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Energetics of Thrombin–Fibrinogen Interaction†

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ABSTRACT: The kinetic mechanism of thrombin–fibrinogen interaction has been elucidated by steady-state measurements of synthetic substrate hydrolysis by human α-thrombin in the presence of human fibrinogen used as a competitive inhibitor and sucrose used as a viscogenic agent. Sucrose greatly affects the $^{F}K_m$ for thrombin–fibrinogen interaction, without altering the intrinsic properties of the system. Under conditions of pH 7.5 and 0.1 M NaCl, fibrinogen behaves like a sticky substrate for thrombin, with acylation being comparable to dissociation in the temperature range 20–37 °C. In the same temperature range, deacylation is much faster than acylation. The van't Hoff enthalpy of binding for thrombin–fibrinogen interaction is -24 ± 3 kcal/mol and the entropy is -55 ± 11 cal mol^{-1} deg^{-1}. A chemical compensation effect is present in the binding of fibrinogen and synthetic amide substrates to thrombin, with the ΔH and ΔG values being linked through a linear relationship.

Thrombin–fibrinogen interaction is the critical step leading to the formation of an insoluble gel of fibrin polymers. The clotting process involves a number of chemical and physical steps. The chemical steps occur earlier and hinge on the interaction of thrombin with fibrinogen with the resulting release of fibrinopeptides A and B, as well as fibrin I and II monomers (Martinelli & Scheraga, 1980; Higgins et al., 1983; Mihalyi, 1988a,b; Naski & Shafer, 1990; Schmitz et al., 1991). Subsequently, fibrin monomers start aggregating in a sequential fashion giving rise to insoluble polymers (Doolittle, 1984; Hantgan & Hermans, 1979; Dietler et al., 1986; Wilf & Minton, 1987). We presently have a quite detailed understanding of the kinetic aspects of both chemical and physical steps, as well as of the thermodynamic components involved in fibrin polymerization (Sturtevant et al., 1955). On the other hand, remarkably little is known on the energetics of the thrombin–fibrinogen interaction which precedes both the chemical and physical steps leading to clot formation. Basic and important questions such as the magnitude of the free energy of binding of fibrinogen to thrombin, as well as the enthalpic and entropic components involved, have so far remained elusive, due to the difficulty of experimentally approaching these aspects in quantitative terms. Berliner and Fenton have long pointed out that thrombin specificity toward fibrinogen is achieved through "bridge-binding" of the natural substrate to the catalytic pocket and an additional site, the fibrinogen recognition site (FRS)[1] (Berliner et al., 1985; Fenton et al., 1988). The same molecular strategy is used by the potent natural inhibitor of thrombin, hirudin (Stone & Hofsteenge, 1986). Recent structural studies have started to reveal the details of thrombin specificity and the spatial organization of the residues of the FRS (Rydel et al., 1990; Bode et al., 1992). Hence, the need for a quantitative understanding of the underlying energetics of the enzyme in its interaction with the natural substrate fibrinogen becomes timely and important. Thrombin–fibrinogen interaction is not only relevant to the pathophysiology of blood coagulation, but it also serves as a paradigm for many other protein–protein interactions and macromolecular recognitions in the coagulation cascade (Davie et al., 1991). The physiological and physicochemical importance of this interaction motivates our approach to the problem in terms of a new strategy. We have recently shown that the $^{F}K_m$ for thrombin–fibrinogen interaction can be obtained under a variety of solution conditions from studies of synthetic substrate hydrolysis in the presence of fibrinogen used as a competitive inhibitor (De Cristofaro & Di Cera, 1992). In this study we extend our strategy to probe the kinetic mechanism of thrombin–fibrinogen interaction and the thermodynamic aspects of fibrinogen binding, along with the underlying enthalpic and entropic components.

