Articles

Chemical Compensation in Macromolecular Bridge-Binding to Thrombin†

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ABSTRACT: The binding energetics of eight synthetic peptides capable of interfering with thrombin function have been studied by steady-state measurements and clotting assays. The synthetic peptides are bifunctional inhibitors consisting of three domains: (i) a fragment of the C-terminus of recombinant hirudin, hir$^{55-65}$, which binds to the fibrinogen-recognition site of thrombin; (ii) a small active site inhibitor, Ac-(DF)PRP, binding to the catalytic pocket of the enzyme, and (iii) a linker spanning these two portions with variable length and chemical composition. All these synthetic peptides are competitive inhibitors of fibrinogen. On the other hand, a linker of at least 13 carbon atoms is required for full competitive inhibition of the hydrolysis by thrombin of small synthetic substrates, which only bind to the catalytic pocket of the enzyme. The best inhibitory effect is observed with a linker of 13 carbon atoms, with a value of K_I in the nanomolar range. Studies conducted as a function of temperature, in the range 15–40 °C, have revealed the enthalpic and entropic components of inhibitor binding to thrombin. Chemical compensation is observed for all synthetic peptides that bridge-bind to the fibrinogen-recognition site and the catalytic pocket of the enzyme thereby inhibiting in a competitive fashion either fibrinogen binding or the hydrolysis of small synthetic substrates. The extrathermodynamic relationship between ΔH and ΔG also includes the enthalpy and free energy of binding for the natural substrate fibrinogen and the potent natural inhibitor hirudin, measured under identical solution conditions. Preferential binding of hirudin over fibrinogen is an entropy-driven process. The existence of chemical compensation suggests that a unique conformational transition of thrombin takes place upon macromolecular bridge-binding to the fibrinogen-recognition site and the catalytic pocket. This transition may have a bearing on the recently discovered Na^+-induced slow → fast transition of human α-thrombin.

Human α-thrombin selectively binds and cleaves fibrinogen triggering the formation of an insoluble gel of fibrin polymers. The basis of thrombin specificity was first recognized by Fenton in terms of a rather peculiar mechanism, defined as "bridge-binding", which seems to have no couterpart in the realm of serine proteases (Fenton, 1981). Fibrinogen bridge-binds to the catalytic pocket (CP)[1] and the fibrinogen-recognition site (FRS), a structural domain that is distinct from the CP and coated by a remarkable number of positively charged residues (Bode et al., 1992). From a thermodynamic point of view, the specificity of thrombin–fibrinogen interaction hinges on the interplay between the CP and FRS. The energetics of this physiologically important interaction can be dissected in terms of site-specific components arising from binding to the CP and FRS separately, along with interaction between these two structural domains. Recent measurements of the equilibrium dissociation constant for thrombin–fibrinogen interaction (Hopfner & Di Cera, 1992) have set the stage for a quantitative understanding of macromolecular recognition by thrombin in a way never before possible. In this study, we extend our investigation of the site-specific components involved in fibrinogen binding by analyzing a number of synthetic peptides specifically tailored to bind to the FRS or to bridge-bind to thrombin in a fashion similar to that of fibrinogen. These peptides have originally been designed to interfere with thrombin–fibrinogen interaction in vivo and are promising candidates for the control of the important role of thrombin in hemostasis (DiMaio et al., 1990). They contain a functional domain formed by a small active site inhibitor, Ac-(DF)PRP, capable of interacting with the CP, and a fragment of hirudin, the potent natural inhibitor of thrombin (Markwardt, 1970), which spans the residues 55–65 and only binds to the FRS (Stubbs et al., 1992). The two domains are connected by a linker of variable length and chemical composition. Quantitative studies of the interaction of these synthetic peptides with thrombin are not only relevant for understanding the physicochemical basis of thrombin–fibrinogen interaction and macromolecular recognition in the thrombin system but also useful to elucidate the components involved in the inhibitory effect of these peptides on important physiological functions of the enzyme.

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[1] Abbreviations: Abu, ω-aminobutyric acid; Ac, acetyl; Aca, ω-aminocaproic acid; Ada, ω-aminododecanoic acid; Aha, ω-aminoheptanoic acid; BOC, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; CP, catalytic pocket; FRS, fibrinogen-recognition site; HPLC, high-performance liquid chromatography; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-2238, H-D-pipecolyl-Arg-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane.

