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Basis for the Reduced Affinity of β_T- and γ_T-Thrombin for Hirudin

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ABSTRACT: Partial proteolysis of human α-thrombin by trypsin results in the formation of β_T-thrombin and γ_T-thrombin which have a reduced affinity for the inhibitor hirudin and the cell-surface cofactor thrombomodulin as well as reduced activity with fibrinogen. The basis of the reduction in affinity of these thrombin derivatives for hirudin has been investigated by examining their kinetics of interaction with a number of hirudin mutants differing in their C-terminal charge properties as well as with a truncated form of hirudin. The results indicate that the reduced affinity of β_T-thrombin for hirudin is most likely due to a decrease in the strength of nonionic interactions between thrombin and the C-terminal region of hirudin. No decrease in the strength of ionic interactions was observed with β_T-thrombin. In contrast, the reduced affinity of γ_T-thrombin was due to a decrease in the strength of both ionic and nonionic interactions. The N-terminal core region of hirudin, which interacts predominantly with the active-site cleft of thrombin, exhibited similar affinities for α-, β_T-, and γ_T-thrombin, indicating that thrombin–hirudin interactions within the active site are largely preserved in β_T- and γ_T-thrombin.

The serine protease thrombin plays a pivotal role in hemostasis. It interacts with a number of substrates, inhibitors, and receptors, and the specificity of these interactions is essential to the hemostatic process (Fenton, 1981). Thrombin's specificity is determined not only by its active site but also by secondary binding sites (Blombäck et al., 1972). The importance of these secondary binding sites to thrombin's interactions with particular ligands has been studied by using proteolytic derivatives of thrombin (Berliner, 1984). Two such derivatives that have proved useful in these studies are β- and γ-thrombin. Cleavage of human α-thrombin between Arg77A and Asn78[1] by trypsin produces β_T-thrombin (Braun et al., 1988b; Bezeaud & Guillin, 1988). Further cleavage by trypsin of the bonds Arg67–Ile68 and Lys149E–Gly150 leads to the formation of γ_T-thrombin (Bing et al., 1977; Fenton et al., 1977a; Braun et al., 1988b). Similar forms of thrombin also result from autolysis (Fenton et al., 1977a,b; Boissel et al., 1984; Chang, 1986). Studies using these derivatives together with peptide-specific antibodies have identified a region of human α-thrombin between Arg67 and Asn78 as important for the interaction of thrombin with the substrate fibrinogen, the inhibitor hirudin, and the cell-surface protein thrombomodulin (Bezeaud et al., 1985; Lewis et al., 1987; Stone et al., 1987; Bezeaud & Guillin, 1988; Hofsteenge et al., 1988; Noé et al., 1988). The region between residues 67 and 78 contains an unusually high proportion of basic residues and occurs in a surface loop in human α-thrombin (Bode et al., 1989). As it contains the cleavage site for the formation of β-thrombin, this loop is conveniently termed the β-loop. This loop is not present in γ_T-thrombin.

The results from several studies suggest that an ionic interaction occurs between the β-loop of thrombin and a region of hirudin comprising the C-terminal 15 amino acids (residues 51–65). Site-directed mutagenesis has demonstrated that the acidic nature of this region of hirudin is important for its interaction with thrombin (Braun et al., 1988a; Stone et al., 1989). Moreover, studies using fragments of hirudin have indicated that the C-terminal region of hirudin interacts with the β-loop of thrombin (Dodt et al., 1990; Dennis et al., 1990; Chang et al., 1990). Peptides corresponding to residues 48–65 and 53–65 of hirudin were able to protect thrombin from cleavage between Arg77A and Asn78 by trypsin. In contrast, truncated forms of hirudin corresponding to residues 1–47 and 1–52 did not protect against cleavage by trypsin (Dodt et al., 1990; Dennis et al., 1990). Hirudin and the C-terminal fragment of hirudin also protected Lys70 from modification by a lysine-specific reagent (Chang, 1989; Chang et al., 1990).

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[1] The sequence numbering of thrombin is that of Bode et al. (1989); "h-" preceding the three-letter code indicates a residue in hirudin.