MATERIALS AND METHODS

Steady-State Measurements. Human α- and γ-thrombin and human fibrinogen were purified and tested for purity and activity as described in detail elsewhere (Di Cera et al., 1991; De Cristofaro et al., 1992a; De Cristofaro & Di Cera, 1992). Steady-state measurements of amidase activity were made using the synthetic chromogenic peptides S-2238 (KabiVitrum, Stockholm, Sweden), Chromozym-TH (Sigma, St. Louis, MO) and Spectrozyme-TH (American Diagnostica, Greenwich, CT) as described elsewhere (Di Cera et al., 1991). The linearity of the initial portion of the progress curve was indicated by very high values of the correlation coefficient for the fit ($r > 0.99$ in all cases) and implied that any complicating effect due to inhibition by *p*-nitroaniline (Hogg & Jackson, 1989) or binding of fibrin I to the enzyme (Naski & Shafer, 1990) was negligible over the time scale of our measurements (De Cristofaro & Di Cera, 1992). All assays were performed using disposable polystyrene cuvettes under solution conditions of 50 mM Tris, 0.1 M NaCl, and 0.1% PEG 8000, pH 7.5 at the desired temperature. The viscosity of the solution was changed by addition of sucrose to the buffer. The viscosity of each solution relative to the buffer containing no viscogenic agent was measured by an Ostwald viscometer over the entire temperature range explored experimentally.

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[1] Abbreviations: FRS, fibrinogen recognition site; Chromozym-TH, tosyl-Gly-Pro-Arg-*p*-nitranilide-AcOH; HPLC, high-performance liquid chromatography; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-2238, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide; Spectrozyme-TH, H-D-hexahydrotyrosyl-Ala-Arg-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane.

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Data Analysis. Competition experiments of synthetic substrate hydrolysis in the presence of fibrinogen can be analyzed in terms of a kinetic scheme where the free enzyme, E, interacts with either the synthetic substrate, S, or fibrinogen, F, as follows:

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

$$E + F \underset{{}^{F}k_{-1}}{\overset{{}^{F}k_1^*}{\rightleftarrows}} EF \xrightarrow{{}^{F}k_2} EF' \xrightarrow{{}^{F}k_3} E + P' \quad (1b)$$

where P and P′ are the products of the two reactions and k_2 and k_3 are the acylation and deacylation rates, while k_1* and k_{-1} are the rate constants for binding and dissociation. The scheme above incorporates the widely accepted kinetic mechanism of action of serine proteases (Fersht, 1985). In the case of thrombin–fibrinogen interaction, the product P′ refers to the fibrinopeptide A and fibrin I, as discussed in detail elsewhere (De Cristofaro & Di Cera, 1992). The steady-state velocity of product formation in the first reaction, $v = d[P]/dt$, is given by

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

where e_T is thrombin concentration. The Michaelis–Menten parameters are functions of the individual rate constants as follows:

$${}^{S}k_{cat} = \frac{{}^{S}k_3\,{}^{S}k_2}{({}^{S}k_3 + {}^{S}k_2)} \quad (3a)$$

$${}^{S}K_m = \frac{{}^{S}k_3({}^{S}k_{-1} + {}^{S}k_2)}{{}^{S}k_1^*({}^{S}k_3 + {}^{S}k_2)} \quad (3b)$$

$${}^{F}K_m = \frac{{}^{F}k_3({}^{F}k_{-1} + {}^{F}k_2)}{{}^{F}k_1^*({}^{F}k_3 + {}^{F}k_2)} \quad (3c)$$

These parameters can be obtained from analysis of measurements of v collected in a matrix of [S] and [F] values (De Cristofaro & Di Cera, 1992). The highest concentration of fibrinogen was 3–8 μM depending upon temperature, with higher concentrations used at high temperature. The range of fibrinogen concentrations was typically $^1/_3$–$^1/_2$ of the measured ${}^{F}K_m$.

The individual kinetic rate constants involved in eq 1 can be determined by steady-state measurements run in solutions with different viscosities. This experimental strategy has been applied successfully in other enzyme systems (Nakatani & Dunford, 1979; Brouwer & Kirsch, 1982; Kurz et al., 1987). Viscosity affects all the steps that involve diffusion, i.e., k_1*, k_{-1}, and k_3 in eq 1. Any kinetic rate can be expressed as $k' = k/\eta_{rel}$ (Nakatani & Dunford, 1979), where η_{rel} is the viscosity of a given solution with added viscogenic agent relative to the reference solution with no viscogenic agent and k is the value of the rate constant in the reference solution. Hence, the exact analytical expressions for the Michaelis–Menten parameters as a function of η_{rel} are given by