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MATERIALS AND METHODS

Thrombin, Fibrinogen, Hirudin, and Synthetic Peptides. Human α-thrombin and human fibrinogen were purified and tested for activity as described in detail elsewhere (De Cristofaro & Di Cera, 1992). Highly purified recombinant hirudin CGP39393 was a generous gift of Dr. Grossenbacher (Ciba-Geigy Pharmaceuticals, Basel, Switzerland). The hirudin concentration was determined using an appropriate extinct coefficient as described elsewhere (De Cristofaro et al., 1992). Alternatively, the concentration of freshly made hirudin solutions was determined by titration of clotting curves carried out with known thrombin concentrations. First, the clotting time, t_c, was measured as a function of thrombin concentration to obtain a calibration curve. The clotting time increases linearly, in a very precise and reproducible way, with the inverse thrombin concentration (De Cristofaro & Di Cera, 1991). Then, aliquots of the hirudin solution of unknown concentration were added to a solution of 4 nM human α-thrombin and the clotting time was determined. At nanomolar concentrations of thrombin, the inhibition by hirudin is stoichiometric and therefore the concentration of hirudin can precisely be determined from the increase in t_c. The free thrombin concentration corresponding to the measured value of t_c is determined from the calibration curve, and the hirudin concentration is derived by difference from the total thrombin concentration, 4 nM, used in the assay. Ada was purchased from Sigma. BOC-Ada was synthesized according to the procedure described by Chaturvedi et al. (1984). BOC-Abu, BOC-Aca, and BOC-Abu were purchased from Bachem. All other amino acid derivatives for peptide synthesis were purchased from Advanced ChemTech. The side chain protecting groups of BOC-amino acids were benzyl for Glu and Asp, tosyl for Arg, and 2-bromobenzyloxycarbonyl for Tyr. BOC-Gln-CH_2-((phenylacetyl)amido)methyl resin (0.73 mmol/g) was purchased from ABI. The solvents for peptide synthesis were obtained from B&J Chemicals and ABI. The peptides were prepared by manual solid-phase technique, using standard BOC synthetic strategy and using diisopropylcarbodiimide or BOP as coupling reagents. Peptides were cleaved from the resin and purified by preparative HPLC as described elsewhere (Szewczuk et al., 1992). Final products were obtained as lyophilizates of 98% purity or higher, as assessed by analytical HPLC. The sequence of purified peptide samples was determined by amino acid analysis on a Beckman Model 6300 high-performance analyzer and molecular weight analysis by a SCIEX API III mass spectrometer. The concentration of purified peptide samples was also determined by amino acid analysis.

Steady-State Measurements. Steady-state measurements of human α-thrombin amidase activity were made using the synthetic chromogenic peptide S-2238 (KabiVitrum, Stockholm, Sweden) as described elsewhere (De Cristofaro & Di Cera, 1992). All assays were performed using disposable polystyrene cuvettes under solution conditions of 50 mM Tris, 0.1 M NaCl, 0.1% PEG 8000, pH 7.5, at the desired temperature. The temperature range in these studies was from 15 to 40 °C. Active thrombin concentration was 1 nM in all assays. Steady-state determinations were collected in a matrix fashion using seven substrate concentrations scaled by a factor of 2 and seven peptide concentrations also scaled by a factor of 2. One curve was measured in the absence of peptide. The increase in absorbance at 405 nm due to the release of *p*-nitroaniline was found to be linear over a time scale of 15–30 s, depending upon solution conditions, and was used to compute the steady-state velocity as a function of peptide and substrate concentration. The relevant kinetic scheme to be used for the inhibition of synthetic substrate hydrolysis is

$$E + S \underset{{}^{s}k_{-1}}{\overset{{}^{s}k_1^*}{\rightleftarrows}} ES \xrightarrow{{}^{s}k_2} EP \xrightarrow{{}^{s}k_3} E + P' \quad (1a)$$

$$E + I \underset{{}^{I}k_{-1}}{\overset{{}^{I}k_1^*}{\rightleftarrows}} EI \quad (1b)$$

where ${}^{s}k_2$ and ${}^{s}k_3$ are the acylation and deacylation rates, while k_1^* and k_{-1} are the rate constants for binding and dissociation for the substrate (S) and the synthetic inhibitor (I). The solution of eqs 1 yields for the time evolution of product formation $v = d[P']/dt$

$$v = e_T \frac{k_{cat}[S]}{K_m(1 + [I]/K_I) + [S]} \quad (2)$$

where e_T is the enzyme concentration, [S] and [I] denote the (free) substrate and inhibitor concentrations, respectively, while $K_m = {}^{s}k_3({}^{s}k_{-1} + {}^{s}k_2)/[{}^{s}k_1^*({}^{s}k_3 + {}^{s}k_2)]$, $k_{cat} = {}^{s}k_3{}^{s}k_2/({}^{s}k_3 + {}^{s}k_2)$, and $K_I = {}^{I}k_{-1}/{}^{I}k_1^*$. The peptide concentration [I] changes significantly upon binding to thrombin when these macromolecular components are present in comparable amounts. In this case, eq 2 needs to be cast in terms of the total, rather than the free amount of peptide, so that (Cha, 1975)

$$v = e_T \frac{k_{cat}[S]}{K_m + [S]} \frac{2K_I'}{I_T - e_T + K_I' + Q} = v_0(Q - K_I' - I_T + e_T)/(2e_T) \quad (3)$$

where $v_0 = k_{cat}[S]/(K_m + [S])$, I_T is the total inhibitor concentration, $K_I' = K_I(1 + [S]/K_m)$, and $Q = \sqrt{(I_T - e_T - K_I')^2 + 4K_I'I_T} = \sqrt{(I_T + e_T + K_I')^2 - 4I_Te_T}$. The entire matrix of steady-state determinations as a function of substrate and inhibitor concentrations was analyzed according to eq 3 to resolve the independent parameters K_m, k_{cat}, and the dissociation constant for the inhibitor K_I. All experimental points were weighted uniformly, and minimization was accomplished by nonlinear least-squares using the Marquardt method.

Steady-state measurements of human α-thrombin amidase activity in the presence of hirudin were carried out using the progress curve method (Stone & Hofsteenge, 1986). At each temperature, 12 progress curves were measured in the presence of 100 pM human α-thrombin and saturating amounts of synthetic substrate, typically 30 times the K_m, as a function of hirudin concentration in the range 0–200 pM. The 12 progress curves were analyzed altogether using appropriate analytical expressions (Cha, 1975) to resolve the values of K_I and ${}^{I}k_1^*$ for hirudin binding.

