The linkage between binding of the C-terminal domain of hirudin and amidase activity in human α -thrombin

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A method derived from the analysis of viscosity effects on the hydrolysis of the amide substrates D-phenylalanylpececolyl-arginine-*p*-nitroaniline, tosylglycylprolylarginine-*p*-nitroaniline and cyclohexylglycylalanylarginine-*p*-nitroalanine by human α -thrombin was developed to dissect the Michaelis–Menten parameters K_m and k_{cat} into the individual rate constants of the binding, acylation and deacylation reactions. This method was used to analyse the effect of the C-terminal hirudin (residues 54–65) [hir-(54–65)] domain on the binding and hydrolysis of the three substrates. The results showed that the C-terminal hir-(54–65) fragment affects only the acylation rate, which is increased approx. 1.2-fold for all the substrates. Analysis of the

dependence of acylation rate constants on hirudin-fragment concentration, allowed the determination of the equilibrium binding constant of C-terminal hir-(54–65) ($K_d \approx 0.7 \mu M$). In addition this peptide was found to competitively inhibit thrombin–fibrinogen interaction with a K_i which is in excellent agreement with the equilibrium constant derived from viscosity experiments. These results demonstrate that binding of hir-(54–65) to the fibrinogen recognition site of thrombin does not affect the equilibrium binding of amide substrates, but induces only a small increase in the acylation rate of the hydrolysis reaction.

INTRODUCTION

Hirudin is a 65-residue polypeptide inhibitor of α -thrombin [1]. It inhibits the enzyme by binding to both the catalytic site and an exosite, referred to as the fibrinogen recognition site (FRS) [2,3], which is responsible for the recognition of several macromolecules such as fibrinogen, thrombomodulin and the platelet receptor [4–6]. The 1–47-residue N-terminal domain of hirudin interacts with the catalytic site of thrombin [7], while the 54–65-residue C-terminal domain binds to the FRS and competitively inhibits the binding of fibrinogen and thrombomodulin [8–10]. Synthetic peptides, corresponding to the primary sequence of the C-terminal domain of hirudin, can interact with the FRS and inhibit binding of fibrinogen to thrombin [11,12].

The catalytic site of thrombin is still available for binding and hydrolysis of small substrates when FRS is bound by the C-terminal hirudin fragment. This fact prompted us to perform an extensive investigation to assess whether the binding of C-terminal hirudin fragment (residues 54–65) [hir-(54–65)] to human α -thrombin might affect the binding and hydrolysis of synthetic amide substrates.

Application of the thermodynamic theory of linkage to the analysis of equilibrium effects of ligands is a well-known procedure by which to quantitatively analyse the energetics of ligand binding [13]. The extension of linkage theory to macromolecular systems working at steady state has been used recently to analyse the effects of protons and ions on the amidase activity of human α -thrombin [14]. This method seemed suitable to give a quantitative description of the effects of C-terminal hir-(54–65) on the functional properties of the catalytic site of thrombin. However, in order to apply the linkage theory we cannot use the Michaelis parameters of thrombin amidase activity, e.g. k_{cat} and K_m , which are composite ratios of kinetic constants of binding, acylation and deacylation processes [15].

Rapid-mixing methods are usually required to separate the rate constants for formation and breakdown of the acyl-enzyme intermediate. However, for this kind of study two necessary requirements have to be met: (a) large quantities of highly purified enzymes must be available; and (b) the deacylation rate constant should be rate limiting in order to build up an appreciable amount of the intermediate.

Because of such limitations of rapid-mixing techniques, a theoretical and experimental strategy, derived from the analysis of viscosity effects, was developed to compute under steady-state conditions the equilibrium binding constants along with the acylation and deacylation rate constants for several amide substrates. These parameters allowed us to assess whether binding of the C-terminal hirudin fragment and binding and hydrolysis of small substrates at the catalytic site of human α -thrombin are linked. The results of the present study open the way to an extensive investigation of the modulatory role played by the FRS in the control of blood coagulation reactions and in the mechanism of platelet activation.

EXPERIMENTAL

Reagents

Plasma barium citrate eluate (containing Factors II, IX and X), *Echis carinatus* venom (ecarin), *p*-aminobenzamidine hydrochloride, *p*-nitroaniline, recombinant hirudin and its fragment hir-(54–65) (non sulphated), gelatin–agarose, lysine–agarose and glycine hydrochloride (ammonia free) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). D-Phenylalanylpececolylarginine-*p*-nitroaniline (D-Phe-Ppc-Arg-pNA; S-2238) was purchased from Kabi (Stockholm, Sweden). Tosylglycylprolylarginine-*p*-nitroaniline (Tos-Gly-Pro-Arg-pNA) was from Boehringer–Mannheim. Cyclohexylglycylalanyl-

Abbreviations used: D-Phe-Ppc-Arg-pNA, D-Phenylalanylpececolylarginine-*p*-nitroaniline; Tos-Gly-Pro-Arg-pNA; Tosylglycylprolylarginine-*p*-nitroaniline; CH-Gly-Ala-Arg-pNA, cyclohexylglycylalanylarginine-*p*-nitroaniline. FRS, fibrinogen recognition site; PEG, polyethylene glycol.