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The recently determined crystal structures of thrombin–hirudin complexes have corroborated the results of the above solution studies (Rydel et al., 1990; Grütter et al., 1990). In these crystal structures, the C-terminal segment of hirudin makes numerous electrostatic interactions with the region around the β-loop of thrombin. In addition, hydrophobic contacts between these regions of thrombin and hirudin were suggested to play an important role in the stabilization of the complex (Rydel et al., 1990).

The affinity of β_T-thrombin for hirudin is reduced by 130-fold in comparison with that of α-thrombin whereas the affinity of γ_T-thrombin for hirudin is reduced by 10^6-fold. The present study examined the basis for these reduced affinities. The results indicate that the reduced affinity of β_T-thrombin is not due to the disruption of ionic interactions between the β-loop and the C-terminal region of hirudin but rather to disturbance of hydrophobic interactions between these regions. In the case of γ_T-thrombin, both ionic and nonionic interactions have been altered.

Experimental Procedures

Materials. The substrate D-Phe-pipecolyl-Arg *p*-nitroanilide (D-Phe-Pip-Arg-pNA)[2] was from Kabi Vitrum, Molndal, Sweden. Human β_T-thrombin and γ_T-thrombin were produced and characterized as described previously (Braun et al., 1988b). Active-site titration (Jameson et al., 1973) indicated that these preparations were greater than 96% active. The β_T-thrombin preparation contained 3% γ-thrombin as determined by active-site labeling with tritiated diisopropyl fluorophosphate followed by autoradiography (Braun et al., 1988b), and the γ_T-thrombin preparation contained negligible amounts of other forms. Native hirudin (hir-SO_3) was a gift from Plantorgan AG, Bad Zwischenahn, FRG, and consisted of a single species with an N-terminal amino acid sequence corresponding to that determined by Bagdy et al. (1976). Wild-type recombinant hirudin (r-hir) and mutant hirudins containing from one to four glutamate to glutamine mutations in the C-terminal region ([Glu62→Gln]hir, [Glu57,58→Gln]hir, [Glu57,58,62→Gln]hir, and [Glu57,58,61,62→Gln]hir that are designated r-hir(1Gln), r-hir(2Gln), r-hir(3Gln), and r-hir(4Gln), respectively) were produced and characterized as described previously (Braun et al., 1988a). The truncated form of hirudin r-hir(1–52) was prepared as described by Dennis et al. (1990).

Amidolytic thrombin assays were performed at 37 °C in Tris-HCl buffer, pH 7.8, containing NaCl and 0.1% poly(ethylene glycol) (M_r 6000) (Stone & Hofsteenge, 1986). The concentrations of Tris and NaCl were adjusted to achieve the desired ionic strength as described previously (Stone et al., 1989). The substrate D-Phe-Pip-Arg-pNA was present at a known concentration of about 100 μM for assays with β_T-thrombin and 50 μM for assays with γ_T-thrombin or r-hir(1–52).

Data Analysis

Hirudin is a slow-binding inhibitor of β_T-thrombin, and the mechanism of the inhibition can be described by Scheme I (Stone et al., 1987) where E, I, and EI represent β_T-thrombin, hirudin, and the β_T-thrombin–hirudin complex, respectively. The dissociation constant for the complex (K_I) can be related to the association rate constant (k_1) and the dissociation rate constant (k_2) by the expression given in the scheme. The slow-binding inhibition of β_T-thrombin by hirudin was followed in the presence of the substrate D-Phe-Pip-Arg-pNA. The progress curve of *p*-nitroaniline formation was analyzed according to the equation for slow-binding inhibition (Morrison, 1982), and the estimates for the apparent dissociation constant ($K_{I'}$) and apparent association rate constant ($k_{1'}$) were calculated as described previously (Stone et al., 1987). Previous studies (Stone et al., 1987) indicated that the estimate of $k_{1'}$ approximated the true value for k_1 and that the true value of the K_I was equal to $K_{I'}/(1 + [S]/K_m)$ where [S] is the concentration of the substrate in the assay and K_m is its Michaelis constant. Estimates of K_m for the substrate D-Phe-Pip-Arg-pNA with β_T- and γ_T-thrombin were determined at each ionic strength used and are given in Table I. The estimates of K_m with β_T-thrombin were used to calculate values of K_I by using the relationship given above.