The availability of K_I values for synthetic peptides and hirudin as a function of temperature, from 15 to 40 °C, makes it possible to characterize the thermodynamic components of binding. The K_I is the same as K_d, the equilibrium dissociation constant for thrombin interaction with a given macromolecular effector, and therefore the standard free energy of binding can be written as

$$\Delta G = RT \ln K_d = RT \ln K_I \quad (4)$$

where R is the gas constant. The dependence of $\ln K_I$ on $1/T$ at constant pressure gives the standard enthalpy of binding

according to the Gibbs–Helmholtz equation

$$(d \ln K_d)/d(1/T) = (d \ln K_I)/d(1/T) = \Delta H/R \quad (5)$$

A linear dependence of $\ln K_I$ versus $1/T$ gives ΔH directly. Under these circumstances, integration of eq 5 yields

$$\ln K_I = \ln K_d = \Delta H/RT - \Delta S/R \quad (6)$$

In a linear van't Hoff plot of $\ln K_I$ versus $1/T$, ΔH and ΔS can be determined from the values of the slope and intercept.

Clotting Assays. The approach based on steady-state measurements was used to obtain the value for K_I for all synthetic peptides behaving as competitive inhibitors of substrate hydrolysis. A different approach was used for those peptides that did not inhibit substrate hydrolysis by thrombin in a competitive fashion and were only competitive inhibitors of fibrinogen binding to thrombin. Clotting curves measured as a function of thrombin and inhibitor concentrations were analyzed altogether according to the equation (De Cristofaro & Di Cera, 1991)

$$t_c = t_\infty + \gamma e_T^{-1}(1 + [I]/K_I) \quad (7)$$

Here t_c is the clotting time, t_∞ is t_c extrapolated at infinite enzyme concentration, γ is a proportionality factor containing the ratio K_m/k_{cat} for the release of fibrinopeptide A, and

$$[I] = \{I_T - e_T - K_I + \sqrt{(I_T - e_T - K_I)^2 + 4K_I I_T}\}/2 \quad (8)$$

Clotting curves were measured as an increase in turbidity at 350 nm as a function of time using a Cary 3 dual-beam spectrophotometer as described in detail elsewhere (De Cristofaro & Di Cera, 1991). All assays were carried out under experimental conditions of 0.25 μM human fibrinogen, 50 mM Tris, 0.1% PEG 8000, pH 7.5, at the desired temperature in the range 15–40 °C. The thrombin concentration was in the range 1–10 nM and peptide concentrations up to 10–50 times the value of K_I were used.

Control Experiments. Reproducible results were obtained by using different preparations of thrombin, fibrinogen, and synthetic peptides. Particular attention was paid to determine the type of inhibition, fast or slow (Cha, 1975), for each synthetic peptide studied. Control experiments were carried out for all peptides over the entire temperature range as follows. Steady-state determinations were carried out by starting the assay with the enzyme and, alternatively, by adding the substrate to solutions of thrombin preincubated for 1–3 h with each synthetic peptide. No difference in the initial linear portion of the progress curve was found in all cases. This result implies that all the peptides analyzed in this study are fast tight-binding inhibitors (Cha, 1975), unlike the potent natural inhibitor hirudin which behaves mostly as a slow tight-binding inhibitor (Stone & Hofsteenge, 1986; De Cristofaro et al., 1992). The results of the control experiments fully justify the use of eqs 3 and 7 for data analysis. The validity of the approach based on measurements of K_I from analysis of the clotting curve was tested with peptides behaving as competitive inhibitors of substrate hydrolysis. For all these peptides, the values of K_I were determined using either steady-state measurements or clotting curves. These values were found to overlap very well (see Results), thereby indicating the validity of the approach based on eq 7 in the study of synthetic inhibitors that only bind to the FRS, and for which steady-state measurements of substrate hydrolysis cannot be used to measure K_I.

RESULTS

A typical set of steady-state determinations of substrate hydrolysis in the presence of a competitive inhibitor (P151) is shown in Figure 1. The global analysis of all curves according to eq 3 yields a very good fit of the data. In particular, the value of k_{cat} derived in the global analysis is practically identical to those derived independently from individual analysis of each curve according to the Michaelis–Menten equation (see Table I), as implied by competitive inhibition. Similar data sets were collected over the temperature range 15–40 °C, and the values of K_I were found to obey a linear relationship in the van't Hoff plot of $\ln K_I$ versus $1/T$. An example of such data is given in Figure 2 for the peptide P53 (○), along with the results obtained independently from analysis of clotting curves (●). The good agreement between values of K_I obtained in terms of two different experimental approaches demonstrates the robustness of the results reported in this study.

