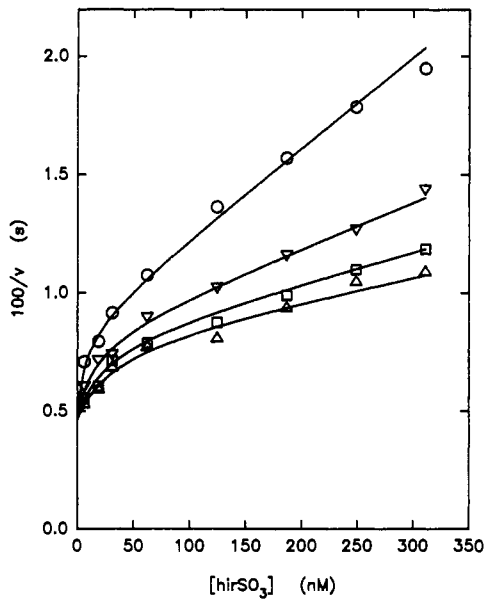

FIGURE 1: Inhibition of thrombin Quick I by $hirSO_3$. Assays were performed under the conditions of the chromogenic assay (see Experimental Procedures) with 50 pM thrombin Quick I. The data are illustrated as Dixon plots of the variation of the reciprocal of the velocity with the concentration of $hirSO_3$ at the following concentrations of D-Phe-Pip-Arg-pNA: 47.8 (○), 95.5 (▽), 143.3 (□), 191 μM (△). Data obtained with 0, 12.4, and 24.9 nM $hirSO_3$ are not shown. The data were fitted to eq 4, and the lines drawn show the fit to this equation.

substrate with both forms. The data of Figure 1 were fitted to eq 4, and the fit of the data to this equation illustrated in the figure indicates that the proposed model involving competitive inhibition of two enzyme forms can adequately explain the data. The data failed to fit to an equation describing a model with three enzyme forms. The analysis of the data according to eq 4 yielded values of 0.44 ± 0.10 and 19.9 ± 4.5 nM for K_{i1} and K_{i2}, respectively (Table I); the ratio of V_{m1} to V_{m2} was 0.8. The estimated values for K_{i1} and K_{i2} indicate that the two forms of the enzyme have affinities for $hirSO_3$ that are respectively 4 and 6 orders of magnitude lower than that observed for α-thrombin. In terms of binding energies, a reduction of 25 and 35 kJ mol^{-1} has occurred with the two forms of thrombin Quick I (Table I).

An assumption made in deriving eq 6 was that the Michaelis constant for the substrate was essentially the same for the two forms of the enzyme. The assumption seems justified since deviations from Michaelis kinetics were not observed over a wide range of substrate concentrations with the three substrates used (D-Phe-Pip-Arg-pNA, D-Ile-Pro-Arg-pNA, and tos-Gly-Pro-Arg-AMC). Moreover, the value of K_m determined from fitting the data of Figure 1 to eq 4 (4.5 ± 1.0 μM) did not differ from that obtained from analysis of initial velocity data in the absence of hirudin (4.4 ± 0.3 μM). A similar agreement between the value of K_m estimated from these two types of experiments was observed in the other inhibition studies presented below. In addition, allowing the K_m values of the two enzyme forms to be different did not significantly improve the fit of the data to the equation.

$HirSO_3$ was also a nonlinear competitive inhibitor of thrombin Quick I under the conditions of the fluorogenic assay. Analysis of the data obtained under these conditions according to eq 4 yielded values of 0.44 ± 0.06 and 24.0 ± 1.2 nM for K_{i1} and K_{i2}, respectively (Table II), and the ratio of V_{m1} to V_{m2} was 1.2. The values of K_{i1} and K_{i2} agree well with those determined under the conditions of the chromogenic assay (Table I).