Table I: Kinetic Parameters for the Reaction of D-Phe-Pip-Arg-pNA with β_T- and γ_T-Thrombin at Different Ionic Strengths[a]

ionic strength (M)	β_T-thrombin		γ_T-thrombin	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
0.05	10.5 ± 0.9	185 ± 9	16.9 ± 1.2	199 ± 10
0.075	10.4 ± 0.2	190 ± 2	11.8 ± 0.8	198 ± 8
0.10	9.2 ± 0.3	180 ± 4	12.2 ± 1.0	216 ± 10
0.125	9.4 ± 0.5	200 ± 6	11.5 ± 1.5	228 ± 5
0.15	8.5 ± 0.2	185 ± 3	11.7 ± 1.0	250 ± 14
0.20	7.1 ± 0.2	197 ± 3	8.9 ± 0.9	231 ± 12
0.25	6.6 ± 0.3	192 ± 3	8.3 ± 0.8	239 ± 11
0.30	6.4 ± 0.4	190 ± 5	7.9 ± 0.3	227 ± 4
0.35	6.5 ± 0.4	195 ± 5	8.0 ± 0.7	239 ± 10
0.40	6.0 ± 0.3	189 ± 3	7.5 ± 0.5	237 ± 8
0.50	5.9 ± 0.3	194 ± 5	8.1 ± 0.8	244 ± 13
0.625	6.3 ± 0.3	204 ± 5	6.8 ± 0.8	225 ± 6

[a] Assays to determine the kinetic parameters were performed as described under Experimental Procedures with the substrate D-Phe-Pip-Arg-pNA being varied over the range 4–43 μM (six points) with 40 pM β_T-thrombin or 4–47 μM with 20 pM γ_T-thrombin. Initial velocities were fitted to the Michaelis–Menten equation by using weighted linear regression to yield estimates of the parameters which are given together with their standard errors (Hofsteenge et al., 1986).

Scheme I

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

The inhibition of γ_T-thrombin by all hirudin variants and of all thrombin derivatives by r-hir(1–52) could be described by the Dixon equation (Segal, 1975). Data were fitted to this equation by weighted linear regression to yield estimates of the apparent dissociation constant ($K_{I'}$); the true value of K_I was calculated by using the relationship given above together with the K_m values given in Table I.

The standard Gibbs free energy for the formation of the thrombin–hirudin complex (ΔG°_b), which is referred to as binding energy in this study, can be calculated from the value of K_I by using the relationship:

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

where T is the absolute temperature and R is the gas constant. Binding energy can be divided into a component due to ionic interactions (ΔG°_{io}) and one due to nonionic interactions (ΔG°_{nio}):

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

[2] Abbreviations: D-Phe-Pip-Arg-pNA, D-Phe-pipecolyl-Arg *p*-nitroanilide; r-hir, recombinant hirudin; r-hir(1–52), fragment of recombinant hirudin comprising residues 1–51 plus a C-terminal homoserine; hir-SO_3, native hirudin which contains a sulfated tyrosine at position 63; r-hir(1Gln), r-hir(2Gln), r-hir(3Gln), and r-hir(4Gln) are used to designate mutant hirudins containing from one to four glutamate to glutamine mutations in the C-terminal region, i.e., [Glu62→Gln]hir, [Glu57,58→Gln]hir, [Glu57,58,62→Gln]hir, and [Glu57,58,61,62→Gln]hir, respectively.

Table II. Kinetic Constants for the Interaction of Different Forms of Hirudin with α-, β_T-, and γ_T-Thrombin[a]

	β_T-thrombin			γ_T-thrombin		α-thrombin	
form of hirudin	$k_1 \times 10^{-6}$ (M^{-1} s^{-1})	K_I (pM)	$-\Delta G_b^\circ$ (kJ mol^{-1})	K_I (nM)	$-\Delta G_b^\circ$ (kJ mol^{-1})	K_I (pM)	$-\Delta G_b^\circ$ (kJ mol^{-1})
hir-SO_3	19.2 ± 0.5	2.9 ± 0.2	68.5	29 ± 1	44.8	0.02	81.1
r-hir	5.6 ± 0.1	11.8 ± 0.1	64.8	55 ± 2	43.1	0.23	75.0
r-hir(1Gln)	3.0 ± 0.1	22.9 ± 0.5	63.1	149 ± 5	40.5	0.55	72.7
r-hir(2Gln)	1.10 ± 0.02	102 ± 2	59.3	173 ± 12	40.1	2.36	69.0
r-hir(3Gln)	0.41 ± 0.01	280 ± 9	56.7	206 ± 7	39.7	8.6	65.6
r-hir(4Gln)	0.18 ± 0.01	698 ± 23	54.3	272 ± 17	39.0	14.1	64.4