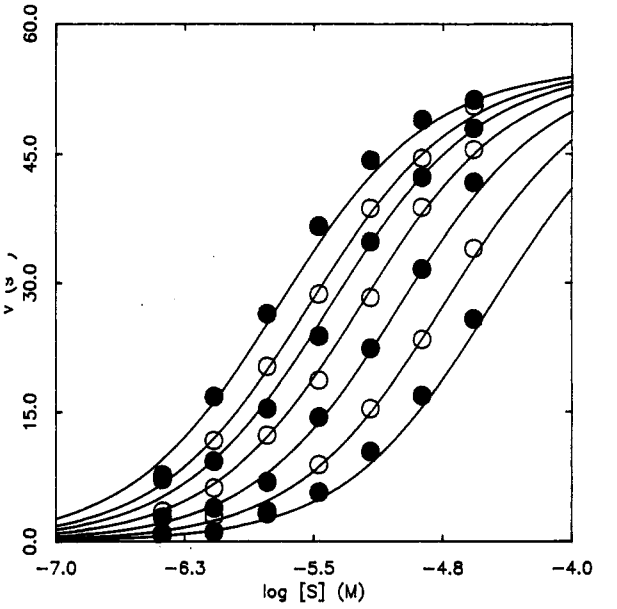

FIGURE 1: Typical steady-state measurements of substrate (S-2338) hydrolysis by human α-thrombin in the presence of a synthetic peptide behaving as a competitive inhibitor. Experimental conditions: 1 nM human α-thrombin, 0.1 M NaCl, 0.1% PEG, pH 7.5, at 25 °C. The concentration of P151 is 50 nM in the curve at the bottom and decreases by a factor of 2 for the other curves. The curve at the top is without P151. The continuous lines were drawn according to eq 3 in the text with the best-fit parameters: $k_{cat} = 55 \pm 1$ s^{-1}, $K_m = 2.1 \pm 0.1$ μM, $K_I = 3.1 \pm 0.2$ nM. The standard error of the fit is 1 s^{-1}.

The experimental determinations of K_m and k_{cat} for the hydrolysis of S-2238 as a function of peptide concentration are listed in Table I. Peptides with linkers of 13 atoms or longer behave as competitive inhibitors of the hydrolysis of S-2238 by thrombin, as documented by the increase in K_m and the constancy of k_{cat}. Peptides with linkers shorter than 13 atoms, i.e., P24, P148, and P149, do not behave as competitive inhibitors. Rather, a significant activation of thrombin is observed upon binding of these peptides, with k_{cat} increasing by 20–30%. On the other hand, the peptides that allosterically activate thrombin catalysis do inhibit fibrinogen binding in a competitive fashion, as demonstrated by the data shown in Figure 3. The same inhibition pattern toward fibrinogen is observed with all other peptides (P201, P239, P151, P145, P53) that inhibit the hydrolysis of S-2238 by thrombin in a competitive fashion. This result supports the "inhibition rule" that competitive inhibition of synthetic substrate hydrolysis by thrombin *necessarily* implies competitive inhibition of fibrinogen binding, although the reverse is *not* necessarily true.

The thermodynamic parameters for the binding of each synthetic peptide to human α-thrombin are summarized in

Table I: Michaelis–Menten Parameters for the Hydrolysis of S-2238 under Conditions of 50 mM Tris, 0.1 M NaCl, 0.1% PEG, pH 7.5, 25 °C, as a Function of the Concentration of the Synthetic Peptides As Indicated[a]

	K_m (μM)	k_{cat} (s^{-1})		K_m (μM)	k_{cat} (s^{-1})
		[P24] (μM), l = 0			
5.00	2.4 ± 0.5	72 ± 2	0.31	2.4 ± 0.3	63 ± 1
2.50	2.6 ± 0.3	72 ± 1	0.16	2.4 ± 0.6	61 ± 2
1.25	2.7 ± 0.3	70 ± 1	0	2.2 ± 0.4	55 ± 1
0.63	2.6 ± 0.3	66 ± 1			
		[P149] (μM), l = 10			
3.40	2.2 ± 0.3	74 ± 6	0.21	2.0 ± 0.1	59 ± 1
1.70	1.9 ± 0.1	68 ± 1	0.11	1.9 ± 0.4	59 ± 3
0.85	1.9 ± 0.2	64 ± 1	0	2.1 ± 0.1	55 ± 1
0.43	1.9 ± 0.2	63 ± 2			
		[P148] (μM), l = 12			
1.00	5.4 ± 0.7	65 ± 2	0.06	3.1 ± 0.3	57 ± 2
0.50	5.0 ± 0.5	62 ± 2	0.03	2.6 ± 0.2	56 ± 1
0.25	4.8 ± 0.4	59 ± 3	0	2.2 ± 0.2	55 ± 1
0.13	3.4 ● 0.2	57 ± 1			
		[P201] (nM), l = 13			
100	35 ± 9	58 ± 4	6	3.4 ± 0.4	58 ± 2
50	13 ± 3	59 ± 3	3	2.8 ± 0.5	57 ± 2
25	6.9 ± 0.8	57 ± 3	0	2.0 ± 0.3	56 ± 1
13	5.0 ± 0.7	56 ± 2			
		[P239] (nM), l = 13			
100	4.2 ± 0.5	58 ± 2	6	2.2 ± 0.2	57 ± 1
50	3.3 ± 0.3	56 ± 3	3	2.3 ± 0.3	58 ± 2
25	2.8 ± 0.3	58 ± 2	0	2.1 ± 0.3	57 ± 1
13	2.5 ± 0.5	59 ± 3			
		[P151] (nM), l = 13			
50	27 ± 2	56 ± 3	3	4.2 ± 0.4	55 ± 2
25	20 ± 2	59 ± 4	2	2.7 ± 0.2	57 ± 1
13	11 ± 1	56 ± 2	0	2.0 ± 0.2	56 ± 1
6	6.6 ± 0.3	57 ● 1			
		[P145] (nM), l = 15			
100	39 ± 9	59 ± 9	6	3.8 ± 0.6	58 ± 3
50	16 ± 4	58 ± 7	3	3.0 ± 0.3	57 ± 2
25	9 ± 1	57 ± 6	0	2.0 ● 0.2	57 ± 2
13	6.8 ± 0.9	58 ± 5			
		[P53] (nM), l = 18			
50	3.5 ± 0.2	58 ± 1	3	2.6 ± 0.2	58 ± 1
25	3.0 ± 0.3	58 ± 2	2	2.3 ± 0.2	58 ± 2
13	2.6 ± 0.2	57 ± 1	0	2.2 ± 0.2	57 ± 2
6	2.7 ± 0.3	58 ± 2			