Table I: Dissociation Constants for the Interaction of Thrombin Quick I with Hirudin under the Conditions of the Chromogenic Assay[a]

form of thrombin	form of hirudin		
	$hirSO_3$	rhir	rhir(1–52)
Quick I			
K_{i1}	0.44 ± 0.10 nM (55.5)	1.41 ± 0.21 nM (52.6)	
K_{i2}	19.9 ± 4.5 nM (45.7)	44.8 ± 3.8 nM (43.6)	
K_i			32.1 ± 1.3 nM (44.4)
α-thrombin	22 ± 1 fM[b] (81.0)	0.23 ± 0.01 pM[b] (75.0)	24.6 ± 1.3 nM[c] (45.1)
β_T-thrombin	2.9 ± 0.2 pM[d] (68.4)	9.7 ± 0.1 pM[c] (65.4)	35.1 ± 4.1 nM[c] (44.2)
γ_T-thrombin	29 ± 1 nM[c] (45.8)	55 ± 2 nM[c] (44.8)	214 ± 12 nM[c] (39.6)

[a] Estimates for the dissociation constants of the different forms of hirudin with thrombin Quick I were determined under the conditions of the chromatogenic assay (see Experimenal Procedures) by fitting the data to the appropriate equation as described in the text. The estimates are reported together with their standard errors as derived from the regression analyses. Values for K_{i1} and K_{i2} represent the dissociation constants for the low- and high-affinity forms of thrombin Quick I. The binding energy (in negative kilojoules per mole) for each interaction was calculated from the dissociation constants with use of the formula $\Delta G_b^\circ = RT \ln (K_i)$ and is given in brackets. [b] From Braun et al. (1988). [c] From Stone and Hofsteenge (1991). [d] From Stone et al. (1987).

Table II: Dissociation Constants for the Interaction of Thrombin Quick I with Hirudin under the Conditions of the Fluorogenic Assay[a]

form of thrombin	form of hirudin		
	$hirSO_3$	rhir	rhir(1–47)
Quick I			
K_{i1}	0.44 ± 0.06 nM (55.5)	1.4 ± 0.8 nM (58.5)	
K_{i2}	24.0 ± 1.2 nM (45.2)	20.4 ± 1.3 nM (45.6)	
K_i			697 ± 55 nM (36.5)
α-thrombin	36 ± 9 fM[b] (79.8)	0.45 ± 0.02 pM[b] (73.2)	420 ± 18 nM[b] (37.8)

[a] Estimates for the dissociation constants of various forms of hirudin with thrombin Quick I were determined under the conditions of the fluorogenic assay (see Experimental Procedures) as described in the text. The estimates are reported together with their standard errors as derived from the regression analyses. Values for K_{i1} and K_{i2} represent the dissociation constants for the low- and high-affinity forms of thrombin Quick I. The binding energy (in negative kilojoules per mole) for each interaction was calculated as described in Table I and is given in brackets. [b] From Schmitz et al. (1991).

Data similar to those presented in Figure 1 were obtained for the inhibition of thrombin Quick I by rhir. Analysis of these data according to eq 6 yielded estimates for K_{i1} and K_{i2} of 1.4 ± 0.2 and 44.8 ± 3.8 nM, respectively, under the conditions of the chromogenic assay (Table I) and 1.4 ± 0.8 and 20.4 ± 1.3 nM, respectively, with the fluorogenic assay (Table II). The ratio of V_{m1} and V_{m2} was about 1.0 in both cases.

The inhibition of thrombin Quick I by truncated forms of hirudin was also examined. In contrast to full-length forms of hirudin, both rhir(1–52) and rhir(1–47) were found to be linear competitive inhibitors (data not shown). A wide concentration range of 61 nM to 3.7 μM rhir(1–52) was used in order to detect any deviation from linearity, but no deviation was seen. Thus, the nonlinear inhibition observed with $hirSO_3$ and rhir appears to involve an interaction between the C-

