Two different proteins that compete for binding to thrombin have opposite kinetic and thermodynamic profiles

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Abstract

Thrombin binds thrombomodulin (TM) at anion binding exosite 1, an allosteric site far from the thrombin active site. A monoclonal antibody (mAb) has been isolated that competes with TM for binding to thrombin. Complete binding kinetic and thermodynamic profiles for these two protein–protein interactions have been generated. Binding kinetics were measured by Biacore. Although both interactions have similar K_D s, TM binding is rapid and reversible while binding of the mAb is slow and nearly irreversible. The enthalpic contribution to the ΔG_{bind} was measured by isothermal titration calorimetry and van't Hoff analysis. The contribution to the ΔG_{bind} from electrostatic steering was assessed from the dependence of the k_a on ionic strength. Release of solvent H_2O molecules from the interface was assessed by monitoring the decrease in amide solvent accessibility at the interface upon protein–protein binding. The mAb binding is enthalpy driven and has a slow k_d . TM binding appears to be entropy driven and has a fast k_a . The favorable entropy of the thrombin–TM interaction seems to be derived from electrostatic steering and a contribution from solvent release. The two interactions have remarkably different thermodynamic driving forces for competing reactions. The possibility that optimization of binding kinetics for a particular function may be reflected in different thermodynamic driving forces is discussed.

Keywords: Surface plasmon resonance; calorimetry; amide $\text{H}/^2\text{H}$ exchange; MALDI-TOF mass spectrometry; hydration; entropy; enthalpy

The thrombin–thrombomodulin (TM) interaction controls an important anticoagulation pathway in the blood, and has been demonstrated to have rapid association and dissociation rates (Baerga-Ortiz et al. 2000). The advantage of such a character is apparent when we consider that thrombin

(max. plasma conc. $2.5 \mu\text{M}$) must be captured from the fast and turbulent blood flow by TM molecules immobilized in the endothelial cell membrane. This recruitment process results in switching of substrate specificity of thrombin towards protein C. The new substrate is already associated to the membrane by its Gla domains and its interaction with the endothelial cell protein C receptor (Esmon 1995). The TM–thrombin complex efficiently activates protein C, which is then released as the first player in the anticoagulant pathway. The TM-bound thrombin is susceptible to inhibition by antithrombin-III, and rapid dissociation of the inhibited thrombin results in efficient reuse of the limited quantities of TM. Because the cessation of blood clotting needs to be a fast process under flow conditions, it is not

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Abbreviations: TM, thrombomodulin; EGF, epidermal growth factor; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MS, mass spectrometry; $\text{H}/^2\text{H}$, hydrogen/deuterium.

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surprising that the thrombin–TM interaction is governed by fast kinetics.

A monoclonal antibody (mAb) has been isolated that competes with TM for binding to thrombin (Dawes et al. 1984). The TM and mAb have overlapping but not identical binding sites (Fig. 1; Baerga-Ortiz et al. 2002). Monoclonal antibodies are selected in vitro for slow dissociation by the washing processes that are used. In a way, the antibody is selected for its function, which is irreversible binding to its target. We thought it would be interesting to compare the binding kinetics and thermodynamics of the mAb and of TM to thrombin because their binding sites are overlapping, but their functions are different.

We have generated kinetic and thermodynamic binding profiles for the thrombin–TM and thrombin–mAb protein–protein interactions to investigate the kinetic profiles and the equilibrium thermodynamic properties of each interaction. We show that TM and mAb have opposite kinetic and thermodynamic profiles. The very different kinetic profiles seem to be reflected in very different thermodynamic contributions to the binding free energy.

Results

Binding kinetics

Binding kinetic data were collected for both the thrombin–TM and thrombin–mAb interactions by flowing thrombin over a surface containing immobilized molecules, of the binding partner, on a BIACORE 3000 surface plasmon resonance instrument (Fig. 2). Specific immobilization of the monoclonal antibody by way of covalent linkage to coupled

Figure 1. Comparison of the binding interfaces determined from H/²H exchange experiments for the interaction of thrombin with (A) the mAb, and with TMEGF45 as determined in Baerga-Ortiz et al. 2002. The mAb binds to thrombin at surface segments that were partially, but not totally solvent excluded by TMEGF45 (red). Two additional surface segments were completely solvent excluded by TMEGF45 but were not protected by the mAb (blue). The thrombin active site residues are colored green.

