Thrombin–thrombomodulin interaction: energetics and potential role of water as an allosteric effector

Raimondo DE CRISTOFARO,* Matilde PICOZZI, Erica DE CANDIA, Bianca ROCCA and Raffaele LANDOLFI

Hemostasis Research Centre, Department of Internal Medicine, Catholic University, Largo F. Vito 1, 00168 Rome, Italy

The interaction of rabbit lung thrombomodulin (TM) and C-terminal hirudin 54–65 fragment (Hir_{54–65}) with human α -thrombin were investigated by exploiting their competitive inhibition of thrombin–fibrinogen interaction. Measurements of K_i values for TM and Hir_{54–65} interactions with human α -thrombin performed over a temperature range spanning from 10 to 40 °C showed a constant enthalpy for both ligands. The enthalpic and entropic contributions to the free energy of binding, however, are different for TM and the hirudin peptide. The calculated values of ΔH and ΔS , in fact, were -47.3 ± 2.51 kJ (-11.3 ± 0.6 kcal)/mol and -42.7 ± 7.9 J (-10.2 ± 1.9 cal)/mol·K for the hirudin peptide, while being -22.9 ± 2.09 kJ (-5.47 ± 0.5 kcal)/mol and 102.50 ± 6.69 J (24.5 ± 1.6 cal)/mol·K respectively for TM binding. These findings indicate that the interaction between thrombin and Hir_{54–65} is largely driven by the enthalpic contribution,

whereas the positive entropy change is the driving force for the formation of the thrombin–TM complex. In other experiments performed in the presence of various concentrations of either sorbitol or sucrose it could be demonstrated that the value of the equilibrium association constant for thrombin–TM interaction increases as a function of the osmotic pressure, while the thrombin–Hir_{54–65} interaction was not affected by the same conditions. Moreover, control experiments showed that no major conformational changes are produced on TM by osmotic pressures used in the present study. From these experiments it was calculated that roughly 35 water molecules are released into the bulk water upon TM binding. Such a phenomenon, which is likely to be responsible for the entropic change described above, indicates the relevance of hydration processes for the formation of the thrombin–TM adduct.

INTRODUCTION

Thrombin is a serine proteinase that plays a central role in many physiological systems, such as the blood-coagulation cascade, the process of platelet activation and the control of mitogenic and chemotactic responses of different cell lines [1–3].

Many functional and structural studies in the past few years have strongly suggested that the thrombin molecule can undergo structural transitions linked to binding of different ligands [4–6].

Recently, evidence of allosteric changes in the α -thrombin molecule have also been derived from thermodynamic data concerning the interaction of the enzyme with many synthetic substrates and reversible competitive inhibitors [6–8].

It is noteworthy that thrombin capacity to undergo conformational changes parallels the specificity constant for the hydrolysis of macromolecular substrates such as fibrinogen and the cleavable platelet receptor [8]. It was also demonstrated that the allosteric transition arises from a thermodynamic linkage between the ligation of the catalytic site (CS) and that of an exosite referred to as the fibrinogen recognition site (FRS), centred roughly 3 nm (30 Å) away from CS [6,8,9]. From all these findings it emerges that a concerted conformational change of CS and FRS could regulate the specificity of thrombin interaction with different substrates, inhibitors and physiological modulators [6,8].

It is well known that thrombin interaction with the endothelial membrane glycoprotein named thrombomodulin (TM) causes a

drastic functional change leading to an enhanced hydrolytic competence of thrombin toward the potent anticoagulant Protein C [4,10].

TM is a single polypeptide chain consisting of five domains: an N-terminal ‘lectin-like’ domain, six epidermal-growth-factor-like (EGF-like) repeats, the glycosylation sites, the transmembrane domain and a cytoplasmic portion [11–13]. The interaction of TM with thrombin involves not only FRS but also additional segments of the thrombin molecule as well [14]. Namely, it has been demonstrated that the EGF-like domains 5–6 of TM interact with thrombin at FRS. Recently, the crystal structure of Phe-Pro-Arg- α -thrombin with a nonadecapeptide from the fifth EGF-like domain of human TM has been determined at 0.3 nm (3 Å) resolution [15], confirming that EGF 5 binds to FRS. On the other hand, EGF 4 domain, responsible for the enhanced specificity for Protein C hydrolysis by α -thrombin, binds to different thrombin exosites [16].