Table III: Dissociation Constants for the Interaction of Thrombin Quick II with Hirudin[a]

form of thrombin	form of hirudin	
	$hirSO_3$	rhir(V1,2S)
Quick II	40 ± 3 pM (61.7)	2.5 ± 0.2 nM (51.0)
α-thrombin	22 ± 1 fM[b] (81.0)	157 ± 9 pM (58.2)
DIP-thrombin	26 ± 6 pM[c] (62.8)	

[a] Values for $hirSO_3$ and for rhir(V1,2S) with thrombin Quick II were calculated as described in the text. The estimates are reported together with their standard errors as derived from the regression analyses. Values for binding energy (in negative kilojoules per mole) calculated from the dissociation constants are given in brackets. [b] From Braun et al. (1988) [c] From Stone et al. (1987).

terminal tails of these molecules and two different forms of thrombin Quick I. The data for inhibition of thrombin Quick I by rhir(1–52) and rhir(1–47) were analyzed according to a linear competitive inhibition model by using weighted linear regression (Segel, 1975; Cornish-Bowden & Endrenyi, 1981). The estimates of the inhibition constant (K_i) obtained from these analyses are given in Tables I and II for rhir(1–52) and rhir(1–47), respectively. The value of 32.1 ± 1.3 nM determined for K_i of rhir(1–52) is in the same range as the values obtained for K_{i2} with $hirSO_3$ and rhir (Table I). The estimate of K_i obtained for rhir(1–47) was 420 ± 18 nM, which is about 20-fold higher than the values obtained for K_{i2} with $hirSO_3$ and rhir (Table II).

Interaction of Thrombin Quick II with Hirudin. It was not possible to measure the affinity of thrombin Quick II for hirudin with an activity assay because of the lack of a suitable substrate for measuring this enzyme's activity (Henriksen & Owen, 1987; Henriksen & Mann, 1989). Therefore, the affinity of thrombin Quick II for $hirSO_3$ was estimated from its ability to compete with α-thrombin for binding to hirudin. The apparent dissociation constant ($K_{i'}$) of $hirSO_3$ with α-thrombin was evaluated by analyzing tight-binding inhibition data as previously described (Stone & Hofsteenge, 1986). The effect of four different concentrations (230–920 nM) of thrombin Quick II was examined. The observed value of $K_{i'}$ for $hirSO_3$ showed a linear dependence on the concentration of thrombin Quick II (data not shown), as would be expected if the two thrombin species were competing for the hirudin molecules. Equation 5 should describe the competitive effect of thrombin Quick II on the observed value of $K_{i'}$, provided that the concentration of the free thrombin Quick II was not significantly depleted by its binding to hirudin

$$K_{i'}(\text{obs}) = K_{i'(0)}(1 + \text{QII}/K_d) \quad (5)$$

where QII and K_d represent the concentration of thrombin Quick II and the dissociation constant of its complex with $hirSO_3$, respectively, $K_{i'}(\text{obs})$ is the observed value of $K_{i'}$ at a particular concentration of thrombin Quick II, and $K_{i'(0)}$ is the value of $K_{i'}$ in the absence of thrombin Quick II. For the data obtained, it could be calculated that the concentration of free thrombin Quick II was at least 90% of the total concentration in all cases. Thus, the data were fitted to eq 5 and a value of 40 ± 3 pM was estimated for K_d. This value is 3 orders of magnitude higher than that obtained with α-thrombin and represents a decrease in binding energy of 19 kJ mol^{-1} (Table III).