$${}^{S}k_{cat} = \frac{{}^{S}k_3\,{}^{S}k_2}{({}^{S}k_3 + {}^{S}k_2\eta_{rel})} \quad (4a)$$

$${}^{S}K_m = \frac{{}^{S}k_3({}^{S}k_{-1} + {}^{S}k_2\eta_{rel})}{{}^{S}k_1^*({}^{S}k_3 + {}^{S}k_2\eta_{rel})} \quad (4b)$$

$${}^{F}K_m = \frac{{}^{F}k_3({}^{F}k_{-1} + {}^{F}k_2\eta_{rel})}{{}^{F}k_1^*({}^{F}k_3 + {}^{F}k_2\eta_{rel})} \quad (4c)$$

All individual kinetic constants pertaining to the synthetic substrate can be resolved from the expressions above by means of studies conducted as a function of relative viscosity. The same does not hold in the case of fibrinogen, since no information is gathered from these studies on the value of ${}^{F}k_{cat}$. However, measurements of ${}^{F}K_m$ as a function of η_{rel} yield the (equilibrium) dissociation constant for thrombin–fibrinogen interaction, ${}^{F}K_d$, as the extrapolation of ${}^{F}K_m$ for $\eta_{rel} = 0$. Therefore, competition experiments can effectively be used to probe the energetics of thrombin–fibrinogen interaction.

Control Experiments. Values of ${}^{F}K_m$ obtained with S-2238 were found to overlap very well with those obtained with Chromozym-TH and Spectrozyme-TH. This is consistent with the expectations embodied by eq 1, since ${}^{F}K_m$ must be independent of the particular synthetic substrate used. We have also tested whether sucrose altered the intrinsic properties of the system by aspecific solvent effects. In the absence of such effects any equilibrium constant must be independent of η_{rel}, since this parameter affects equally the on and off rates for binding and dissociation (Nakatani & Dunford, 1979). Measurements of K_I for competitive inhibitors of thrombin activity, such as hirudin and *p*-aminobenzamidine, have yielded the same value (within experimental error) in 0 ($\eta_{rel} = 1$), 0.4 ($\eta_{rel} = 1.44$) and 0.8 M sucrose ($\eta_{rel} = 2.34$). We have also run experiments in the presence of 0.13 mM Ficoll ($\eta_{rel} = 2.38$). Ficoll is a polymer of sucrose (400 000 MW) that only alters the macroscopic viscosity and has no effect on the diffusion properties of macromolecular components and small substrates (Kurz et al., 1987). In our control experiments, Ficoll buffers have yielded no change in K_m and k_{cat} for synthetic substrates, as expected. These control experiments prove that the change in viscosity of the medium, with either sucrose or Ficoll, does not alter the intrinsic properties of the system through aspecific solvent effects. The role of the FRS was examined by studies involving γ-thrombin, where the FRS is perturbed by a proteolytic cleavage (Bing et al., 1977). Competition experiments as a function of substrate and fibrinogen concentration were carried out at 25 °C in the presence of 10 μM fibrinogen.

RESULTS

The results of our measurements of ${}^{F}K_m$ as a function of η_{rel} in the temperature range 20–37 °C are shown in Figure 1. In this temperature range scattering due to fibrin polymerization was low enough to allow for precise and reproducible estimates of all parameters. The validity of the kinetic scheme in eq 1 and eq 2 is demonstrated by the overlap between data obtained in the presence of S-2238 (●) and Chromozym-TH (○). The values of ${}^{F}K_m$ increase linearly with η_{rel} and follow a unique trend at each temperature examined here regardless of the particular synthetic substrate used. This observation has a bearing on the kinetic mechanism