Figure 2. (A) Biacore sensorgrams for the binding at 298 K and 150 mM NaCl of thrombin to (A) mAb (300 RU) or (B) TMEGF456 (570 RU) immobilized on the sensor chip. The thrombin concentrations were 0.78 nM (red), 1.56 nM (blue), 3.12 nM (green), 6.25 nM (purple), 12.5 nM (pink), 25 nM (cyan). The data were globally fit to a 1 : 1 binding model (black lines in both).

protein G gave (at 298 K and 150 mM NaCl) a measured association rate constant of $k_a = 6.4 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ and a dissociation rate constant of $k_d = 0.001 \text{ sec}^{-1}$ under the same conditions (Table 1). Specific immobilization of an N-terminally biotinylated TM fragment, TMEGF456, and subsequent flowing of thrombin through the flow channel at 298 K and 150 mM NaCl, resulted in a measured association rate constant of $k_a = 2.0 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$ and a dissociation rate constant of $k_d = 0.037 \text{ sec}^{-1}$ (Table 1). The k_d was much slower for the thrombin–mAb interaction than for the thrombin–TM interaction. In fact, to make sure that the thrombin–mAb complex was fully dissociated before the next injection of thrombin, all injections were immediately followed by the injection of 10 mM glycine (pH 2.0), whereas no regeneration of the surface was required for the thrombin–TM interaction, which fully dissociates within 1 min. The dramatic difference in association rate constants is qualitatively obvious from visual examination of the sensorgrams. Figure 2 shows that the thrombin–mAb interaction does not reach flowing equilibrium by the end of the injection at 225 sec, whereas after a 40 sec injection of thrombin, the thrombin–TM complex has already reached a state of flowing equilibrium on the surface. The huge difference in dissociation rate constants was nearly completely compensated for by an equal and opposite difference in association rate constants so that the binding dissociation

Table 1. Binding kinetics for thrombin binding to thrombomodulin and mAb

Temperature (K)	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	R_{max} (R.U.)	K_D (M) ^a	χ^2 ^b
A. Thrombomodulin					
279 K	7.82×10^6	2.80×10^{-2}	23.2	3.58×10^{-9}	0.36
293 K	3.78×10^7	7.16×10^{-2}	10.1	1.89×10^{-9}	0.55
298 K	2.00×10^7	3.74×10^{-2}	25.8	1.87×10^{-9}	0.49
313 K	2.89×10^7	5.45×10^{-2}	19.5	1.89×10^{-9}	1.05
B. Monoclonal Antibody					
279	4.22×10^5	9.1×10^{-5}	73.7	2.16×10^{-10}	0.34
288	1.13×10^5	5.42×10^{-5}	25.9	4.78×10^{-10}	0.26
293	4.34×10^5	3.52×10^{-4}	23.5	8.11×10^{-10}	0.18
298	6.40×10^5	1.05×10^{-3}	21	1.64×10^{-9}	0.144
303	9.24×10^5	1.68×10^{-3}	27.4	1.82×10^{-9}	0.72
310	9.33×10^5	2.00×10^{-3}	19.5	2.15×10^{-9}	0.38
313	1.06×10^6	1.95×10^{-3}	25.9	1.84×10^{-9}	0.40

Values were obtained from global fitting of data from five to six different concentrations of thrombin (typically 0.78 nM, 1.56 nM, 3.13 nM, 6.25 nM, 12.5 nM, 25 nM) using a 1:1 Langmuir fit. See Figure 4 for more details.

^a The K_D is calculated from the globally fit k_d and k_a since $K_D = k_d/k_a$.

^b A χ^2 value of less than 2 is generally considered an excellent fit.

constant of the thrombin–mAb interaction (K_{DmAb}) of 1.6 ± 0.3 nM nearly equaled that of the thrombin–TM interaction (K_{DTM}) of 1.9 ± 0.5 nM under the same conditions of 298 K and 150 mM NaCl.

Enthalpy of binding

The observed enthalpy change (ΔH_{obs}) upon binding was directly measured by isothermal titration calorimetry (ITC).

The binding isotherm for the thrombin–mAb interaction (Fig. 3A,B) is characteristic of an exothermic single binding site interaction with low nM binding affinity. A ΔH_{obs} of -8.2 ± 0.9 kcal per mole of thrombin was calculated from the average of two separate experiments. These experiments show that the thrombin–mAb interaction is dominated by favorable ΔH , at 298 K, with a smaller contribution coming from a favorable observed entropy change (ΔS_{obs})

Figure 3. Isothermal titration calorimetry of thrombin binding to either mAb or TMEGF45. (A) Raw data showing the heat released during binding of thrombin to mAb (black trace) and the heat of dilution control experiment (blue trace). (B) Integrated heat of binding for the thrombin–mAb interaction at 298 K. Data were fit to a single binding site model, assuming an effective concentration of binding sites for the antibody, after subtracting the heat of dilution data. Raw (C) and integrated (D) data for the thrombin–TMEGF45 interaction showing no heat change was observed during binding at 298 K.

($T\Delta S_{\text{obs}} \approx 2.5 \pm 0.7$ kcal per mole of mAb binding sites). We carried out ITC binding experiments for the thrombin–TM interaction in an identical manner to the thrombin–mAb experiments. However, no detectable ΔH of binding was observed (Fig. 3C,D) at 298 K, and we were unable to carry out experiments over a wider temperature range, due to limitations of our available instrumentation.

The temperature dependence of the dissociation constant (K_D) was also determined from k_d/k_a by Biacore to provide a separate means to assess ΔH of binding by van't Hoff analysis. The temperature dependence of the thrombin–mAb interaction was qualitatively observed from the raw sensorgrams (Fig. 4), but no temperature dependence was observed for the thrombin–TM interaction (data not shown because all sensorgrams looked like those of Fig. 2).