Recent studies seem to indicate the module structures around the active site of thrombin as possible candidates for this additional TM binding site [14]. It was demonstrated that there are two distinct sites in the catalytic groove of α -thrombin, which change their conformation upon TM binding: one in the proximity of the active-site Ser-195 and the other located at roughly 1.5 nm (15 Å) away from Ser-195. The change in the latter site is presumably induced by interaction with TM EGF 4 domain [14,16].

The change in thrombin’s capacity to hydrolyse Protein C

Abbreviations used: CS, catalytic site; EGF-like, epidermal-growth-factor-like; FRS, fibrinogen recognition site; Hir_{54–65}, C-terminal hirudin peptide 54–65 (Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln); NPGB, nitrophenyl guanidinobenzoate; PEG, poly(ethylene glycol); P₁, P₂ and P₃ are the substrate residues N-terminal to the scissile peptide bond; TM, thrombomodulin (numbering of thrombin residues was done according to the chymotrypsinogen nomenclature [1]).

*To whom correspondence should be addressed.

seems to be related to the alleviation of the inhibitory influence exerted by Asp residues at the P3 and P3' positions in the Protein C molecule. Namely, it was demonstrated that Glu-192 and to a minor extent Glu-39 have an unfavourable interaction with substrates having acidic residues at the P3 and P3' positions [17–20]. When TM binds to thrombin, the negative influence of these acidic residues is cancelled out through a conformational change of the thrombin molecule [21].

The present study was aimed at investigating the energetics of the thrombin–TM interaction. The thermodynamics of thrombin–TM interaction was also compared with that pertaining to the interaction of thrombin with the C-terminal hirudin peptide 54–65 (Hir_{54–65}). This molecule binds solely to FRS and does not cause an enhancement of thrombin activity toward Protein C. We placed a particular emphasis on evaluating the possible role of water as an allosteric effector linked to TM binding and thus to the process of Protein C activation by human α -thrombin.

MATERIALS AND METHODS

Materials

Sucrose, Hepes, D-sorbitol, nitrophenyl guanidinobenzoate hydrochloride (NPGB), and BSA (fatty acid-free) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lubrol was from ICN Biomedicals. TM from rabbit lung was purchased from American Diagnostica (Greenwich, CT, U.S.A.). Purified fibrinogen [plasmin(ogen)-free] was from Calbiochem Biochemicals (San Diego, CA, U.S.A.).

Purification of proteins

Human α -thrombin was purified and characterized as previously reported [22]. SDS/PAGE showed that the purified material consisted only of α -thrombin, without any contamination by autolysed forms of the enzyme. The active-site titration with NPGB showed that the enzyme was 100% active.

TM was free from other contaminating proteins as judged by SDS/PAGE. Its concentration was measured spectrophotometrically using an absorption coefficient ($A_{280,1\text{ cm}}^{0.1\%}$) of 0.88 [21].

Measurement of K_i for thrombin–TM and thrombin–Hir_{54–65} interactions

The equilibrium dissociation constant, K_i , of both TM and Hir_{54–65} binding to human α -thrombin was measured by exploiting the competitive inhibition effects of these ligands on thrombin–fibrinogen interaction, evaluated by the clotting curve. The theoretical aspects of this experimental approach were previously detailed [23]. Accordingly, the apparent clotting time, t_c , measured as a function of the competitive inhibitors, gives the following relationship:

$$t_c = t_\infty + \alpha(K_m/V_{\max})[1 + (I/K_i)] \quad (1)$$

where t_∞ is the clotting time at infinite thrombin concentration, α is a constant, I is the competitive-inhibitor concentration and K_i is the equilibrium dissociation constant of inhibitor binding to thrombin. The value of t_∞ was preliminarily derived from measurements of t_c as a function of thrombin concentration under the same experimental conditions, as previously detailed [23].

In the case of Hir_{54–65}, the apparent t_c was linearly correlated with the total inhibitor concentration, whereas for TM this is not so, since the clotting curve was performed under experimental

conditions where the binding of TM to α -thrombin caused a significant decrease of free TM concentration [24,25]. The clotting data for TM were thus fitted to a modified version of eqn. (1) in which the free TM concentration, instead of the total inhibitor concentration, was introduced into the equation [24] as follows:

$$I = [Q + \sqrt{(Q^2 + 4K_i I_T)}]/2 \quad (2)$$

where I and I_T are the free and total TM concentrations and $Q = I_T - e_T - K_i$ [24] (e_T is total enzyme concentration).