[a] The peptides behave either as allosteric activators (P24, P149, P148) or as competitive inhibitors (P201, P239, P151, P145, P53) of substrate hydrolysis. The chemical composition of each peptide is given in Table II. The linker length, l, refers to the number of atoms spanning the two functional domains binding to the CP, Ac-(DF)PRP, and the FRS, hirudin$^{55-65}$.

Table II. The free energy of binding depends on both the length and chemical composition of the linker. At least 13 atoms are needed to link the two domains of the synthetic inhibitors in such a way that the resulting bridge-binding to the CP and FRS effectively prevents the synthetic substrate from interacting with the enzyme. If this condition is not met, the peptides can still inhibit fibrinogen binding in a competitive fashion by binding to the FRS but are not long or rigid enough to provide full inhibition of binding to the CP. Indeed, the free energy of binding of P24, the hirudin fragment 55–65 which only binds to the FRS, is remarkably similar to that of P149 and P148 which have a linker of 10 and 12 carbon atoms, respectively, and so are the enthalpic and entropic components. It should be pointed out that P24 activates substrate hydrolysis upon binding to the FRS in an allosteric fashion and changes the value of k_{cat} for S-2238 of 20–30% (see Table I). This finding is consistent with the results of previous studies (Hortin & Benutto, 1990). Increasing the length of the linker from 12 to 13 atoms results in a significant increase in the binding affinity and gives rise to competitive inhibition also of synthetic substrate hydrolysis. The peptide P151 is the best inhibitor with a K_I = 5 nM at 37 °C. The presence of an amide bond between the Aha and Abu portions of the linker seems to be of particular importance in stabilizing the binding of P151 to thrombin. In fact, removal of the amide bond while keeping the length of the linker to 13 atoms, as done for P201, results in a loss of 0.72 kcal/mol in the free energy of binding. More interestingly, shifting the position of the amide bond by two atoms, as done with P239 where the linker Aha-Abu is inverted in Abu-Aha, results in a loss of 2.53 kcal/mol in the free energy of binding. Presently it is not clear what type of favorable interactions the amide bond would bring about, positioned as it is five atoms away from Asp55 of the hir$^{55-65}$ portion of the synthetic peptide fragment. This amide bond should mimic the position of the amide bond between Asn52 and Asn53 of rhir, but neither one of these residues is involved in ionic or hydrogen bond interactions with thrombin (Rydel et al., 1991). The importance of the amide bond in P151 is also demonstrated by the smaller loss in free energy of binding, about 0.5 kcal/mol, observed with P145 where the portion Abu of P151 is replaced by a Aca sequence. The addition of two atoms clearly does not shift the position of the amide bond and probably guarantees, at the same time, binding of the Ac-(DF)PRP portion to the CP. However, the optimal length of the linker has not only a lower bound (13 atoms), but also an upper

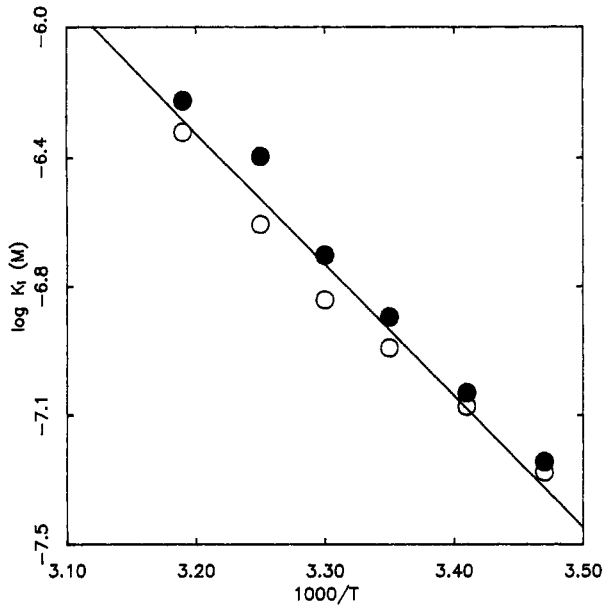

FIGURE 2: van't Hoff plot of log K_I versus the inverse temperature for the synthetic peptide P53. Values refer to results obtained from analysis of steady-state measurements (○) or clotting curves (●), as discussed in the text. The two experimental approaches yield results that overlap extremely well.