Figure 4. Biacore sensorgrams for the binding of mAb (300 RU immobilized) to thrombin at 150 mM NaCl and (A) 288 K or (B) 298 K or (C) 310 K. The thrombin concentrations are 0.78 nM (red), 1.56 nM (blue), 3.12 nM (green), 6.25 nM (purple), 12.5 nM (pink), and 25 nM (cyan). The data were globally fit to a 1 : 1 binding model (black lines). For the 288 K data set, no binding was observed at 0.78 nM, and for the 310 K data set, the binding at 25 nM was left out of the fit. The sensorgrams represent one of the two data sets used in the van't Hoff analysis presented in Figure 5.

Figure 5. van't Hoff plots for the temperature dependence of $\ln(K_D)$ determined by Biacore for the (A) the thrombin–mAb interaction and (B) thrombin–TM interaction. The error bars on both plots are the standard deviation for two independent determinations of each K_D . The open squares on the plot for the thrombin–TM interaction are the data obtained from binding competition experiments carried out previously (Vindigni et al. 1997). For the thrombin–mAb interaction, an estimate of ΔH was obtained from the slope of the line. The error bars are the standard deviation from two independent data sets, one of which is given in Table 1 and a part of which is shown in Figure 4. The data from the 279 K study of the thrombin–mAb interaction was not used in the final data analysis because the binding became so slow that global fitting of the data resulted in an overestimate of the R_{max} (Table 1).

Consistent with the ITC, the thrombin–mAb showed a linear dependence of $\ln K_D$ with $1/T$ indicating a favorable ΔH for interaction (Fig. 5A). The ΔH estimated from the slope was -10.6 ± 1.7 kcal/mole. As has been observed by others, when Biacore experiments are carefully done, the value from Biacore agrees well with that from the ITC (Day et al. 2002). Because ITC is a direct measure not requiring assumptions of a linear model, we take it as a more reliable method for determining the enthalpy when it can be measured this way. In confirmation of the ITC results, the thrombin–TM interaction showed no significant trend in $\ln K_D$ over the temperature range studied (one data set is presented in Table 1; both data sets are presented in Fig. 5B [closed squares]). In previous work using competition binding experiments to measure the temperature dependence of K_D , a similar broadly smiling van't Hoff plot was obtained (open squares in Fig. 5B; Vindigni et al.

1997). Thus, in three separate experiments using three different binding measures, a lack of an enthalpic contribution to the binding of thrombin at 298 K to TM was observed.

Eyring analysis showed that the temperature dependence of the K_D for the thrombin–mAb interaction is due mainly to the temperature dependence of the k_d constant and to a significantly lesser extent k_a (Fig. 6A,B). A similar result has been obtained for at least one other antibody–antigen interaction (Zeder-Lutz et al. 1997). Eyring analysis was not carried out for the thrombin–TM interaction because it did not show any temperature dependence in the range we could measure.

Electrostatic steering

Given the favorable observed ΔG_{bind} of the thrombin–TM interaction (−11.9 kcal /mole) and the negligible contribution from ΔH , we sought to determine contributions to favorable ΔS of interaction. According to recent theories, electrostatic steering contributes to a favorable entropy of interaction by maximizing the frequency of productive encounters (Janin 1997). A linearized model has been proposed for estimating the contribution of ΔG due to electro-

Figure 6. Eyring plots represent the temperature dependence of the kinetic constants (A) k_d and (B) k_a determined by Biacore for the thrombin–mAb interaction. The error bars on both plots are the standard deviation for two independent determinations of each kinetic constant.

Figure 7. Ionic strength dependence of the k_a is represented by Debye–Hückel plots for (A) the thrombin–mAb interaction and (B) the thrombin–TM interaction. The ionic strength was varied by changing the concentration of NaCl (circles) or $(\text{CH}_3)_4\text{NCl}$ (diamonds). The error bars on both plots are the standard deviation for two independent determinations of each k_a . The data plotted in Figure 7B are from previously published work (Baerga-Ortiz et al. 2000).

static steering from the ionic strength dependence of the k_a . To apply this model, we measured the k_a for both interactions at different ionic strengths, again using Biacore. For these experiments, the same conditions were used as for the data in Table 1 except that the concentration of NaCl in the flowing and sample buffers was varied between 100 and 300 mM. Thrombin concentrations were adjusted so that at least five sensorgrams gave binding as described previously (Baerga-Ortiz et al. 2000). Two independent experiments were performed on different days so that the errors in the determination of the k_a could be assessed. The plots of k_a versus ionic strength, or Debye–Hückel plots, for the thrombin–mAb and thrombin–TM interactions are shown in Figure 7, A and B. In the case of the thrombin–TM interaction, the ionic strength was also adjusted using $(\text{CH}_3)_4\text{NCl}$ in addition to NaCl. In each case a similar result was observed, demonstrating that the ionic strength dependence was not due to a specific cation binding effect. The data were fit to equation 1:

$$\log k_a = \log k_0 + 2 Z_A Z_B \sqrt{I} \quad (1)$$

where the slope $2Z_A Z_B$ is the product of the effective charges of interacting proteins A and B. The linearized model of