The binding of thrombin Quick II to the hirudin mutant rhir(V1,2S) was also examined. However, because of the reduced affinity of rhir(V1,2S) for α-thrombin, it was necessary to use concentrations of rhir(V1,2S) and thrombin Quick II that were of the same order, and the assumption that the concentration of thrombin Quick II was not significantly reduced by its binding to hirudin could not be made. In the absence of thrombin Quick II, the inhibition of α-thrombin by rhir(V1,2S) in the presence of 19.3 μM D-Phe-Pip-Arg-pNA could be described by the Dixon equation

$$v = \frac{V'}{1 + I/K_{i'}} \quad (6)$$

where I is the concentration of free rhir(V1,2S) and V' is the velocity in the absence of rhir(V1,2S). Estimates for V' and $K_{i'}$ of 178 ± 4 nM/s nM^{-1} and 0.99 ± 0.06 nM were determined. The Dixon equation (eq 6) can be rearranged to provide an expression (eq 7) with which it was possible to

$$I = K_{i'}\left(\frac{V'}{v} - 1\right) \quad (7)$$

calculate the concentration of free rhir(V1,2S). With use of the estimate for free rhir(V1,2S) calculated from eq 7, the concentrations of the thrombin Quick II–rhir(V1,2S) complex and free thrombin Quick II could be calculated from the known total concentrations of thrombin Quick II and rhir(V1,2S). Data for the variation of the concentration of complex with the concentration of thrombin Quick II or rhir(V1,2S) were fitted to the standard binding equation

$$C = \frac{C_m L_f}{K_d + L_f} \quad (8)$$

where C is the concentration of complex at a particular concentration of free ligand (L_f) and C_m is the amount of complex formed at saturating concentrations of ligand, i.e., the maximum amount of complex that can be formed. For the analysis of the data, the value for C_m was fixed at the known concentration of the nonvaried ligand. The effect of five different concentrations of thrombin Quick II (0.46–3.68 nM) was examined at six different concentrations of rhir(V1,2S) (0.24–1.41 nM) in the presence of a fixed concentration of 20 pM α-thrombin. Thus, a total of 11 sets of data were fitted to eq 8: six with thrombin Quick II as the varied ligand and five with rhir(V1,2S) varied. The 11 estimates of the dissociation constant of the thrombin Quick II–rhir(V1,2S) complex ranged from 1.3 ± 0.3 to 3.6 ± 0.4 nM with a weighted average of 2.5 ± 0.2 nM (Table III). This estimate for the dissociation constant is only 16-fold higher than that observed with α-thrombin (Table III).

Discussion

The data obtained for the inhibition of thrombin Quick I by $hirSO_3$ and rhir were consistent with the hypothesis that thrombin Quick I exists in two forms that have different affinities for hirudin. The primary structural defect in thrombin Quick I is the substitution of a cysteine for Arg67. This residue is located in a surface loop that forms one side of the "anion-binding exosite" (Bode et al., 1989). This exosite has been shown to bind the C-terminal tail of hirudin (Rydel et al., 1990; Grütter et al., 1990), and it is also thought to be involved in the binding of the substrate fibrinogen (Fenton, 1981; Bode et al., 1989). Arg67 makes several hydrophobic contacts with h-Ile59 (Rydel et al., 1990), but the loss of these contacts would not cause the large decrease in affinity observed with thrombin Quick I, nor would their loss explain the observed nonlinear inhibition. It seems possible, however, that the replacement of Arg67 by cysteine has resulted in an alteration of the conformation of the anion-binding exosite such that the binding of the C-terminal tail of hirudin to the exosite has been disrupted. Arg67 is flanked by two loop regions that make important interactions with hirudin. Within the loop comprising residues 59–61 located at the N-terminal side of Arg67, interactions with Trp60D would seem to be particularly