[a] Assays were performed and data were analyzed as described under Experimental Procedures. The kinetic constants are as defined in Scheme I, and the estimates for these constants obtained from the analyses are given together with their standard errors. Values for ΔG_b° were calculated by using the relationship given in eq 1. Values obtained previously (Braun et al., 1988a) for K_I and ΔG_b° with α-thrombin are given for comparison.

Table III: Estimates of the Contributions of Ionic and Nonionic Interactions to the Binding Energy for the α-, β_T-, and γ_T-Thrombin–Hirudin Complexes[a]

	α-thrombin		β_T-thrombin		γ_T-thrombin	
form of hirudin	$-\Delta G_{nio}^\circ$ (kJ mol^{-1})	$-\Delta G_{io0}^\circ$ (kJ mol^{-1})	$-\Delta G_{nio}^\circ$ (kJ mol^{-1})	$-\Delta G_{io0}^\circ$ (kJ mol^{-1})	$-\Delta G_{nio}^\circ$ (kJ mol^{-1})	$-\Delta G_{io0}^\circ$ (kJ mol^{-1})
r-hir	62.2 ± 2.4	25.1 ± 0.4	50.9 ± 4.3	24.4 ± 0.9	37.5 ± 1.5	8.8 ± 1.2
r-hir(4Gln)	63.3 ± 0.1	9.2 ± 0.2	53.6 ± 0.2	8.0 ± 1.0	ND[b]	ND

[a] Estimates for ΔG_{nio}° and ΔG_{io0}° for β_T-thrombin were calculated from variation of k_1 and ΔG_b° with ionic strength as described under Data Analysis whereas the values for γ_T-thrombin were calculated from the variation of ΔG_b°. The values for α-thrombin were determined previously (Stone et al., 1989), and the values of ΔG_{io0}° for β_T-thrombin represent the weighted mean values of the estimates obtained from the analysis of the effect of ionic strength on k_1 and K_I. [b] Not determined.

The component of the binding energy due to ionic interactions will be dependent on the ionic strength (I), and eq 3 has been

$$\Delta G_b^\circ = \Delta G_{nio}^\circ + \Delta G_{io0}^\circ \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (3)$$

shown to describe the ionic strength dependence of ΔG_b° for the interaction of hirudin with human α-thrombin (Stone et al., (1989) where ΔG_{io0}° is the component of binding energy due to ionic interactions at zero ionic strength and C_1 is a constant that is related to the Debye–Hückel screening parameter and contains also a distance term (Stone et al., 1989). Values of ΔG_b° were calculated by using eq 1 from estimates of K_I obtained at a number of ionic strengths. The values of ΔG_b° were weighted according to the inverse square of their standard errors and fitted to eq 3 by weighted nonlinear regression. This analysis yielded estimates for ΔG_{nio}° and ΔG_{io0}°.

For ionic interactions, the association rate constant (k_1) will also be dependent on ionic strength (Laidler, 1987), and eq 4 has been shown to describe this dependence for the α-

$$\ln k_1 = \ln k_{1\infty} + \frac{\Delta G_{io0}^\circ}{RT} \frac{\exp(-C_1\sqrt{I})}{1 + C_1\sqrt{I}} \quad (4)$$

thrombin–hirudin interaction (Stone et al., 1989) where $k_{1\infty}$ is the value of k_1 at infinite ionic strength, i.e., under conditions where the contribution of ionic interactions will be negligible. The values of k_1 obtained at different ionic strengths were weighted according to the inverse square of their standard errors and fitted to eq 4 by weighted nonlinear regression. This analysis yielded a second estimate for ΔG_{io0}° for β_T-thrombin.