In a typical experiment the clotting curve was measured at 350 nm using a Cary 2200 spectrophotometer, thermostatically controlled by means of a Haake bath thermostat. Thrombin was used over the concentration range 0.25–10 nM under the solution conditions of 10 mM Hepes, 0.15 M NaCl, 0.1% poly(ethylene glycol) (PEG), 0.1% BSA, pH 7.50, at all studied temperatures. For the experiments using TM, 0.01% Lubrol was added to the above buffer. Assays were initiated by adding 50 μ l of thrombin to 1.15 ml of a solution containing fibrinogen at concentrations ranging from 0.3 to 0.4 μ M and different concentrations of the inhibitors. TM was used at concentrations ranging from 0.125 to 8 nM, whereas Hir_{54–65} was used at concentrations over the range 62.5 nM–4 μ M.

The values of K_i for the thrombin–TM and thrombin–Hir_{54–65} interactions, measured over the temperature range 10–40 °C, were then fitted to the van't Hoff equation to compute the values of enthalpy and entropy of binding. All the calculations were carried out by the software SigmaPlot (Jandel Scientific).

Osmotic-stress experiments

The osmotic pressure of the solution was changed by adding to buffer solutions neutral solutes such as sorbitol and sucrose at concentrations ranging from 0.1 to 0.8 M. The solution osmolality was measured by using an OSMOSTAT OM-6020 osmometer (Kagaku CO., Ltd., Kyoto, Japan).

The use of osmotic stress method to study the influence of water activity on the energetics of intermolecular reactions is described in detail in a large number of papers (see, for example [26–28]) and will not be discussed here. Briefly, the values of K_i for the thrombin–TM interaction were analysed as a function of osmotic pressure of the solution as follows [27]:

$$\ln K_i^{\text{app}} = \ln K_i^\circ + (\Pi_{\text{osm}} V_w / RT) \quad (3)$$

where K_i^{app} and K_i° are the equilibrium dissociation constants for TM binding, measured at osmotic pressures $\Pi \neq 0$ and $\Pi = 0$ respectively, V_w is the molar water volume associated to TM binding, R is the gas constant and T is the absolute temperature. The use of two different neutral solutes as sorbitol and sucrose allowed us to rule out the possibility that the observed effects were due to direct solute binding. In fact, osmotic effects should be insensitive to the chemical nature of the solute [27], whereas direct solute binding strictly depends on its own chemical structure. In addition, in the case of real osmotic effects, a plot of $\ln K_i$ as a function of Π_{osm} should give a straight line whose slope is equal to:

$$d \ln K_i / d \Pi_{\text{osm}} = d V_w / RT \quad (4)$$

Taking the partial molar volume of water as 18 ml $\cdot$ mol^{−1}, it was possible from eqn. (4) to calculate the number of water molecules linked (uptaken or released) to molecular interactions.

A possible sorbitol and sucrose effect on TM structure, monitored by its intrinsic fluorescence, was also evaluated. Briefly, a quartz cuvette containing 2 ml of 100 nM TM solution in 10 mM Hepes/0.15 M NaCl/0.1% PEG 6000/0.01% Lubrol, pH 7.50, at 0, 0.5 and 0.8 M sorbitol and sucrose, was placed at

25 °C in the cell holder of a Perkin–Elmer LS5B spectrofluorimeter. The excitation and emission wavelengths were set at 280 and 340 nm respectively, and 5 and 10 nm slit widths were used for excitation and emission beams. At each co-solvent concentration the fluorescence was zeroed using the same solution without TM.