important. In the loop segment 70–80, Arg73, Thr74, Arg75, and Tyr76 make numerous close intermolecular contacts (Rydel et al., 1990). The results obtained with hirSO_3 and rhir could be explained by proposing that the exosite in thrombin Quick I exists in two conformations that have different affinities for the C-terminal tail of hirudin and that do not interconvert during the time of the assay (30 min). This hypothesis was tested by examining the inhibition of thrombin Quick I by truncated forms of hirudin. These forms lack the C-terminal tail and, therefore, do not interact with the exosite. Linear inhibition was observed with these forms of hirudin over a wide concentration range. Thus, the nonlinear inhibition observed with hirSO_3 and rhir appears to be due to the interaction of the C-terminal tail of these molecules with two different conformations of the anion-binding exosite of thrombin Quick I. Moreover, the affinity of thrombin Quick I for the truncated forms of hirudin approximated that of α-thrombin (Tables I and II), which suggests that the interaction of the core of hirudin with the active-site cleft of thrombin is not altered in thrombin Quick I. The value of the inhibition constant for rhir(1–52) was in the same range as the values obtained for K_{i2} with hirSO_3 and rhir (Table I). This result suggests that, in the low-affinity form of thrombin Quick I, interactions of the C-terminal tail with the anion-binding exosite do not make any contribution to binding energy. The dissociation constant for rhir(1–47) was about 20-fold higher than the values of K_{i2} observed under the same conditions for hirSO_3 and rhir (Table II). It is noteworthy that the value of K_i for rhir(1–47) with α-thrombin is also 20-fold higher than that for rhir(1–52). These results suggest that the interactions between hirudin residues 48–51 and thrombin make some contribution to binding energy in both the α-thrombin and thrombin Quick I complexes. This hypothesis is consistent with the contacts observed between hirudin residues 49, 50, and 51 and thrombin in the X-ray crystal structures (Rydel et al., 1990).

Each molecule of thrombin Quick I contains a free cysteinyl residue. Therefore, it seemed possible that the low-affinity form of thrombin Quick I was a disulfide-bonded dimer. Polyacrylamide gel electrophoresis (Laemmli, 1970), however, indicated that thrombin Quick I has a molecular mass identical with that of α-thrombin under both reducing and nonreducing conditions (data not shown). Thus, the formation of a covalently bonded dimer is not the explanation for the inability of the anion-binding exosite in the low-affinity form of thrombin Quick I to interact with the C-terminal region of hirudin.

Comparison of the relative affinities of thrombin Quick I and β_T- and γ_T-thrombin for hirudin yields some information about the degree of the disruption of the structure of the anion-binding exosite in the two forms of thrombin Quick I. The affinity of γ_T-thrombin for hirSO_3 and rhir approximates that of the low-affinity form of thrombin Quick I (Table I). The conversion of α- to γ_T-thrombin results in a major alteration in the structure of the anion-binding exosite; one side of the exosite has been completely removed (Boissel et al., 1984). Thus, it can be concluded that the structure of the exosite in the low-affinity form of thrombin Quick I has been drastically altered. Compared with γ_T-thrombin, β_T-thrombin possesses an anion-binding exosite that appears not to be as markedly altered. β_T-Thrombin still binds hirudin with a relatively high affinity (Table I). The strength of binding of hirudin to the high-affinity form of thrombin Quick I lies in between that observed for β_T- and γ_T-thrombin. Thus, it appears that the structural integrity of the anion-binding exosite in the high-affinity form lies somewhere between the almost totally disrupted form seen in γ_T-thrombin and the less altered form of β_T-thrombin.