Results

Inhibition of β_T- and γ_T-Thrombin by Hirudin Mutants with Altered C-Terminal Charge Properties. In order to investigate to what extent the reduced affinities of β_T- and γ_T-thrombin for hirudin are due to weaker ionic interactions with the C-terminal region of hirudin, the kinetics of inhibition of these thrombin derivatives by a number of hirudins with altered C-terminal charge properties have been examined at an ionic strength of 0.125 M. r-hir has one less negative charge than native hirudin (hir-SO_3) because h-Tyr63, which is sulfated in native hirudin, is not sulfated in the recombinant molecule (Dodt et al., 1986; Braun et al., 1988a; Riehl-Bellon et al., 1989). Removal of this negative charge caused a decrease in binding energy (ΔG_b°) of 3.1 kJ mol^{-1} with β_T-thrombin. Removal of additional negative charges by mutation of glutamyl to glutaminyl residues caused further losses in binding energy ranging from 2.3 to 3.8 kJ mol^{-1} for the removal of a negative charge (Table II). These reductions in binding energy are similar to those previously observed with α-thrombin (Table II). In addition, the reduction in affinity observed with β_T-thrombin was primarily due to a decrease in the association rate constant (k_1) for the mutant hirudins as was also the case with α-thrombin (Braun et al., 1988a).

Removing negatively charged residues from hirudin had a less pronounced effect on the interaction with γ_T-thrombin. In contrast to β_T-thrombin where the removal of five negatively charged residues resulted in a decrease of 240-fold in affinity, the removal of these charges caused only a 9-fold reduction in affinity for γ_T-thrombin (Table II). The decrease in binding energy for the removal of the five charges was 14.2 kJ mol^{-1} for β_T-thrombin and 5.8 kJ mol^{-1} for γ_T-thrombin (Table II).

Evaluation of the Relative Contributions of Ionic and Nonionic Interactions to the Binding Energies of Hirudin Variants with β_T- and γ_T-Thrombin. The fact that removal of negative charges from the C-terminal region of hirudin caused similar reductions in binding energies with α- and β_T-thrombin suggests that the ionic interactions involving this region of hirudin and the β-loop of thrombin are quantitatively similar for both these thrombin species. In contrast, the results of Table II suggest that the ionic interactions of the C-terminal of hirudin have been markedly altered with γ_T-thrombin. In order to examine these proposals in more detail, the relative contributions of ionic (ΔG_{io0}°) and nonionic (ΔG_{nio}°) interactions to binding energy were determined for r-hir and for the mutant with four Glu→Gln mutations [r-hir(4Gln)] by examining the effect of ionic strength on the interactions (Stone et al., 1989). Binding energies for these two hirudins were determined at 12 different ionic strengths between 0.05 and 0.625 M with β_T- and γ_T-thrombin (Figure 1), and analysis of these data yielded values for ΔG_{nio}° and ΔG_{io0}°. Similar data were obtained for the effect of ionic strength on the logarithm of the asso-

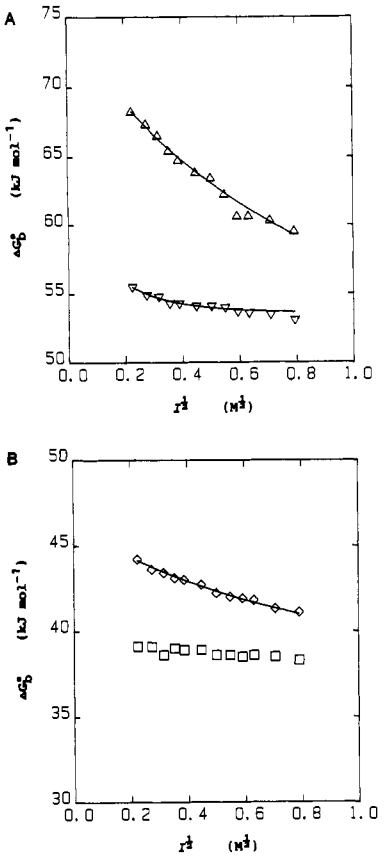

FIGURE 1: Effect of ionic strength on the binding energy of the thrombin–hirudin complex. Binding energies (ΔG°_b) for β_T-thrombin (A) with r-hir (▲) and r-hir(4Gln) (▽) and for γ_T-thrombin (B) with r-hir (◇) and r-hir(4Gln) (□) are plotted against the square root of ionic strength (I). The data were analyzed according to eq 3 and the lines drawn represent the fit of the data in this equation.

ciation rate constant (k_1) for β_T-thrombin (data not shown). It was not possible to obtain an estimate of k_1 with γ_T-thrombin. The results of these experiments (given in Table III) indicate that the reduced affinity that resulted from the conversion of α- to β_T-thrombin was due to a decrease in the contributions of nonionic interactions to binding energy. For both forms of hirudin, the contributions of ionic interactions were about the same for both α- and β_T-thrombin whereas the nonionic portions of binding energy were lower with β_T-thrombin (Table III).