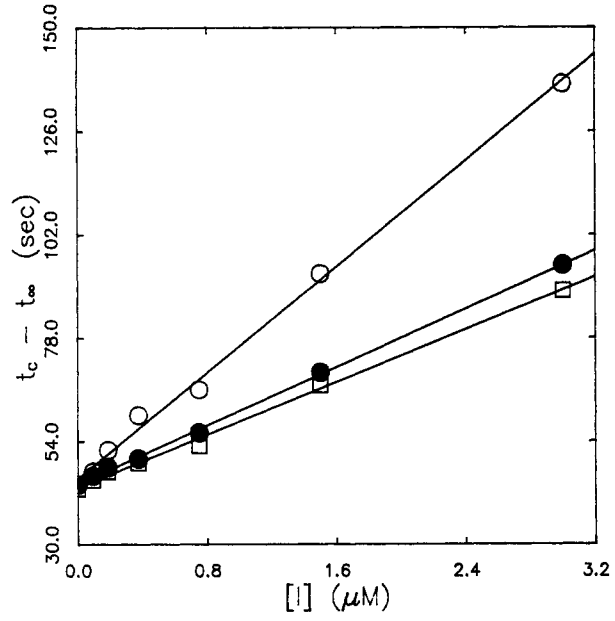

FIGURE 3: Effect of P24 (○), P148 (●), and P149 (□) concentration on the clotting time. The linear increase of t_c with [I] proves that these synthetic peptides inhibit fibrinogen binding in a competitive fashion. Notice that these peptides are allosteric activators of the hydrolysis of S-2238 by thrombin (see Table I). The best-fit values derived from analysis of the data according to eq 7 in the text are $\gamma/e_T = 45 \pm 1$ s, $K_I = 1.5 \pm 0.1$ μM (P24); $\gamma/e_T = 44 \pm 1$ s, $K_I = 2.6 \pm 0.3$ μM (P148); $\gamma/e_T = 43 \pm 1$ s, $K_I = 2.8 \pm 0.2$ μM (P149). Experimental conditions: 4 nM human α-thrombin, 0.25 μM human fibrinogen, 0.1 M NaCl, 0.1% PEG, pH 7.5, at 35 °C.

bound, and P145 may actually happen to be at this cutoff. Increasing the length of the linker brings about other factors that may interfere with the binding affinity. In particular, a linker longer than necessary may have an abnormal flexibility that may prevent optimal orientation of the two functional domains binding to the CP and FRS. This feature might explain the small decrease in binding affinity observed with P145 and the substantial loss of free energy of binding in P53, where the linker composed by natural amino acids is 18 atoms long.

DISCUSSION

The properties of the synthetic peptides analyzed in this study can be considered in connection with other bifunctional inhibitors called hirulogs (Maraganore et al., 1990). Hirulogs have been designed using a similar molecular strategy, by linking two functional domains interacting with the CP and the FRS with a polyGly spacer. The structure of thrombin complexed with hirulog-3 has recently been solved (Qiu et al., 1992). The only two similarities between hirulogs and the synthetic peptides analyzed here are that a minimum length of the spacer is required for optimal binding and inhibition of synthetic substrate hydrolysis by thrombin and that optimal binding occurs with a K_I in the nanomolar range. A number of differences are noteworthy. First, the minimum length of the spacer is 13 atoms for our peptides and only three Gly residues, or else 9 atoms, for hirulogs. Second, hirulogs are noncompetitive inhibitors of the hydrolysis of synthetic substrates by thrombin, since they increase K_m and decrease k_{cat}. No such effect is observed with any of our peptides. Third, the mechanism of action of hirulogs is even more complex than originally reported since the effect of hirulog-1, which carries a spacer of four Gly residues, on the hydrolysis of synthetic substrates by thrombin is concentration-dependent. At low concentrations it activates the hydrolysis in an allosteric fashion and at higher concentrations it inhibits the hydrolysis in a noncompetitive fashion. This biphasic effect is observed with different synthetic substrates under conditions close to those examined in this study (Wells and Di Cera, unpublished results). No such biphasic effect is seen with our synthetic peptides.

The results reported in this study should be discussed in connection with the important mechanism of bridge-binding whereby thrombin derives its unique specificity. Measurements of the free energy of binding of the various synthetic peptides as a function of temperature have yielded the thermodynamic components involved, as listed in Table II. Although different peptides have different free energies and enthalpy of binding to thrombin, a remarkable feature is observed in a Krug–Hunter–Grieger plot (Krug et al., 1976a,b) of ΔH versus ΔG computed at the geometric mean of experimental temperatures (T_h = 27.3 °C). A simple quadratic expression interpolates all values referring to the peptides that bridge-bind to thrombin and inhibit substrate hydrolysis in a competitive fashion, as shown in Figure 4. This chemical compensation effect also includes the natural substrate of thrombin, fibrinogen, and the potent competitive inhibitor hirudin. The peptides that do not inhibit substrate hydrolysis in a competitive fashion, or only bind to the FRS, are not subject to chemical compensation. The effect depicted in Figure 4 is peculiar of macromolecular bridge-binding to thrombin and proves the existence of an extrathermodynamic dependence of ΔH on ΔG for any macromolecule binding to the CP and FRS in a concerted fashion. Due to the widely different nature of the peptides analyzed in this study and, most notably, to the enormous structural difference between these peptides and fibrinogen, one has to conclude that the enzyme plays the major role in setting the rules for the energetics of bridge-binding and macromolecular recognition. There seems to be a unique code that sets a balance between enthalpic and entropic components for macromolecular bridge-binding, regardless of the particular effector or substrate involved. The code for bridge-binding implies that the higher the affinity, the smaller the temperature dependence of binding. In other words, macromolecular bridge-binding is optimized by an entropy-driven process.