The primary structural defect in thrombin Quick II is the substitution of a valine for Gly226 (Henriksen & Mann, 1989). In α-thrombin, Gly226 forms part of the primary specificity pocket. It has been proposed that the replacement of Gly226 by valine in thrombin Quick II results in a restricted access to this pocket and that this is the cause of the lack of activity of the enzyme with fibrinogen and low molecular weight substrates (Henriksen & Mann, 1989). If this were the case, hirudin would be expected to bind with equal affinities to α-thrombin and thrombin Quick II, since hirudin does not occupy the primary specificity pocket of thrombin. The results presented in Table III, however, indicate that hirSO_3 bound to thrombin Quick II with a 10^3-fold lower affinity. The affinity of thrombin Quick II for hirSO_3 was about equal to that of thrombin inactivated with diisopropyl fluorophosphate (DIP-thrombin; Table III). In DIP-thrombin, important contacts with Ser195 and His57 are expected to be blocked as well as access to the primary specificity pocket (Stone et al., 1987). Thus, it seems probable that the structure of additional regions of the active site of thrombin Quick II have been altered. Molecular modeling based on the structure of the D-Phe-Pro-Arg-CH_2-thrombin (Bode et al., 1989) indicates that replacement of Gly226 by a valine results in unfavorable contacts between the side chains of Val226 and Asp189. Although Gly226 and Asp189 do not make any direct contacts with hirudin, other residues nearby in the thrombin polypeptide chain make important interactions with hirudin, e.g., Glu192, Gly216, Glu217, Arg221A, and Lys224 (Rydel et al., 1990). Thus, a movement of the polypeptide chain to accommodate the valine substitution at position 226 could change the structure of the active site such that interactions with the N-terminal region of hirudin are disturbed. In this case, interactions of the active site of thrombin Quick II would make a smaller contribution to binding energy than active-site interactions with α-thrombin. Consequently, a change to a less than optimal N-terminal structure of hirudin could be expected to have a smaller effect with thrombin Quick II because of the lower contribution of interactions with this region of hirudin to binding energy. For the mutant hirudin rhir(V1,2S), interactions with the active site of thrombin contribute less to binding energy than such interactions with natural hirudin or rhir (Wallace et al., 1989). The decrease in the binding energy for this mutant compared with that for hirSO_3 was 23 kJ mol^{-1} for α-thrombin, whereas a decrease of only 11 kJ mol^{-1} was observed with thrombin Quick II (Table III). Thus, the results with rhir(V1,2S) are consistent with the hypothesis that the structures of other regions of the active site of thrombin Quick II in addition to that of the primary specificity pocket are altered.

ACKNOWLEDGMENTS

We thank Dr. W. Bode for helpful discussions.

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An Aspartate Residue in Yeast Alcohol Dehydrogenase I Determines the Specificity for Coenzyme†

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Received February 13, 1991; Revised Manuscript Received April 11, 1991

ABSTRACT: In the three-dimensional structures of enzymes that bind NAD or FAD, there is an acidic residue that interacts with the 2′- and 3′-hydroxyl groups of the adenosine ribose of the coenzyme. The size and charge of the carboxylate might repel the binding of the 2′-phosphate group of NADP and explain the specificity for NAD. In the NAD-dependent alcohol dehydrogenases, Asp-223 (horse liver alcohol dehydrogenase sequence) appears to have this role. The homologous residue in yeast alcohol dehydrogenase I (residue 201 in the protein sequence) was substituted with Gly, and the D223G enzyme was expressed in yeast, purified, and characterized. The wild-type enzyme is specific for NAD. In contrast, the D223G enzyme bound and reduced NAD^+ and $NADP^+$ equally well, but, relative to wild-type enzyme, the dissociation constant for NAD^+ was increased 17-fold, and the reactivity (V/K) on ethanol was decreased to 1%. Even though catalytic efficiency was reduced, yeast expressing the altered or wild-type enzyme grew at comparable rates, suggesting that equilibration of NAD and NADP pools is not lethal. Asp-223 participates in binding NAD and in excluding NADP, but it is not the only residue important for determining specificity for coenzyme.

The structure and mechanism of alcohol dehydrogenase (EC 1.1.1.1, ADH)[1] have been extensively studied (Pettersson, 1987; Eklund & Brändén, 1987). Most alcohol dehydrogenases from different sources use NAD rather than NADP as coenzyme. The three-dimensional structure of horse liver ADH suggests that Asp-223 is important in determining

† This work was supported by Grant AA06223 from the National Institute on Alcohol Abuse and Alcoholism, U.S. Public Health Service.
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[1] Abbreviations: ADH, alcohol dehydrogenase; *Sc*ADH, alcohol dehydrogenase I from *Saccharomyces cerevisiae*; D223G, substitution of Asp-223 with Gly-223; L187A, substitution of Leu-187 with Ala-187 etc.

0006-2960/91/0430-6397$02.50/0