The above treatment of ionic strength effects that was used to obtain estimates of ΔG°_{nio} and ΔG°_{io0} assumes that changes in ionic strength have specifically affected the ionic interactions that occur between thrombin and hirudin. It seems possible, however, that the structures of thrombin and hirudin are also dependent on ionic strength and that, consequently, nonionic interactions might also be affected by salt concentration. Such secondary salt effects are difficult to exclude. These secondary effects would lead to an under- or overestimation of the value of ΔG°_{io0} depending on whether the salt-induced structural changes favored or hindered complex formation. Over the range of ionic strengths used, large changes in the values of k_{cat} and K_m for D-Phe-Pip-Arg-pNA were not observed with either β_T- or γ_T-thrombin (Table I). These results suggest that the effect of salt concentration on the active site of thrombin was minor. The effect of salt on the structure of other regions of thrombin and on the structure of hirudin cannot easily be assessed. However, the possibility that the constant value of ΔG°_{io0} observed with α- and β_T-thrombin has been caused by equal and opposite secondary salt effects in the α- and β_T-thrombin interactions seems remote. Therefore, it appears likely that the contribution of ionic interactions to binding energy is the same for α- and β_T-thrombin. Given the possibility of secondary salt effects, the quantitative significance of the values obtained for ΔG°_{nio} and ΔG°_{io0} is not clear. In previous studies, however, changes in these parameters with different mutations have been qualitatively consistent with the nature of the mutation. When mutations were made that altered ionic interactions, only ΔG°_{io0} was affected while ΔG°_{nio} remained constant (Stone et al., 1989), whereas mutations that altered hydrophobic interactions resulted in a change in ΔG°_{nio} without affecting ΔG°_{io0} (Wallace et al., 1989).

In contrast to the results obtained with β_T-thrombin, those obtained with γ_T-thrombin suggest that the reduction in affinity of γ_T-thrombin for hirudin is due to a decrease in the strength of both ionic and nonionic interactions. Analysis of the data for r-hir with γ-thrombin (Figure 1B) yielded values of −8.8 and −37.5 kJ mol^{-1} for ΔG°_{io0} and ΔG°_{nio}, respectively, which are substantially lower than the values obtained with α-thrombin (Table III). The variation of ΔG°_b for r-hir(4Gln) with ionic strength was insufficient to obtain an estimate for ΔG°_{io0} (Figure 1B).

Inhibition of β_T- and γ_T-Thrombin by the Truncated Form of Hirudin, r-hir(1–52). The reduction in the contribution of nonionic interactions to the binding energy of the hirudin with β_T- and γ_T-thrombin could be due to a decrease in the strength of these interactions with the C-terminal domain of hirudin and/or with the N-terminal core of hirudin. In order to examine these possibilities, the inhibitory properties of a hirudin molecule in which the C-terminal domain had been deleted [r-hir(1–52)] were determined at an ionic strength of 0.125 M. It can be assumed that the N-terminal domain of hirudin in this truncated molecule binds to the active site of thrombin in essentially the same manner as it does in the entire molecule. The sum of the binding energies for the N-terminal and C-terminal fragments of hirudin was found to be equal to the binding energy of the entire molecule (Dennis et al., 1990). This suggests that the mode of binding of the two domains does not depend on their being joined together. The truncated molecule [r-hir(1–52)] displayed a similar affinity for both α- and β_T-thrombin; the dissociation constant for β_T-thrombin (35.0 ± 4.1 nM) was only 1.4 times that observed for α-thrombin (24.4 ± 1.0 nM). The dissociation constant for γ_T-thrombin (214 ± 12 nM) was about 9-fold higher than that for α-thrombin. From the results obtained with r-hir(1–52), it can be concluded that the interaction of the N-terminal core of hirudin with thrombin is essentially the same for both α-thrombin and β_T-thrombin and only slightly altered with γ_T-thrombin. For r-hir(1–52), a difference of only 5.6 kJ mol^{-1} in binding energy was observed between α- and γ_T-thrombin, whereas the difference for native hirudin was 36.3 kJ mol^{-1}.