Table II: Chemical Composition, Kinetic Properties, and Standard Thermodynamic Parameters of the Synthetic Peptides Analyzed in This Study[a]

peptide	chemical composition	kinetic behavior	K_d	ΔG	ΔH	ΔS
P24	Ac-DFEEIPEEYLQ(Ac-hir$^{55-65}$), no linker	allosteric	1.6 ± 0.1 μM	−8.22 ± 0.02	−13.6 ± 0.7	−17 ± 2
P149	Ac-(DF)PRP(Abu-Abu)hir$^{55-65}$, linker length = 10	allosteric	2.8 ± 0.4 μM	−7.89 ± 0.09	−10.5 ± 1.8	−8 ± 3
P148	Ac-(DF)PRP(Aca-Abu)hir$^{55-65}$, linker length = 12	allosteric	2.7 ± 0.2 μM	−7.92 ± 0.05	−12.3 ± 2.1	−14 ± 4
P201	Ac-(DF)PRP(Ada)hir$^{55-65}$, linker length = 13	competitive	17 ± 3 nM	−11.02 ± 0.12	−12.4 ± 2.6	−5 ± 5
P239	Ac-(DF)PRP(Abu-Aha)hir$^{55-65}$, linker length = 13	competitive	330 ± 30 nM	−9.21 ± 0.06	−18.7 ± 2.4	−31 ± 5
P151	Ac-(DF)PRP(Aha-Abu)hir$^{55-65}$, linker length = 13	competitive	5.4 ± 0.4 nM	−11.74 ± 0.04	−11.3 ± 1.7	1 ± 2
P145	Ac-(DF)PRP(Aha-Aca)hir$^{55-65}$, linker length = 15	competitive	12 ± 3 nM	−11.23 ± 0.13	−12.9 ± 2.8	−5 ± 6
P53	Ac-(DF)PRP-hir$^{49-65}$, linker length = 18	competitive	480 ± 24 nM	−8.98 ± 0.03	−18.7 ± 1.4	−31 ± 3
	fibrinogen	competitive	4.7 ± 0.7 μM	−7.56 ± 0.09	−24.3 ± 3.0	−54 ± 11
	rhir	competitive	684 ± 68 fM	−17.28 ± 0.06	−14.8 ± 0.9	8 ± 3

[a] The kinetic behavior refers to the effect on the hydrolysis of S-2238 by thrombin. The values of $\Delta G = RT \ln K_d = RT \ln K_i$ refer to 37 °C, under conditions of 50 mM Tris, 0.1 M NaCl, 0.1% PEG, pH 7.5. ΔG and ΔH are in kcal/mol, while ΔS is in cal/(mol·deg). The linker length refers to the number of atoms spanning the two functional domains binding to the CP, Ac-(DF)PRP, and the FRS, hirudin$^{55-65}$. The thermodynamic parameters for fibrinogen and hirudin are also given for comparison. The values for hirudin have been determined in this study, while those for fibrinogen are from Hopfner and Di Cera (1992).

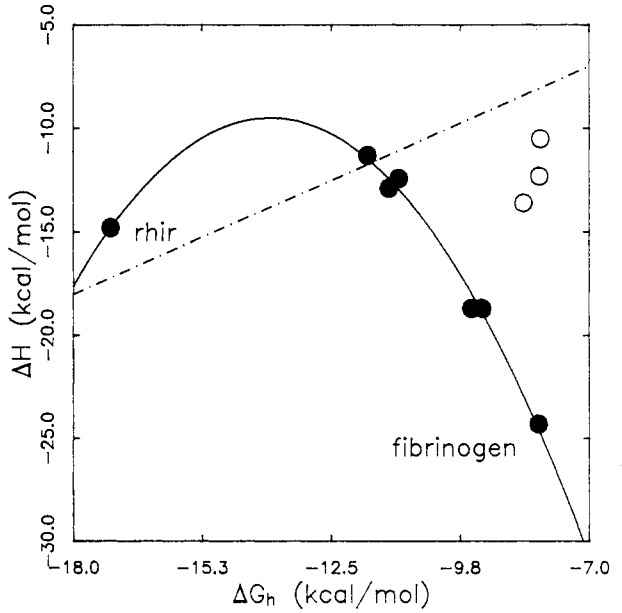

FIGURE 4: Krug–Hunter–Grieger plot of ΔH versus ΔG computed at the geometric mean of experimental temperatures (T_h = 27.3 °C) for macromolecular bridge-binding to thrombin. The plot includes peptides that behave as competitive inhibitors (●) or allosteric effectors (○) of the hydrolysis of S-2238 by thrombin. The continuous line is the best-fit of all data points depicted by a black circle according to the extrathermodynamic relationship $\Delta H = \alpha + \beta(\gamma - \Delta G)^2$, where α = −9.5 ± 0.4 kcal/mol, β = −0.46 ± 0.03 mol/kcal, γ = −13.8 ± 0.1 kcal/mol. The discontinuous line depicts the contour for which ΔS = 0. Above this line binding occurs with an increase of entropy (e.g., hirudin), and below this line binding occurs with a decrease of entropy (e.g., fibrinogen). Competitive inhibitors (●), from top to bottom: P151, P201, P145, P53, P239. Allosteric effectors (○), from top to bottom: P149, P148, P24.