Janin proposes that a quantitative estimation of the contribution of electrostatic enhancement to ΔG can be obtained from equation 2 (Janin 1997):

$$\Delta G_{\text{long-range}} = RT \ln (k_0/k_\infty) \quad (2)$$

The value of k_a extrapolated to an ionic strength $I = 0$ (k_0) was obtained from the y-intercept of the Debye-Hückel plots (Fig. 7). The extrapolated values of k_0 for thrombin binding to TM and mAb were $7.0 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$ and $1.6 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$, respectively. An estimate of k_∞ was taken from the k_a at the highest NaCl concentration (300 mM NaCl), which was $2.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ and $5.6 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ for TM and mAb, respectively. Using these values, the contribution to the overall ΔG_{bind} from electrostatic steering for the thrombin-TM interaction was -3.3 ± 0.1 kcal/mole, while the contribution of this term to thrombin-mAb is -0.6 ± 0.06 kcal/mole.

Solvent exclusion at the interface

Although electrostatic steering did appear to contribute to the favorable ΔG_{bind} , a significant proportion of the highly favorable ΔG_{bind} for the thrombin-TM interaction remained unaccounted for. Another source of favorable entropy in protein-protein interactions is the release of H_2O molecules from the interface upon binding because the entropy of H_2O increases when it is transferred from the surface of the protein to the bulk (Brady and Sharp 1997a,b). The number of H_2O molecules released can be estimated by addition of osmolytes, or by measuring changes in volumetric properties (Xavier et al. 1997; Chalikian and Breslauer 1998). We were unable to attain the high-protein concentrations necessary to make these measurements, so instead, we measured the number of amides at each protein interface that were rendered solvent inaccessible upon protein-protein interaction and related this to the number of H_2O molecules released (Mandell et al. 2001). To measure the change in amide exchange, the rates of off-exchange of deuterium from backbone amides on the surface of thrombin, both free and in complex with its binding partner, were measured (Fig. 8A). The regions of thrombin where backbone amides become protected upon interaction with TM were previously identified (Mandell et al. 1998a, 2001). The thrombin-mAb interface was also previously identified (Baerga-Ortiz et al. 2002).

The amide H^2H exchange experiments showed that both the mAb and TMEGF45 protected surface amides from exchange for the length of the lifetime of the complex (Fig. 8). Although TM and the mAb compete for binding, the surface regions of thrombin that contained solvent inaccessible amides upon protein complex formation were not identical (Fig. 1). The mAb rendered amides within residues 139–149 solvent inaccessible while amides within residues 97–117

were rendered partially inaccessible. TM rendered amides within two segments of thrombin, residues 54–61 and 97–117 solvent inaccessible while amides within residues 139–149 were rendered only partially inaccessible. The kinetic plot for off-exchange of deuterium from residues 139–149 for the thrombin-mAb complex is shown in Figure 8B. For the thrombin-mAb interaction, this region contained the most slowly exchanging amides. Residues 54–61 contained one inaccessible amide in the thrombin-TM complex (Fig. 7C). Residues 97–117 were highly protected from amide exchange in the thrombin-TM complex (Fig. 7D).

The number of solvent-inaccessible amides in both complexes were obtained from the exponential fits of the off-exchange plots of data from experiments performed at pH 7.9 (Fig. 8B–D). Considering only amides with exchange rates at the interface that are lower than 0.1 min^{-1} , the number of solvent-inaccessible amides at each protein-protein interface were determined (Table 2). To relate the number of solvent-inaccessible amides to the number of H_2O molecules that may have been released into the bulk, the hydration shell around thrombin was modeled. After each of five segments of 0.5 psec of dynamics, the structure was minimized, and the H_2O molecules within 4 Å, which encompasses the first hydration shell, were enumerated (Garcia and Hummer 2000). Then, the number of H_2O molecules associated with each region of thrombin was multiplied by the fraction of amides that were protected over the total number of amides that exchanged with deuterium. This calculation resulted in an estimate of the number of H_2O molecules released from each interface (Table 2). In the thrombin-mAb interface, one amide was excluded and this predicted the release of six H_2O molecules. Swaminathan et al. (1999) have reported similar numbers of H_2O molecules released from other antibody combining sites using a coupled osmotic stress and ITC measurement. In the thrombin-TM interface, four amides were excluded and these predicted 27 H_2O molecules released.