Discussion

Results from solution studies as well as crystallographic studies indicate that the C-terminal tail of hirudin interacts with the β-loop of human α-thrombin (Dodt et al., 1990; Dennis et al., 1990; Chang et al., 1990; Rydel et al., 1990; Grütter et al., 1990). Human β_T-thrombin, which contains a single cleavage between Arg77A and Asn78, has a 130-fold reduced affinity for hirudin compared with α-thrombin, and it has been proposed that the ionic interactions that normally occur between the β-loop and hirudin are disrupted by the altered structure of the β-loop in β_T-thrombin (Stone et al., 1987). In this case, there would be a reduction in the contribution of these interactions to the binding energy, and removal of negative charges from the C-terminal region of hirudin would be expected to have a smaller effect with β_T-

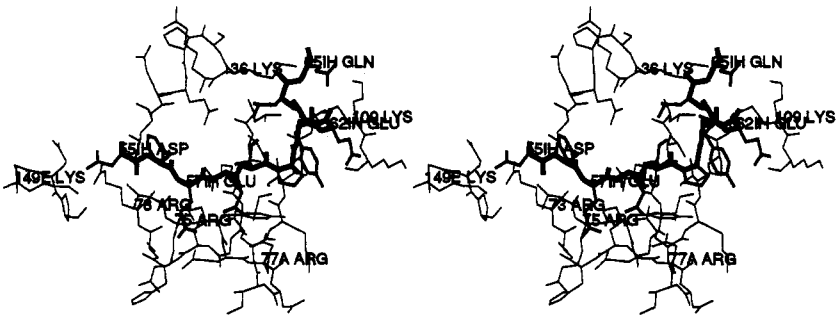

FIGURE 2: Stereopair of the binding of the C-terminus of hirudin to human α-thrombin. The C-terminal region of hirudin (residues 55–65) is shown in thick lines while thrombin residues are in thinner lines. The structure shown is that determined by Rydel et al. (1990).

thrombin than with α-thrombin. However, the results given in Table II indicate that the removal of negative charges has the same effect with both α- and β_T-thrombin. Moreover, the relative contribution of ionic interactions to binding energy was essentially the same for α- and β_T-thrombin (Table III). Thus, the decrease in affinity of β_T-thrombin for hirudin appears to be due to a decrease in the contribution of nonionic rather than ionic interactions to binding energy. The β-cleavage would be expected to alter the structure of the β-loop region and possibly affect nonionic interactions with this region. Alternatively, this cleavage could also affect the numerous hydrophobic interactions that occur between the N-terminal region of hirudin and the active-site cleft of thrombin (Rydel et al., 1990; Grütter et al., 1990). There is evidence from studies on the kinetics of β_T-thrombin with substrates that the β-cleavage affects the structure of the active site (Hofsteenge et al., 1988). The N-terminal domain of hirudin [r-hir(1–52)] binds with almost the same affinity to α- and β_T-thrombin. Thus, the reduction in the affinity of β_T-thrombin for hirudin appears not to be due to an altered interaction of the N-terminal core of hirudin with the active site but rather to a decrease in the strength of the nonionic interactions that occur between the C-terminal tail of hirudin and the β-loop region of thrombin. This conclusion is consistent with the numerous nonionic contacts made between the β-loop region of thrombin from Leu65 to Ile82 and residues in the C-terminal region of hirudin as illustrated in Figure 2. In the C-terminal tail of hirudin from h-Asp55 to h-Gln65, 5 out of 11 of the residues are hydrophobic or aromatic, and all 5 of these residues participate in nonpolar thrombin–hirudin interactions (Rydel et al., 1990). Strong hydrophobic interactions depend on the close packing of two complementary surfaces, and the β-cleavage has presumably caused a change in the conformation of thrombin that has disrupted the complementarity of the two surfaces.