The chemical compensation for macromolecular bridge-binding to thrombin reported here draws attention to the thermodynamic components underlying the difference between the natural substrate fibrinogen and the natural inhibitor hirudin. The importance of our recent measurements of the K_d for thrombin–fibrinogen interaction for the first time (Hopfner & Di Cera, 1992) is readily appreciated in this connection. No such comparison between the energetics of fibrinogen and hirudin binding has been possible in the past due to lack of any estimates of the free energy and enthalpy of fibrinogen binding to thrombin. Although fibrinogen and hirudin bridge-bind to thrombin in a similar fashion, the free energy of binding differs by almost 10 kcal/mol (see Table II). Hirudin binds with a much higher affinity, but its enthalpy of binding is much smaller than that of fibrinogen. Clearly, the driving force for preferential binding of hirudin to thrombin is entropic. Thrombin and hirudin gain entropy when they form a complex. Such an effect may be related to the enormous number of contacts between thrombin and hirudin and the richness of ionic and hydrophobic interactions (Rydel et al., 1991). The precise architecture of hydrogen bonds, ion pairs, and hydrophobic contacts of the C-terminal domain of hirudin (hir$^{55-65}$) has recently been revealed by crystallographic analysis (Rydel et al., 1991; Stubbs et al., 1992). Asp55 of hirudin is ion paired to Lys149E and Arg73 of the FRS, Glu57 is hydrogen bonded to Tyr76 and Thr74 of thrombin, while Glu58 is ion paired to Arg77A of the FRS. These three hirudin residues are engaged in 48 of the 217 total contacts less than 0.4 nm apart of the thrombin–hirudin complex. Yet, isosteric replacement of these residues (Asp55Asn, Glu57Gln, Glu58Gln) changes the free energy of binding respectively by 0.6, 1.4, and 1.3 kcal/mol at 37 °C (Betz et al., 1991a) and by 0.9, 0.8, and 0.4 kcal/mol at 25 °C (Ayala and Di Cera, unpublished results). The small effect observed in isosteric replacements may well be a consequence of the existence of multiple binding modes for the hirudin tail that may coexist in a shallow potential well. The high degeneracy of these modes may give rise to the increase in entropy experienced by thrombin and hirudin upon formation of the complex. In addition, large positive changes in ΔS upon binding of hirudin stress the importance of hydrophobic interactions. The transfer of nonpolar compounds from water to nonpolar solvents is linked to large positive values of ΔS and small values of ΔH (Kauzmann, 1959). Hydrophobic contacts play an important role in stabilizing hirudin binding to the FRS, as demonstrated by structural (Rydel et al., 1991) and functional (Betz et al., 1991b) studies. The binding strategy used by fibrinogen must be very different from that of hirudin. There are some differences between the contacts formed by fibrinopeptide A and the N-terminal compact domain of hirudin with the CP of the enzyme (Rydel et al., 1992; Stubbs et al., 1992), but some of the most important differences are likely to be found in the way fibrinogen and hirudin bind to the FRS. In the case of the natural substrate, destabilization of the bond to be cleaved is more important than abnormally tight binding, which would be incompatible with biological function. Hence, hydrophobic interactions may play for fibrinogen a less important role than other components, such as electrostatic interactions. Given the structural details available on the interaction of the C-terminal domain of hirudin with residues of the FRS, it is likely that hirudin, unlike fibrinogen, has evolved to optimize the strong electrostatic coupling with charged residues of the FRS using tight hydrophobic interactions. This feature may be responsible for the small increase in entropy upon binding to thrombin.

Fibrinogen binding to thrombin is accompanied by a loss of entropy. This suggests that a certain degree of rigidity, or tension, may be generated upon binding to the FRS. The tension may be generated by the combination of favorable and unfavorable electrostatic contacts and be released after fibrinopeptide A is cleaved and fibrin I monomer released. In this picture, the tension would provide the actual driving force for dissociation of fibrin I from thrombin, which would then regenerate the free form of the enzyme.

It is quite interesting to note that the behavior of synthetic peptides that bridge-bind to thrombin allows one to draw a connection between the binding energetics of fibrinogen and hirudin to thrombin, notwithstanding the enormous differences in structure. This fact strongly supports the idea that a major conformational transition of the enzyme takes place upon macromolecular bridge-binding and that it is thrombin, not the particular ligand, that sets the rules for bridge-binding and macromolecular recognition. Indeed, preliminary findings indicate that the slow $\rightarrow$ fast conformational transition of thrombin induced by Na^+ binding (Wells & Di Cera, 1992) is a key step of macromolecular bridge-binding to thrombin since the fast form binds fibrinogen and hirudin with an affinity 20 times higher than that of the slow form (Ayala and Di Cera, unpublished results). The results reported here also indicate, quite clearly, that the development of synthetic inhibitors of thrombin function has not only potential relevance for therapeutical purposes, but also provides a way of dissecting the basic physicochemical aspects of the enzyme function. The study of synthetic inhibitors and hirudin mutants that bridge-bind in the free energy range from −14 to −17 kcal/mol would help to further test the validity of the conclusions drawn in this study and elucidate the molecular components underlying chemical compensation in macromolecular bridge-binding to thrombin.

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