Discussion

Thermodynamic contributions to binding free energy

Both TM and the mAb bind to thrombin with similar binding affinities at 298 K and physiological ionic strength, $1.9 \pm 0.5 \text{ nM}$ versus $1.6 \pm 0.3 \text{ nM}$. Although these two thrombin-binding proteins compete for the same site, the interactions are driven by very different thermodynamic forces. The thrombin-TM interaction is rapidly reversible, while the thrombin-mAb interaction is slow and essentially irreversible. The majority of ΔG_{bind} for the thrombin-mAb interaction is due to a favorable ΔH at 298 K, whereas the thrombin-TM interaction has a negligible observable contribution from ΔH , and must be entropy driven (Table 3). Several previous reports show that it is common that anti-

Figure 8. Time courses for the retention of deuterium at the interface regions of thrombin reveal areas with solvent inaccessible amides. (A) An example of the raw data used to calculate the number of amides from which interface H_2O molecules were released. (B) Kinetic plots showing the retention of deuterium on amides within residues 139–149 of thrombin alone (filled squares) compared to thrombin bound to the mAb (filled circles). (C) Kinetic plots showing the retention of deuterium on amides within residues 54–61 of thrombin alone (filled squares) compared to thrombin bound to TM (filled circles). (D) Same as C, but for residues 97–117 of thrombin. Data were fit to biexponential or triexponential models as required.

body–antigen interactions are enthalpy driven (Murphy et al. 1993; Shick et al. 1997; Pierce et al. 1999). The fact that the thrombin–TM interaction shows no ΔH of interaction is also not unique (Baker and Murphy 1997).

Because the ΔG_{bind} for the thrombin–TM interaction could not be accounted for by favorable ΔH , we sought to identify contributions to a favorable ΔS of binding. These are expected to include an increase in configurational entropy resulting from increased backbone or side chain mobility in the protein–protein complex, electrostatic steering, and release of solvent water molecules from the protein surface into the bulk. We were not able to accurately measure the change in configurational entropy upon binding; however, NMR evidence points to an ordering, and therefore, an unfavorable configurational entropy change upon TM–thrombin binding (Zídek et al. 1999; Wood et al. 2000). A linearized model was used to estimate the contribution of electrostatic steering to the overall binding free energy model, the electrostatic steering contribution was estimated to be -0.6 kcal/mole for the thrombin–mAb in-

teraction and -3.3 kcal/mole for the thrombin–TM interaction (Janin 1997). Thus, electrostatic steering clearly contributes to the favorable ΔG_{bind} of both interactions, but more strongly to the thrombin–TM interaction (Table 3).

A third favorable component of the overall entropy of binding may be the return of surface H_2O molecules into the bulk solution (Brady and Sharp 1997a,b). We obtained an estimate of the number of H_2O molecules released from the number of amides at each protein interface that were rendered solvent inaccessible upon protein–protein interaction. The entropy gain for release of a single H_2O molecule has been measured at -0.69 ± 0.48 (Habermann and Murphy 1996). This value is in agreement with other estimates of between -0.2 to -0.7 kcal/mole of favorable ΔG_{bind} for each H_2O molecule, and it is expected that there will be a range of values because each H_2O molecule will be different. Nevertheless, estimating the contribution to the ΔG_{bind} for each H_2O at -0.69 kcal/mole gives a ΔG_{bind} of the thrombin–mAb interaction is approx. -4 kcal/mole, essentially accounting for the remaining binding energy required. The

Table 2. Analysis of amides excluded at the interface

Region	Total amides	Number fast	Number intermediate	Number slow	Total H ₂ O	Excluded H ₂ O
A. Thrombin-mAb interaction						
97-117						
Thrombin	20	12.8	0	2.6		
mAb	20	12.8	0	2.6		
Interfacial excluded amides				0	118 ± 4	0
139-149						
Thrombin	9	5.5	0	1.1		
mAb	9	4.5	0	2.3		
Interfacial excluded amides				1	52 ± 1	6 ± 1
B. Thrombin-TM interaction						
54-61						
Thrombin	6	4.7	0	1.3		
TM45	6	3.9	0	2.1		
Interfacial excluded amides				1	58 ± 1	9 ± 1
97-117						
Thrombin	20	12.8	0	2.6		
TM45	20	5.4	4.3	5.6		
Interfacial excluded amides				3	118 ± 4	18 ± 1

contribution to the ΔG_{bind} of the thrombin-TM interaction is -18.6 kcal/mole, which would be more than enough to account for the remaining ΔG_{bind} required (Table 3). The experimental measurement carries with it a high error, but regardless of the exact value for the $\Delta G_{\text{bind}}/\text{H}_2\text{O}$, it can be seen that the major contribution to ΔG_{bind} for the thrombin-TM interaction appears to be from H_2O release.

Is there a functional link between binding kinetics and thermodynamic driving forces?