The interaction of γ_T-thrombin with hirudin can be distinguished from that of β_T-thrombin in that a significant decrease in the strength of ionic interactions has occurred with γ_T-thrombin. The reason for the reduction in the strength of ionic interactions is clear from Figure 2. In γ_T-thrombin, the β-loop from Ile68 to Arg77A has been removed with a result that all the strong ionic interactions that occur in this region between α-thrombin and hirudin are no longer possible. Many important hydrophobic interactions that occur with Leu65, Arg73, Thr74, Tyr76, and Ile82 will also be lost, and this loss would account, at least partially, for the observed decrease in the strength of nonionic interactions (Table III). Although the strong ionic interactions between negatively charged side chains in the C-terminal region of hirudin and the β-loop are no longer possible with γ_T-thrombin, removal of negative charges from the C-terminal of hirudin still resulted in a small decrease in binding energy (Table II). The most probable reason for this is the existence of long-range ionic interactions. The C-terminal tail of hirudin is bound in a surface groove on thrombin that is lined by basic residues. In addition to the basic residues in the β-loop, Arg35, Lys36, Lys109, and Lys110 seem well placed to make a contribution to ionic interactions with the negatively charged residues in the C-terminal tail of hirudin (Rydel et al., 1990). The 9-fold decrease in affinity of γ_T-thrombin for r-hir(1–52) indicates a slight change in the structural elements of thrombin that interact with the N-terminal core of hirudin. The main region of interaction of the N-terminal core is the active site, and a number of studies have indicated an altered architecture in the active site of γ-thrombin [see, for example, Berliner (1984), Sonder and Fenton (1986), Lottenberg et al. (1982), Bezeaud et al. (1985), Lewis et al. (1987), and Hofsteenge et al. (1988)]. It should be noted, however, that the difference in affinities of α-, β_T-, and γ_T-thrombin for r-hir(1–52) is small which suggests that the interactions that occur within the active site between hirudin and α-thrombin are largely preserved with β_T- and γ_T-thrombin.

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Registry No. D-Phe-Pip-Arg-pNA, 83377-26-8; thrombin, 9002-04-4; hirudin, 8001-27-2.

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Deuterium Quadrupole Coupling in *N*-Acetylglycine and Librational Dynamics in Solid Poly(γ-benzyl-L-glutamate)†

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ABSTRACT: To study the dynamics of peptide groups in solid proteins, we have accurately determined the principal components and molecular orientation of the electric field gradient tensor for the exchangeable deuterons in monoclinic *N*-acetylglycine by single-crystal deuterium nuclear magnetic resonance. These results are compared with the principal components of the amide deuterons in solid poly(γ-benzyl-L-glutamate) measured in powder samples over a wide temperature range (140-400 K). The comparison indicates that in the solid polypeptide the N-D bonds undergo a small-amplitude torsional reorientation (libration) perpendicular to the peptide plane. To estimate dynamic rates, longitudinal relaxation times (T_1 values) are reported for *N*-acetylglycine and poly(γ-benzyl-L-glutamate). T_1 values for the carboxyl and amide deuterons in *N*-acetylglycine are $\sim$100 s, whereas for the amide deuterons in the polypeptide $T_1 \sim 1$ s, also indicating that the N-D bonds are not stationary in the polypeptide. We determine from the reduced quadrupole coupling tensor the mean-square amplitude for the libration and show that it increases linearly with temperature. A simple qualitative theory for the relaxation times is presented on the basis of the assumption that the N-D reorientation is described either as a diffusion process in a square well or as a damped Langevin oscillator with a harmonic restoring force. The conclusion is that the short relaxation times of the polypeptide amide deuterons result from substantial frictional effects on reorientation that increase with temperature.

We have used deuterium magnetic resonance to study monoclinic crystals of amide- and carboxyl-deuterated *N*-acetylglycine (NAG)[1] and amide-deuterated polycrystalline poly(γ-benzyl-L-glutamate) (PBG). Once the quadrupole coupling or electric field gradient (efg) tensor principal components and molecular orientation are known for the amide

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[1] Abbreviations: efg, electric field gradient tensor; T_1, longitudinal relaxation time; NAG, *N*-acetylglycine; PBG, poly(γ-benzyl-L-glutamate).

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