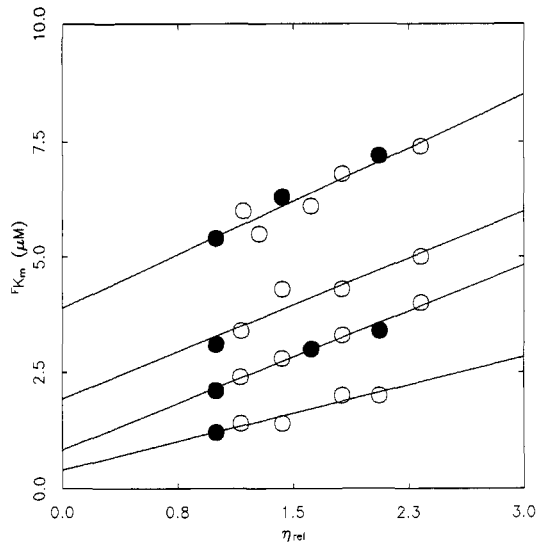

FIGURE 1: Results of steady-state measurements of synthetic substrate hydrolysis in the presence of human fibrinogen as a function of relative viscosity. Experimental conditions are 1 nM human α-thrombin, 50 mM Tris, 0.1% PEG 8000, 0.1 M NaCl, pH 7.5, and temperature of 37, 30, 25, and 20 °C from top to bottom. The synthetic substrates used are (●) S-2238 and (○) Chromozym-TH. Continuous lines were drawn according to eq 5 in the text with $\beta = 0$ in all cases. Inclusion of this parameter in the minimization did not affect the goodness of the fit within 95% confidence. The best-fit parameter values are (37 °C) $^{F}K_d = 3.9 \pm 0.3$ (μM), $\alpha = 0.40 \pm 0.09$; (30 °C) $^{F}K_d = 1.9 \pm 0.4$ (μM), $\alpha = 0.70 \pm 0.29$; (25 °C) $^{F}K_d = 0.8 \pm 0.1$ (μM), $\alpha = 1.59 \pm 0.36$; (20 °C) $^{F}K_d = 0.4 \pm 0.2$ (μM), $\alpha = 2.04 \pm 1.50$.

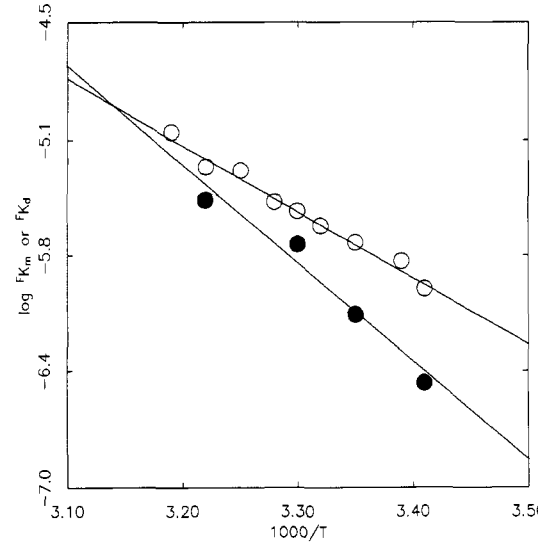

FIGURE 2: van't Hoff plot of $^{F}K_d$ (●) and $^{F}K_m$ (○) values for thrombin–fibrinogen interaction, under experimental conditions of 0.1 M NaCl, pH 7.5. The enthalpy value for fibrinogen binding to thrombin is −24 ± 3 kcal/mol. Notice how the $^{F}K_d$ and $^{F}K_m$ values progressively diverge as temperature decreases.

of thrombin–fibrinogen interaction. In fact, eq 4c can be rewritten as

$$^{F}K_m = {}^{F}K_d \frac{(1 + \alpha\eta_{rel})}{(1 + \beta\eta_{rel})} \quad (5)$$

where $^{F}K_d = {}^{F}k_{-1}/{}^{F}k_1{}^{*}$, $\alpha = {}^{F}k_2/{}^{F}k_{-1}$, and $\beta = {}^{F}k_2/{}^{F}k_3$. The results depicted in Figure 1 imply that $1 \gg \beta\eta_{rel}$ up to $\eta_{rel} = 3$ or else that deacylation is much faster than acylation in the temperature range 20–37 °C. The values of α change from 0.40 ± 0.09 at 37 °C to 2.04 ± 1.50 at 20 °C, thereby indicating that acylation occurs on a time scale comparable to that for dissociation. The intercept for $\eta_{rel} = 0$ in Figure 1 gives the value of $^{F}K_d$ at any given temperature. These values are shown in Figure 2 as a van't Hoff plot, along with the values of $^{F}K_m$ obtained in the same temperature range. The plot shows quite clearly how the values of $^{F}K_d$ and $^{F}K_m$ diverge progressively as temperature decreases. The enthalpy of fibrinogen binding to thrombin is −24.3 ± 3.3 kcal/mol, and the entropy is −55 ± 11 cal mol^{-1} deg^{-1}. The thermodynamic components for fibrinogen binding to thrombin can be compared to analogous quantities obtained for synthetic amide substrates. Figure 3 shows a plot of ΔH versus ΔG values for two such substrates and fibrinogen computed at the harmonic mean of the experimental temperatures following the Krug–Hunter–Grieger procedure (Krug et al., 1976a,b). The three points are linked by a linear relationship, which indicates the existence of an extrathermodynamic dependence of ΔH on ΔG and hence chemical compensation in macromolecular binding to thrombin. The importance of the FRS in thrombin–fibrinogen interaction is demonstrated by results obtained with γ-thrombin, a derivative perturbed at the level of the FRS by proteolytic digestion (Bing et al., 1977) which has a drastic reduction of clotting activity. As shown in Figure 4, competition experiments using human fibrinogen show a modest effect of fibrinogen on the hydrolysis of Chromozym-TH. The value