The two thrombin-binding interactions studied here have very different functions. The thrombin-TM interaction needs to be rapid and reversible, while the thrombin-mAb interaction needs to be highly specific and irreversible. The functional difference is reflected in the binding kinetics. The reversible interaction (fast k_d) is required to have a rapid k_a to achieve tight binding, while the irreversible interaction has an optimized k_d . These kinetic differences are not seen

in the overall equilibrium constant, or in the ΔG_{bind} . The kinetic differences appear to be reflected in the degree to which the interaction is driven by enthalpy. Theoretical studies point out that highly specific interactions are expected to have smooth, funneled energy landscapes, while proteins that bind more than one target have broader recognition specificity and a rugged funnel (Tsai et al. 1999; Shoemaker et al. 2000). Studies of antibody antigen interactions show a strong dependence of the k_d on temperature (Eyring analysis), suggesting that the microscopic events that result in favorable ΔH are rate limiting for the dissociation reaction and take place once the complex is formed (Zeder-Lutz et al. 1997; Day et al. 2002). It makes sense, then, that interactions with a slow k_d will be enthalpy driven. On the other hand, rapidly reversible interactions, if they are to achieve tight binding, must evolve rapid k_a s. The thrombin-TM interaction association has significant electrostatic steering, which contributes to a favorable entropy of binding. The largest contribution to the favorable ΔG_{bind} , however, appears to be release of H_2O molecules from the interface. It will be interesting to see if rapidly reversible protein-protein interactions are generally driven by entropy, and whether release of H_2O molecules is generally a dominant contribution to ΔG_{bind} in these interactions.

Materials and methods

Proteins

The mouse monoclonal antibody against human thrombin (herein referred to as mAb) first reported by Dawes et al. (1984), was obtained from Haematologic Technologies and used without fur-

Table 3. Contributions to ΔG

Contribution (kcal/mole)	TM	mAb
ΔH	0 ± 1.0	-8.2 ± 0.9
Electrostatic steering	-3.3 ± 0.1	-0.6 ± 0.06
H_2O release ^a	-18.6 ± 13	-4 ± 2.9
ΔG_{sum}	-21.9 ± 14	-12.8 ± 3.9
ΔG_{bind}	-11.9 ± 3	-12.0 ± 2

^a For H_2O release, we report an estimate based on a value of -0.69 ± 0.48 kcal/mole · H_2O (Habermann and Murphy 1996) multiplied by the number of excluded H_2O molecules (from Table 2).

ther purification. The fully active thrombomodulin fragments, TMEGF45 (used for the mass spectrometry experiments and ITC experiments) and TMEGF456 (used for all Biacore experiments) were expressed in *Pichia pastoris* yeast as previously described (White et al. 1995). Both proteins were first purified by anion-exchange chromatography (QAE Sephadex followed by HiLoad 26/10 Q Sepharose) followed by HiLoad 16/60 Superdex 75 size-exclusion chromatography (Pharmacia Biotech). TMEGF45 was further purified and desalted by reverse-phase HPLC as described, and TMEGF456 was exchanged into water by centrifugation with a Centricon-10 filter (Millipore Corp.; Wood and Komives 1999). Both TMEGF45 and TMEGF456 protect the same regions of thrombin because the sixth domain does not contact thrombin (Fuentes-Prior et al. 2000; Mandell 2000). TMEGF45 has a k_d of 0.37 sec^{-1} , and as a result, binds thrombin 10-fold less tightly than TMEGF456 (Baerga-Ortiz 2002). TMEGF456 is used in Biacore experiments because the slower k_d enables more accurate kinetic determinations. Because TMEGF456 aggregates, experiments such as ITC and mass spectrometry that require high concentrations of protein, were performed with TMEGF45. Differences in binding affinities were corrected for in each experiment, and we have previously shown that the thermodynamics of binding of TMEGF456 and of TMEGF45 to thrombin are qualitatively similar (Vindigni et al. 1997).

Human thrombin was purified from plasma barium citrate eluate purchased from Sigma according to the method of Ni et al. (1990) and purified by chromatography on a MonoS 10/10 column using a gradient of 100 mM to 500 mM NaCl in NaKPO4 buffer, pH 6.5 (Ni et al. 1990). Thrombin eluted from the column at approx. 250 mM NaCl, and was concentrated to 1.5 mg/mL and stored in small portions at -70°C . Active thrombin was stored frozen at -70°C for less than 3 weeks before use. For Biacore, thrombin was thawed, treated with PPACK, purified by chromatography on a Mono S 10/10 column as described above, stored at -70°C , and used within 1 week. For mass spectrometry, thrombin was exchanged into 12.5 mM potassium phosphate buffer, 25 mM NaCl, pH 6.5, using a Centricon-10 filter. Aliquots containing 435 pmole of thrombin, 375 mM K_2PO_4 , and 750 mM NaCl were lyophilized and stored at -20°C until use. TMEGF45 aliquots contained 3.18 nM.

Protein concentrations were determined by the bicinchoninic (BCA) assay (Pierce) and spectrophotometrically using an $\epsilon_{280} = 74,140 \text{ M}^{-1} \text{ cm}^{-1}$ for human and bovine thrombin, $\epsilon_{280} = 204,500 \text{ M}^{-1} \text{ cm}^{-1}$ for mAb and $\epsilon_{280} = 3840 \text{ M}^{-1} \text{ cm}^{-1}$ for TMEGF45.