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arginine-*p*-nitroaniline (CH-Gly-Ala-Arg-pNA) was purchased from Immuno (Vienna, Austria).

Purification of α -thrombin

Barium eluate (0.5 units) was suspended in 5 ml of buffer containing 20 mM EDTA, 20 mM trisodium citrate and 20 mM NaCl, pH 7.4, (buffer 1) and dialysed for 45 min against 1 l of the same buffer. The dialysis was continued against buffer 1 without EDTA for 3 h with a change of buffer every 30 min and then continued overnight at 4 °C. The dialysed material was gel-filtered on Econo-Pac 10 DG columns (Bio-Rad, Richmond, CA, U.S.A.) equilibrated in 50 mM Tris/HCl, 0.1 M NaCl, pH 7.4, at 37 °C. Ecarin and CaCl₂ were then added to the solution (final concentrations of 0.2 mg/ml and 10 mM respectively) and the activation was allowed to proceed for 75 min. The solution was then gel-filtered in the above-mentioned columns equilibrated in 20 mM Bis-tris, 0.1 % polyethylene glycol (PEG) 6000, pH 6.5, at 25 °C (buffer 2). Purification of α -thrombin was performed by h.p.l.c. (Perkin-Elmer, model LC10) on a Bio-Gel MA7S preparative cation-exchange column. An aliquot (500 μ l) of 100 μ M crude thrombin solution was injected on the column. α -Thrombin was eluted by a gradient of 0–500 mM NaCl in buffer 2 over a period of 45 min at a flow rate of 2 ml/min. The enzyme was eluted at the end of the chromatographic run. Purity, concentration and active-site titration were carried out as previously described [16]. Thrombin solution was divided into 50 μ l aliquots at a concentration of 3 μ M and stored at –80 °C until use.

Theory of viscosity effects

The catalytic activity of thrombin is described by the classical kinetic scheme of serine proteases [15]:

where E, EP₂, S and P₂ are the free and acylated enzyme forms, the substrate and the final product respectively. The steady-state parameters, e.g. k_{cat} and K_m are given by [14]:

$$k_{\text{cat}} = \frac{k_2 k_3}{(k_2 + k_3)} \quad (1)$$

$$K_m = \frac{k_3(k_{-1} + k_2)}{k_{+1}(k_2 + k_3)} \quad (2)$$

The rate constants of diffusion-limited reactions are to a first approximation inversely proportional to the relative viscosity, η_{rel} , of the solution [17–19]. In Scheme 1 the rate constants of bimolecular processes of binding and dissociation, i.e. k_{+1} , k_{-1} and k_3 should be inversely proportional to the relative viscosity of the solution [20]. On the other hand the acylation step, that is the formation of the tetrahedral intermediate, is an intramolecularly catalysed reaction and thus k_2 should not be affected by solution viscosity. Apparent k_{cat} and K_m measured as a function of solution viscosity (k_{cat}^* , K_m^*) are indeed given by:

$$k_{\text{cat}}^* = \left(\frac{k_2 k_3}{\eta_{\text{rel}}} \right) \left(k_2 + \frac{k_3}{\eta_{\text{rel}}} \right) \quad (3)$$

$$K_m^* = \frac{\frac{k_3}{\eta_{\text{rel}}} \left(\frac{k_{-1}}{\eta_{\text{rel}}} + k_2 \right)}{\left(\frac{k_{+1}}{\eta_{\text{rel}}} \right) \left(k_2 + \frac{k_3}{\eta_{\text{rel}}} \right)} \quad (4)$$

Rearrangement of eqns. 3 and 4 produces the following equations:

$$k_{\text{cat}}^* = \frac{k_2 k_3}{(k_2 \eta_{\text{rel}} + k_3)} \quad (5)$$

$$K_m^* = \frac{k_3(k_{-1} + k_2 \eta_{\text{rel}})}{k_{+1}(k_2 \eta_{\text{rel}} + k_3)} \quad (6)$$

Eqns. (5 and 6) provide exact expressions for k_{cat} and K_m as a function of the relative solution viscosity without any assumption about the relative magnitude of k_2 and k_3 . The steady-state velocity of substrate hydrolysis, v , is indeed provided by a modified version of the Michaelis–Menten equation, as follows:

$$v = \frac{k_{\text{cat}}^* S}{K_m^* + S} \quad (7)$$

where k_{cat}^* and K_m^* are given by eqns. (5 and 6).