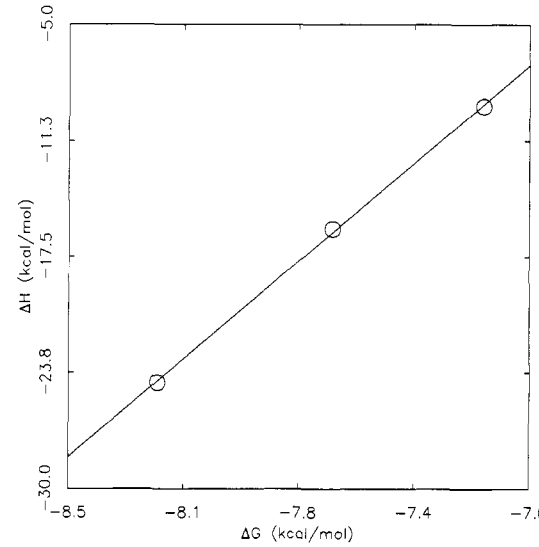

FIGURE 3: Krug–Hunter–Greiger plot of ΔH and ΔG values for binding to thrombin in the case of fibrinogen (bottom left), S-2238 (center), and Spectrozyme-TH (top right). The values were computed at the harmonic mean of the experimental temperatures (28 °C in this case). The linearity of the plot indicates the presence of chemical compensation and an extrathermodynamic relationship between ΔH and ΔG that connect the energetics of fibrinogen and synthetic substrates. The continuous line was drawn according to the expression $\Delta H = a + b\delta G$, with $a = 91 \pm 2$ kcal/mol and $b = 14.1 \pm 0.3$.

of $^{F}K_m$ for human γ-thrombin is 49 ± 17 μM and is 1 order of magnitude higher than that found in the case of the native enzyme.

DISCUSSION

The results presented in this study provide new details on the kinetic mechanism of fibrinogen hydrolysis by thrombin. Since the release of fibrinopeptide A precedes that of fibrinopeptide B (Higgins et al., 1983; Mihalyi, 1988b; Schmitz et al., 1991) and the latter reaction is certainly negligible over the time scale of 15–30 s employed in our measurements, the results reported here are mostly pertaining to the hydrolysis of fibrinogen to yield fibrin I and the fibrinopeptide A, as implied by eq 1. In this respect, it should be pointed out that