Measurement of binding kinetics

Surface plasmon resonance experiments were performed using a BIACORE 3000 surface plasmon resonance instrument (Biacore, Inc.) as described in detail elsewhere (Baerga-Ortiz et al. 2000). Biotin-labeled TMEGF456 (570 response units) was coupled to an SA (streptavidin) sensor chip on a BIACORE 3000 instrument. For the TMEGF456 experiments, the blank channel was biotin treated. All sensorgrams shown are subtractions of data from a blank channel subtracted from a TMEGF456 channel. The mAb (1 mg/mL, 50 mM NaBorate pH 9.0, 3 M NaCl flowed to attain 300 response units on the surface) was covalently linked to protein G that was first covalently linked to a CM5 chip using ethyl diethyl carbodiimide: N-hydroxysuccinimide (EDC/NHS) coupling according to manufacturer's instructions. The mAb was crosslinked to the protein G using 20 mM dimethylpimelimidate (Pierce Chemicals, in 50 mM NaH_2BO_3 pH 9.0, 3 M NaCl) injected for 30 min at a flow rate of $5 \mu\text{L}/\text{min}$. The cross-linking reaction was quenched with 1 M ethanolamine pH 8.0 and the noncovalently linked mAb was

removed from the surface by washing with 10 mM Glycine pH 1.7. For the mAb experiments, the blank channel also contained protein G coupled at the same levels, and sensorgrams were data from a protein G channel subtracted from data from a mAb channel.

Sensorgrams were collected for PPACK-thrombin (0.78 nM, 1.56 nM, 3.125 nM, 6.25 nM, 12.5 nM, 25 nM prepared by serial dilution) in 10 mM HEPES buffer, 150 mM NaCl, 2.5 mM CaCl_2 (pH 7.4). The thrombin-TMEGF456 experiments were carried out at a flow rate of $100 \mu\text{L}/\text{min}$ and at a sampling rate of 5 Hz on a BIACORE 3000. The thrombin-mAb experiments were carried out at a flow rate of $20 \mu\text{L}/\text{min}$ and the same sampling rate. No surface regeneration was required for the thrombin-TMEGF456 interaction, but due to the slow dissociation of the thrombin-mAb complex, regeneration of the surface was accomplished by injection of 10 mM glycine, pH 2.0 for 30 sec. A typical experiment used the six thrombin concentrations with a regeneration step in between and regeneration was assessed as complete in each case by the fact that the baseline returned to zero (see Fig. 2A or Fig. 4 for baseline assessment). Rate constants for association (k_a) and dissociation (k_d) and the dissociation constant (K_D) were obtained by globally fitting the data from five or six injections of different concentrations of thrombin using the BIAevaluation software version 3.0 using the simple 1 : 1 Langmuir binding model. Statistical analysis of the curve fits or both dissociation and association phases of the sensorgrams show low χ^2 values (Table 1).

For the temperature dependence studies, the same surfaces were used but the temperature was set at different values within the BIACORE range of 279 K to 313 K and allowed to equilibrate overnight. For each experiment, at least two independent sets of experiments were performed, and the average of the measured values (K_D for the van't Hoff analysis, and k_a or k_d for the Eyring analysis) and the standard deviation of the mean was determined. The data from the 279 K study of the thrombin-mAb interaction was not used in the final data analysis because the binding became so slow that global fitting of the data resulted in an overestimate of the R_{max} (Table 1).

Studies of the ionic strength dependence of the interaction were carried out by equilibrating the instrument overnight in identical buffers with varying concentrations of NaCl (from 100–300 mM) as described previously (Baerga-Ortiz et al. 2000). In the previous studies, we also showed that the thrombin-TMEGF456 interaction showed identical ionic strength variation whether the salt added was NaCl or $(\text{CH}_3)_4\text{NCl}$, demonstrating a general ionic strength dependence and not a specific cation effect. For each experiment, at least two independent sets of experiments were performed, and the average of the measured values of k_a from global fitting were used in the Debye-Huckel analysis.

Isothermal titration calorimetry

Experiments were carried out using an MCS ITC unit (Microcal Inc.). Samples were prepared by dialyzing all interacting components extensively into 150 mM NaCl, 25 mM KH_2PO_4 (pH 6.5). Titrations were performed as described elsewhere (Wiseman et al. 1989; Ladbury and Chowdhry 1996). In a typical experiment 19 injections of $15 \mu\text{L}$ each of human or bovine thrombin (70 μM), spaced 300 sec apart, were made into mAb (3 μM) or TMEGF45 (9 μM), respectively, in the cell. Heats of dilution of the human and bovine thrombin into buffer were determined in separate experiments and subtracted prior to the analysis of the titration. The data were analyzed using the ORIGIN software supplied with the instrument according to a single binding site model. The antibody binding sites were assumed to be independent and an effective

concentration of antibody binding sites was assumed. Due to the high affinity of the thrombin–mAb complex and the slow aggregation of the mAb within the ITC instrument K_{obs} could not be obtained with high certainty. The concentration of active antibody was therefore adjusted based on a 1 : 1 stoichiometry of interaction. However, this does not affect the value of the ΔH determined, which is dependent only on the concentration of thrombin in the syringe.