Interesting linear relationships may be derived from eqn. (6) if we make some assumptions about the relative magnitudes of k_2 and k_3 . If $k_2 \ll k_3$ eqn. (2) reduces to $K_m = (k_{-1} + k_2)/k_{+1}$ so that the apparent value of K_m under different solution viscosity is given by:

$$K_m^* = K_s + \left(\frac{k_2}{k_{+1}} \right) \eta_{\text{rel}} \quad (8)$$

where K_s is the equilibrium dissociation constant of substrate binding. In this case a plot of apparent K_m versus η_{rel} should provide the value of the equilibrium constant of the binding reaction. This result might be very useful in calculating the binding constant when $k_2 \ll k_3$ and only the K_m of the reaction is experimentally measured, as recently reported for thrombin–fibrinogen interaction [21]. If $k_2 \gg k_3$ then:

$$K_m^* = \left(\frac{K_s}{\eta_{\text{rel}}} \right) \left(\frac{k_3}{k_2} \right) + \frac{k_3}{k_{+1}} \quad (9)$$

It is interesting to point out that in the latter case a relationship of inverse proportionality does exist between K_m and relative viscosity. This is just the opposite of what can be observed when $k_2 \ll k_3$ and thus one could deduce very simply whether acylation or deacylation is the rate-limiting step of the hydrolysis reaction.

On the other hand the dependence of the ratio K_m/k_{cat} on the relative viscosity in all cases ($k_2 \ll k_3$ or $k_2 \gg k_3$) is given by:

$$\frac{K_m}{k_{\text{cat}}} = \frac{\eta_{\text{rel}}}{k_{+1}} + \frac{k_{-1}}{k_{+1} k_2} \quad (10)$$

as recently shown for thrombin amidase activity [22].

Amidolytic assays

Viscosity of solutions was changed by adding sucrose and viscosity was measured at 25 °C by means of a Brookfield model LVT thermostated viscometer (Stoughton, MA, U.S.A.). Sucrose was found to affect the extinction coefficient of *p*-nitroaniline at 405 nm, according to the relationship $\epsilon_{\text{obs}} = 9771 + 951 [\text{Suc}]$, where ϵ_{obs} is the observed absorption coefficient at any sucrose concentration, [Suc], in a range from 0 to 1 M and 9771 is the absorption coefficient of *p*-nitroaniline under the standard conditions (see below). The relationship was used to calculate the value of the hydrolysis rate at all sucrose concentrations. Concentration of the amide substrates was measured at 342 nm, using the absorption coefficient of 8270 M^{–1}·cm^{–1} [23]. We studied the effect of sucrose under solution conditions of 50 mM

Tris/HCl, 0.1 M NaCl, 0.1 % PEG 6000, pH 8.00, at 25 °C. Eight curves were measured, using seven substrate concentrations decreasing by a factor of 2 in a range typically from 0.8 μ M to 50 μ M. One curve was measured in the absence of sucrose and seven others were determined in the presence of sucrose concentrations scaled by a factor of 1.25, starting from 0.8 μ M. Thrombin was used at a concentration of 0.5 nM. When the effect of the hirudin fragment was studied, hirudin peptide was added in a concentration range spanning 30 nM–4 μ M. *p*-Nitroaniline release was monitored using a Varian Cary 2200 spectrophotometer and initial velocity measurements of substrate hydrolysis were calculated by using the KINETICS program of the spectrophotometer. Experimental points (56; eight curves) of each data set obtained at each hirudin fragment concentration were simultaneously fitted to eqn. (7). Minimization was accomplished by means of a non-linear least-squares method using the Marquardt method to compute all the parameters involved in eqn. (7). Confidence intervals on the parameters were computed by *F*-testing at the cutoff of one S.D. (68 %). In all cases a unique set of best-fit parameter values was obtained using different starting estimates. No systematic departure from random distribution of residuals was observed for all of the sets of data. From the values of k_{-1} and k_{+1} the equilibrium dissociation constant of substrate binding was calculated using the relation $K_s = k_{-1}/k_{+1}$.