RESULTS

Energetics of thrombin-TM and thrombin-hirudin peptide interactions

The effects of TM and Hir_{54–65} on fibrinogen clotting time are shown in Figures 1 and 2. In the case of TM, the clotting time is not linearly correlated with the total inhibitor concentration, the tight binding being responsible for a change in the free TM concentration. However, the simultaneous use of eqns. (1) and (2) permitted us to analyse correctly the experimental data

Figure 1 Inhibition of fibrinogen clotting by the C-terminal Hir_{54–65} under the experimental conditions of 10 mM Hepes, 0.15 M NaCl, 0.1% PEG, 0.1% BSA, pH 7.50 at 25 °C

The fibrinogen concentration was 0.4 μM. The straight line is drawn according to eqn. (1) with the best-fit parameter values: $K_i = 1.02 \pm 0.11 \mu\text{M}$; intercept = 20.7 ± 1.4 s. The value of t_∞ was preliminarily calculated by the intercept of the plot of t_c versus the inverse of thrombin concentration, as described in the text.

Figure 2 Inhibition of fibrinogen clotting by TM under the experimental conditions of 10 mM Hepes, 0.15 M NaCl, 0.1% PEG, 0.1% BSA, pH 7.50 at 25 °C

The fibrinogen concentration was 0.3 μM. TM concentration refers to the total inhibitor concentration. The analytical expression of the free concentration was provided by fitting the data to eqn. (2). The continuous line was drawn according to eqns. (1) and (2) with the best-fit parameter values: $K_i = 0.39 \pm 0.07$ nM; intercept = 16.2 ± 2.55 s.

Figure 3 Analysis of the experimental values of K_i of Hir_{54–65} binding to human α -thrombin as a function of temperature

The experimental data were fitted to the van't Hoff equation with the best fit ΔH and ΔS values equal to -47.3 ± 2.51 kJ (-11.3 ± 0.6 kcal)/mol and -42.7 ± 7.9 J (-10.2 ± 1.9 cal)/mol K respectively.

Figure 4 Analysis of the experimental values of K_i of TM binding to human α -thrombin as a function of temperature

The experimental data were fitted to the van't Hoff equation with the best fit ΔH and ΔS values equal to -22.9 ± 2.09 kJ (-5.47 ± 0.5 kcal)/mol and 102.50 ± 6.69 J (24.5 ± 1.6 cal)/mol K respectively.

pertaining to the inhibitory effect of TM. The clotting-time method provided a simple and reliable tool to study these interactions under different experimental conditions.

Measurements of K_i values for both TM and Hir_{54–65} as a function of temperature indicated that the enthalpy is constant over the temperature range 10–40 °C. The enthalpic and entropic contributions to the free energy of binding are different for TM and the hirudin peptide, as emerged from a van't Hoff plot of data shown in Figures 3 and 4. The calculated values of ΔH were -47.2 ± 2.51 kJ (-11.3 ± 0.6 kcal)/mol and -22.9 ± 2.09 kJ (-5.47 ± 0.5 kcal)/mol for the hirudin peptide and TM respectively. The value of ΔS for the hirudin peptide is -42.7 ± 7.9 J (-10.2 ± 1.9 cal)/mol·K, whereas the same parameter value for TM binding was found equal to 102.50 ± 6.69 J (24.5 ± 1.6 cal)/mol·K.

These findings indicate that the positive entropy change is the driving force for the formation of the thrombin–TM complex, whereas the enthalpic contribution mostly drives the interaction between the enzyme and the C-terminal hirudin peptide.

Figure 5 Values of K_i for the thrombin–TM interaction plotted as a function of solution osmotic pressure, changed with sorbitol, under the experimental conditions described in the text

The straight line was drawn according to eqn. (3) with the best-fit parameter values: $\ln K_i^0 = -21.7 \pm 0.05$ (M); slope = $-2.796 \pm 0.2229 \text{ kPa}^{-1}$ ($-0.0276 \pm 0.0022 \text{ atm}^{-1}$). The number of water molecules released into solution was calculated to be 37 ± 4 .

Figure 6 Values of K_i for the thrombin–TM interaction plotted as a function of solution osmotic pressure, changed with sucrose, under the experimental conditions reported in the text

The straight line was drawn according to eqn. (3) with the best-fit parameter values: $\ln K_i^0 = -21.5 \pm 0.02$ (M); slope = $-2.533 \pm 0.0088 \text{ kPa}^{-1}$ ($-0.025 \pm 0.00087 \text{ atm}^{-1}$). The calculated number of water molecules released into solution was 34 ± 1 .