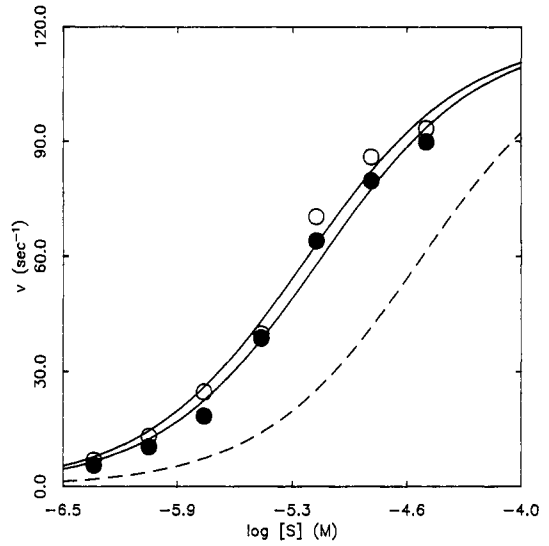

FIGURE 4: Steady-state measurements of synthetic substrate (Chromozym-TH) hydrolysis by human γ-thrombin in the presence of human fibrinogen. The data are shown as velocity normalized by thrombin concentration, as a function of the logarithm of substrate concentration. Data points were collected in a 7 × 8 matrix of substrate and fibrinogen concentration as discussed in the text. Only two curves are shown for the sake of clarity. Experimental conditions are 1.25 nM human γ-thrombin, 50 mM Tris, 0.1 M NaCl, 0.1% PEG, pH 7.5 and 25 °C. The concentration of human fibrinogen is (○) 0 and (●) 10 μM. Continuous lines were drawn from eq 2 using the best-fit parameter values ${}^{S}k_{cat} = 118 \pm 6\ s^{-1}$, ${}^{S}K_m = 6.6 \pm 0.9\ \mu M$, and ${}^{F}K_m = 49 \pm 17\ \mu M$. The discontinuous line depicts the effect that would be observed if ${}^{F}K_m$ had the same value as that obtained for the native enzyme under identical solution conditions.

the value of ${}^{F}K_m$ obtained in this study at 37 °C agrees very well with that determined independently by HPLC in the case of the fibrinopeptide A (Higgins et al., 1981; Schmitz et al., 1991), and with the value obtained in previous competition experiments (De Cristofaro & Di Cera, 1992). Fibrinogen behaves as a sticky substrate for thrombin, with the acylation rate being comparable to dissociation. Deacylation is faster than acylation over the entire temperature range explored in this study, from 20 to 37 °C. The mechanism is similar to that seen in the hydrolysis of several amide substrates by serine proteases obeying Briggs–Haldane kinetics (Fersht, 1985). In this respect, our findings embody a result that could have qualitatively been anticipated by general considerations on the hydrolysis of amide bonds. The strategy introduced here yields quantitative information on the equilibrium aspects of thrombin–fibrinogen interaction for the first time. The dissociation constant ${}^{F}K_d$ is in the micromolar range and is extremely sensitive to temperature, with $\Delta H = -24 \pm 3$ kcal/mol. The value of the dissociation constant is comparable to that of fibrin I when it allosterically affects the properties of the enzyme by binding to the FRS and leaving the active site free (Naski & Shafer, 1990). More interestingly, the dissociation constant of fibrinogen is similar to that of fibrin II, which only binds to the FRS (Liu et al., 1979; Berliner et al., 1985; Kaminsky & McDonagh, 1987), as well as to those of a number of synthetic substrates and competitive inhibitors of substrate hydrolysis, such as *p*-aminobenzamidine, that only bind to the catalytic pocket of the enzyme. The comparison with fibrin II shows that the fibrinopeptides A and B provide little contribution to fibrinogen binding to thrombin, as first suggested by Shafer (Naski & Shafer, 1990). The value of the enthalpy of binding for fibrinogen is significantly more exothermic than that found for synthetic substrates. A linear relationship is observed betwen ΔH and ΔG values of binding to thrombin for three amide substrates, including fibrinogen. This unexpected result draws attention to the existence of a true chemical compensation which is probably a manifestation of a unique "code" for macromolecular binding to thrombin that is exploited by fibrinogen as well as synthetic substrates. This code may be indicative of conformational transitions triggered by substrate and fibrinogen binding that scale with the free energy of binding. Alternatively, chemical compensation may imply involvement of similar structural components, such as hydrophobic interactions and hydrogen bonds, that contribute to the overall scaled energetics.