Control experiments

Since viscosity effects were studied at high sucrose concentrations it was important to check that the observed change of k_{cat} or K_m did not arise from effects deriving from change of water activity and/or change of enzyme conformation. Control experiments were performed to calculate the K_i of competitive inhibitors of thrombin, namely *p*-aminobenzamidine and hirudin. The effect of sucrose on the inhibition constant of *p*-aminobenzamidine and hirudin was studied under solution conditions of 50 mM Tris/HCl, 0.1 M NaCl, 0.1 % PEG, pH 8.00, at 25 °C and 37 °C respectively, as reported previously [24,25]. The inhibition constants were not found to be significantly different at concentrations of 0 M and 0.8 M sucrose ($115 \pm 9 \mu$ M and $105 \pm 11 \mu$ M respectively for *p*-aminobenzamidine; 0.78 ± 0.23 pM and 0.9 ± 0.26 pM respectively for hirudin). These results demonstrate that both the association and dissociation constants are similarly affected by the change in the solution viscosity, as predicted by the theory, so that the equilibrium constant is kept constant. These experiments demonstrate that no direct sucrose effect can be held responsible for the change in the Michaelis parameters of substrate hydrolysis.

Fibrinogen experiments

Fibrinogen was purified from human plasma by means of the glycine method [26]. Contaminating fibronectin and plasmin were eliminated by affinity chromatography on gelatin-agarose and lysine-agarose columns respectively. Purity of this preparation was assessed by 4–20 % SDS/PAGE gels. Concentration of fibrinogen was calculated spectrophotometrically as reported previously [20]. The K_m of the thrombin–fibrinogen interaction was calculated by means of competition experiments at pH 8.00, at 25 °C in the above-mentioned buffer solution, using S-2238 as described [20]. The competitive effect of the hirudin fragment was analysed according to the following relationship:

$$K_m = K_m^0 (1 + [\text{Hir}-(54-65)]/K_i) \quad (11)$$

where K_m^0 is the K_m in the absence of hir-(54–65) fragment and K_i is the binding equilibrium constant of the hirudin fragment.

RESULTS

A typical set of steady-state velocity measurements plotted as a function of the sucrose concentration is shown in Figure 1 (a–c). The computational method described above (see the Experimental section) allowed the calculation of the four independent

Figure 1 Effect of viscosity on amidase activity of human α -thrombin

Hydrolysis of D-Phe-Ppc-Arg-pNA (a), Tos-Gly-Pro-Arg-pNA (b) and CH-Gly-Ala-Arg-pNA (c) as a function of relative viscosity, under experimental conditions described in the text. For each data set only four curves are shown for the sake of clarity ($\square$, $\eta_{rel} = 1$; $\triangle$, $\eta_{rel} = 1.24$; $*$, $\eta_{rel} = 1.46$; $+$, $\eta_{rel} = 2.34$). Continuous lines were drawn by using eqn. (7) and the best-fit parameter values are listed in Table 1.

Table 1 Parameters of binding and hydrolysis by human α -thrombin of amide substrates D-Phe-Ppc-Arg-pNA, Tos-Gly-Pro-Arg-pNA and CH-Gly-Ala-Arg-pNA

The values are calculated by using eqn. (7). Values of k_{+1} are given in terms of $10^{-8} \text{ M}^{-1} \cdot \text{s}^{-1}$; all the other parameters are given in s^{-1} . σ , S.D. of the fit.

Substrate	Rate constants				σ
	k_{+1}	k_{-1}	k_2	k_3	
D-Phe-Ppc-Arg-pNA	1.09 ± 0.24	311.2 ± 118.2	130.2 ± 5.6	642.1 ± 93.8	2.09
Tos-Gly-Pro-Arg-pNA	0.69 ± 0.12	219.6 ± 83.90	159.6 ± 6.6	2183.8 ± 844.1	3.14
CH-Gly-Ala-Arg-pNA	0.73 ± 0.14	380.2 ± 131.0	178.5 ± 7.5	940.5 ± 143.2	2.61

Table 3 Effect of C-terminal hir-(54–65) fragment on the equilibrium and kinetic constants calculated using eqn. (7) and involved in thrombin interaction with Tos-Gly-Pro-Arg-pNA under experimental conditions reported in the text

σ , S.E.M. of the fit.