Effect of osmotic pressure on the thrombin–TM interaction

As shown in Figures 5 and 6, the change of the osmotic pressure by means of sorbitol and sucrose brought about a change of the apparent affinity of the thrombin–TM interaction. Namely, the higher the osmotic pressure the higher was the affinity between the enzyme and TM. This result was obtained with two different lots of TM and different enzyme preparations. Since the relationship between K_i and osmotic pressure was linear, by using eqn. (3) it was possible to calculate the number of water molecules released into the bulk water upon the formation of the thrombin–TM complex. This number was found to be 37 ± 4 and 34 ± 1 water molecules in sorbitol and sucrose experiments respectively.

The good agreement between the two values, obtained by using different solutes, indicated that a differential hydration of the thrombin–TM complex, with respect to the unligated proteins, could be responsible for the increase of binding affinity as a function of the osmotic pressure. On the other hand, no

significant change was observed for the K_i of the thrombin–Hir_{54–65} reaction when the osmotic pressure was changed up to 3.54 MPa (35 atm.) using sorbitol or sucrose (results not shown). In addition, no significant change of the intrinsic fluorescence of TM was observed. This suggests that no apparent change occurs in environments of tryptophan residues in the TM molecule upon addition of the above cosolvents.

DISCUSSION

The study of the energetics of the thrombin–TM and thrombin–Hir_{54–65} interactions showed that, although these macromolecular ligands have a partial sharing of the same binding site on the thrombin molecule [15], the driving forces of these interactions are different. In fact, in the case of the C-terminal hirudin peptide, the enthalpic contribution to the free energy of binding is predominant, whereas the increment of entropy mostly drives the formation of the thrombin–TM complex. This fact demands a reasonable explanation.

It is obvious that, once two macromolecules such as thrombin and TM form a complex, there is a relevant loss in their translational and rotational entropy. The positive entropy change, experimentally observed [$104.6 \text{ J (25 cal)}/\text{mol} \cdot \text{K}$], should thus arise from a release of water molecules, previously ‘structured’ on to protein surfaces, into the bulk water.

One cannot obviously predict the macromolecular source (thrombin and/or TM) of these water molecules, since the thermodynamic data refer to the thrombin–TM adduct and not to the individual proteins.

The results obtained from the osmotic-stress experiments strikingly agreed with the hypothesis that water molecules are released into bulk solution upon TM binding to thrombin. The use of different solutes, such as sorbitol and sucrose, in fact gave very similar results. This demonstrated that the increase of the affinity of thrombin–TM formation as a function of the osmotic pressure is indeed specifically linked to a change of the water activity and is not due to direct solute effects [27]. In addition, control experiments showed that osmotic stress and/or direct solute effects do not seem to cause major conformational transitions in free TM. Also the analysis of the osmotic-stress-method experiments does not allow us to assign the molecular source of the water molecules released into the bulk water. These experiments, however, demonstrated that roughly 35 water molecules are released into solution, once the thrombin–TM complex is formed.

The fact that some thrombin residues likely change their orientation upon TM binding, as in the case of Glu-192 [17], might suggest that discrete areas of the thrombin molecule could change their hydration pattern [29], releasing water molecules into the bulk water.

For the sake of argument it must be noted, however, that the decrease of the K_i values for thrombin–TM interaction as a function of sorbitol and sucrose concentration could also arise from a reduction of the dielectric constant of the medium with a subsequent strengthening of the electrostatic interactions. The lack of significant effects of both sorbitol and sucrose on thrombin–Hir_{54–65} interaction, however, argue against this hypothesis.

It is noteworthy that the relevance of hydration to the energetics of molecular assembly and enzyme catalysis has been recognized for a long time [30–33]. More recently, an increasing mass of experimental data has actually demonstrated the effect of water activity on the allosteric transitions of several proteins and enzymes, such as haemoglobin, cytochrome oxidase, hexokinase and ion gate channels [27,28,34,35]. On the basis of the findings

reported in the present study, the thrombin–TM interaction could be another biochemical example, where the change of water activity strongly contributes to modulation of the binding process. Whether the binding-release cycle of water affects mostly the structure and function of either thrombin or TM is an intriguing issue which remains to be addressed.

R.D.C. gratefully acknowledges Dr. Silvia Baroni for the use of the OSMOSTAT OM-6020 osmometer. This study was financially supported in part by the grant no. 94.02636.CT04 from the National Research Council (CNR) of Italy.

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