Mass spectrometry

Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager DE STR (PE Biosystems). Data were acquired as described elsewhere (Mandell et al. 1998b). All reported masses are theoretical monoisotopic MH^+ masses unless otherwise noted. The matrix used was 5 mg/mL α -cyano-4-hydroxycinnamic acid (Sigma), in a solution containing 1 : 1 : 1 acetonitrile, ethanol, and 0.1% TFA and was adjusted to pH 2.2 with 2% TFA using an InLab 423 pH electrode (Mettler Toledo). The identification of each peptide in the digest mixture was described previously (Mandell et al. 1998a).

H^2H exchange experiments for the mAb–thrombin interaction were carried out using mAb that was coupled to a solid support as described elsewhere (Baerga-Ortiz et al. 2002). In these experiments, 750 pmole of thrombin were resuspended from the solid state in 3 μL of D_2O . The resuspended sample had total concentration of 50 mM NaH_2PO_4 /20 mM Tris pH 7.9 and 50 mM NaCl and was allowed to exchange with D_2O for 10 min. The mAb beads were washed in D_2O 50 mM NaH_2PO_4 pH 6.5 to a final volume of 27 μL . After 10 min the deuterated mAb beads were mixed with the deuterated thrombin in a total volume of 30 μL , and the complex was allowed to form for another 10 min. The complexes were allowed to back exchange with water by adding 270 μL of water. During the back exchange time, the samples were centrifuged for 20 sec, decanted, and another 270 μL of H_2O was added and the sample was decanted to 30 μL . After back exchange times of 0, 1, 2, 5, 10, 20, and 30 min, the reaction was quenched and the thrombin eluted from the mAb beads by adding 30 μL of a solution containing 1 : 1 (0.1% TFA pH 2.25 : 1-propanol). The mAb beads were spun down and discarded, while the supernatant containing the thrombin was mixed with 100 μL containing 50 μL of immobilized pepsin for 5 min. After pepsin digestion, the immobilized pepsin was removed by centrifugation and the supernatant containing the peptic fragments of thrombin was flash frozen in liquid nitrogen and saved in the -70°C freezer.

Controls without the mAb were subjected to the same treatment as the mAb samples. Thrombin was again suspended in 3 μL D_2O for 20 min and back exchange was done by adding 30 μL of water for the same amounts of time as the mAb samples. A solution of 1 : 1 (0.1% TFA pH 2.5 : 1-propanol) 30 μL , was mixed with the 30 μL of thrombin solution and 30 μL of this mixture was mixed with 100 μL of pepsin. The rest of the experiment was carried out in exactly the same way for the controls as for the mAb sample.

The way in which the thrombin–TMEGF45 amide H^2H exchange measurements were obtained and analyzed is described in detail in Mandell et al. (2001). The two lyophilized proteins (with buffer) were each dissolved in 6 μL of D_2O containing 25 mM Tris base (for pH 7.9) and allowed to exchange for 8 min. The two protein solutions were mixed and allowed to complex for 2 min, and then diluted 10 fold into H_2O for off-exchange. Control samples contained the same amount of thrombin in 12 μL of D_2O . After varying times, the off-exchange reaction was quenched by addition of 2% TFA to a final pH of 2.2, rapidly chilled to 0°C , and

digested with immobilized pepsin. The digested mixture was frozen in liquid N_2 until analysis by MALDI-TOF the next day.

Frozen samples were quickly (<30 sec) defrosted to 0°C , mixed 1 : 1 with 0°C matrix, and 0.5 μL was spotted onto a chilled MALDI target. The target was vacuum dried in ~ 1 min, transferred as quickly as possible (<10 sec) to the mass spectrometer, and analyzed. Data were collected for 256 scans over 256 sec (Mandell et al. 1998b). Mass spectra were calibrated and the average mass of a peptide was calculated by determining the centroid of its isotopic envelope as previously described (Mandell et al. 1998b). The difference between the average masses of the deuterated and nondeuterated peptide gave the number of deuterons incorporated. A back-exchange correction factor based on the amount of deuterium of solvent-accessible peptides after long times was applied to all data. Kinetic data were fit to multiexponential models implemented in Kaleidagraph 3.0 (Synergy Software, Inc.; Mandell et al. 2001). A three-exponential model was required for the region from 97–117 because some amides showed intermediately slowed exchange rates on the order of 0.5 min^{-1} and others were essentially inaccessible with exchange with rates lower than 0.1 min^{-1} . A two-exponential model was used to fit the other curves.

Molecular modeling of solvent association

The simulation of the water molecules that contact thrombin was carried out using Insight II version 98 (Accelrys). The crystal structure of free thrombin was soaked with a hydration shell of 5 Å. The soaked model was then subjected to 2.5 psec of molecular dynamics at 298 K. During the dynamics simulation, energy minimizations of 10,000 steepest descents were carried out every 0.5 psec and a snapshot of the soaked model was saved at the same time interval. The water molecules contacting a region of thrombin were defined as those within 4 Å of the amino acid stretch that defines the region. These water molecules were counted and averaged over the five snapshots taken during the simulation.

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