The existence of chemical compensation is a most interesting feature of thrombin since it indicates the presence of a connection between the energetics of synthetic amide substrates and fibrinogen. Synthetic substrates are small and bind to the catalytic pocket alone. Fibrinogen, on the other hand, is 10 times bigger than the enzyme and bridge-binds to the catalytic pocket and the adjacent FRS. The FRS is the structural domain whereby the enzyme derives its unique specificity. The importance of the FRS in thrombin–fibrinogen interaction is supported by the fact that γ-thrombin has a drastic reduction of clotting activity. The same effect is observed with selective phosphopyridoxylation of lysyl residues of the FRS (Griffith, 1979; Church et al., 1989). These observations strongly suggest that perturbation of the FRS translates into impaired fibrinogen binding to thrombin. Our results on γ-thrombin strongly support this hypothesis since the value of ${}^{F}K_m$ for γ-thrombin is 10 times bigger than that of the native enzyme. On the other hand, the structural perturbation of the FRS produces only a 1.5–3-fold increase in the K_m of synthetic substrates (Lottenberg et al., 1982; De Cristofaro et al., 1992b). Therefore, although a perturbation of the catalytic pocket cannot be excluded in γ-thrombin, it seems reasonable to conclude that the perturbation of the FRS is responsible for most of the loss in free energy of binding of fibrinogen. This conclusion is supported by a recent suggestion by Bode that cleavage of the Trp148 loop in γ-thrombin translates into partial unfolding of the loop with a consequent disruption of the FRS (Bode et al., 1992). Having demonstrated the importance of the FRS in fibrinogen binding, a paradox seems to arise as to how the natural substrate can bind to thrombin with an affinity comparable to that of synthetic substrates that only bind to the catalytic pocket. Furthermore, how can the natural substrate bind to thrombin with an affinity comparable to that of fibrin II monomer that only binds to the FRS (Liu et al., 1979; Berliner et al., 1985; Kaminsky & McDonagh, 1987)? From a thermodynamic standpoint, bridge-binding can be decoupled into separate contributions arising from the catalytic pocket (CP), the FRS, and any interaction between them. The relevant thermodynamic expression is

$$\Delta G_{bb} = \Delta G_{FRS} + \Delta G_{CP} + \Delta G_{int} \qquad (6)$$

where ΔG_{bb} is the (standard) free energy of bridge-binding and ΔG_{FRS} and ΔG_{CP} are the analogous free energy changes for binding to the FRS and the catalytic pocket (CP), while ΔG_{int} is the free energy of interaction between the FRS and the CP. The energetic dissection of ΔG_{bb} demands knowledge of the site-specific components ΔG_{FRS} and ΔG_{CP}, along with stabilization ($\Delta G_{int} < 0$) or destabilization ($\Delta G_{int} > 0$) effects due to interaction between the sites. In this respect, bridge-binding can be considered as a *global* property of the system arising from *local* site-specific binding processes and its thermodynamic description can be cast in terms of site-specific

linkage theory (Di Cera, 1990). Estimates of ΔG_{CP} can reasonably be taken from studies involving synthetic substrates and are in the range from −8 to −7 kcal/mol. This is the same range of ΔG_{bb} for fibrinogen, so that one necessarily has for the natural substrate that

$$\Delta G_{bb} \approx \Delta G_{CP} \quad (7)$$

$$\Delta G_{FRS} + \Delta G_{int} \approx 0 \quad (8)$$

Hence, the interaction between the FRS and the CP opposes binding to the FRS and acts to cancel out the free energy term arising from this structural domain with a destabilization effect. Analogous considerations apply in the case of fibrin II binding to the FRS alone, which also occurs with a free energy change of about −7 kcal/mol. Hirudin, a potent natural inhibitor of thrombin, bridge-binds to the enzyme with a binding free energy in the range from −17 to −15 kcal/mol (Stone & Hofsteenge, 1986; De Cristofaro et al., 1992b). The origin of such a high affinity may be due to the lack or drastic decrease of destabilization effect for this inhibitor. The foregoing argument provides a simple and rigorous explanation for the effects observed experimentally in the case of thrombin–fibrinogen interaction. The presence of a destabilization effect scales down the energetics of fibrinogen binding to the level of synthetic amide substrates, which may account for the linear chemical compensation shown in Figure 3. The destabilization also explains why fibrinogen and hirudin bridge-bind to thrombin in a similar fashion but with largely different affinities. Different ligands may exploit different energetics of bridge-binding based on different values of ΔG_{int} in eq 6. The experimental strategy introduced in this study and the powerful thermodynamic approach on site-specific energetics dealt with above open the way to a quantitative investigation of the energetics of thrombin–fibrinogen interaction under a variety of conditions of interest. The site-specific energetics of the FRS and the CP can be explored by synthetic substrates and inhibitors specifically tailored to interact with either structural domain. When information is gathered on all these aspects it will be possible to understand how separate domains of the enzyme participate in its important physiological function.

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