Hir-(54–65) (μM)	K_s (μM)	k_2 (s^{-1})	k_3 (s^{-1})	σ (s^{-1})
0.031	3.31 ± 1.84	160.7 ± 6.8	2226.6 ± 881.5	3.14
0.062	3.45 ± 1.90	161.7 ± 6.6	2298.6 ± 910.9	3.21
0.125	3.32 ± 1.00	162.6 ± 4.2	1858.4 ± 322.3	2.60
0.250	3.88 ± 3.40	164.8 ± 7.0	1609.9 ± 450.3	3.83
0.500	4.13 ± 2.32	169.5 ± 7.1	2261.9 ± 860.6	3.35
1.000	3.96 ± 3.15	171.3 ± 8.9	1947.4 ± 775.0	4.20
2.000	3.11 ± 2.00	173.9 ± 7.3	2320.8 ± 885.1	3.44
4.000	3.50 ± 2.35	177.7 ± 8.9	1333.9 ± 305.1	3.76

Figure 2 Dependence of K_m of Tos-Gly-Pro-Arg-pNA hydrolysis as a function of relative solution viscosity

The values of apparent K_m of Tos-Gly-Pro-Arg-pNA as a function of relative viscosity are fitted to eqn. (8) with the best-fit parameter values: K_s , $3.39 \pm 0.32 \mu\text{M}$; k_2/k_{+1} , $1.77 \pm 0.22 \times 10^{-6}$. The S.E.M. of the fit is $\sigma = 2.46 \times 10^{-7}$ (M).

Table 2 Effect of C-terminal hir-(54–65) fragment on the equilibrium and kinetic constants calculated using eqn. (7) and involved in thrombin interaction with D-Phe-Ppo-Arg-pNA under experimental conditions reported in the text

σ , S.E.M. of the fit.

Hir-(54–65) (μM)	K_s (μM)	k_2 (s^{-1})	k_3 (s^{-1})	σ (s^{-1})
0.031	2.66 ± 1.71	126.3 ± 5.4	622.7 ± 90.7	2.02
0.062	2.96 ± 1.62	126.2 ± 4.9	539.8 ± 62.4	1.71
0.125	3.47 ± 1.92	128.0 ± 5.0	457.7 ± 44.6	1.54
0.250	2.85 ± 0.71	132.4 ± 3.6	679.1 ± 65.4	2.07
0.500	2.60 ± 1.13	134.8 ± 4.7	748.1 ± 99.1	2.02
1.000	2.56 ± 1.53	143.5 ± 6.4	648.0 ± 90.7	2.29
2.000	2.21 ± 1.12	141.9 ± 5.7	726.9 ± 103.2	2.15
4.000	2.80 ± 1.70	145.9 ± 6.3	681.8 ± 95.2	2.30

parameters (k_{+1} , k_{-1} , k_2 and k_3) involved in eqn. (7). It is noteworthy that we tried to exploit other models in which: (a) k_2 and k_3 are not affected by viscosity; (b) k_2 and k_3 are affected by

viscosity; or else (c) k_2 only is affected by viscosity. The equations derived from these models failed to fit to experimental data. These results unequivocally demonstrate the validity of the theoretical aspects expressed by eqns. (5) and (6).

Estimates of k_{+1} , the rate of formation of the ES complex, were approx. $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for all the substrates (see Table 1). This rate is typical for a diffusion-controlled bimolecular encounter [15].

For all the substrates the rate-limiting step was found to be the acylation process, although the ratio k_2/k_3 is slightly different in the three cases. This ratio was equal to $0.20 (\pm 0.04)$, $0.07 (\pm 0.03)$ and $0.19 (\pm 0.04)$ for S-2238, Tos-Gly-Pro-Arg-pNA and CH-Gly-Ala-Arg-pNA respectively (see Table 1).

Since it was possible to measure both k_{cat} and K_m for the three substrates, computation of the parameters listed in Table 1 was carried out by using eqn. (7), without any assumption about the rate-limiting step of the catalysis and thus without any approximation. However, the phenomenological observation that the K_m of all the substrates increases linearly as a function of relative viscosity implies $k_2 < k_3$ as demonstrated previously in the Experimental section. This result is shown in Figure 2 where apparent K_m values of Tos-Gly-Pro-Arg-pNA hydrolysis as a function of relative viscosity are fitted to eqn. (8). The calculated values of K_s and in k_2/k_{+1} are in excellent agreement with the values reported in Table 1 and calculated independently using eqn. (7). This result corroborates the validity of the theoretical and experimental strategy.

The ratio k_2/k_{-1} , which reflects the 'stickiness' of the substrate [21] (or in other words the tendency of the substrate to dissociate from the Michaelis complex more slowly than this adduct undergoes catalysis), is approx. 0.5 for the three substrates and indicates that all of these substrates are moderately 'sticky' under these experimental conditions.

The best-fit values of the kinetic and equilibrium constants for all the substrates as a function of concentration of C-terminal hir-(54–65) fragment are listed in Tables 2–4. The results show that the only parameter which slightly, but significantly, changes as a function of hir-(54–65) is k_2 for all the substrates. On the other hand the values of K_s and k_3 did not indicate a precise trend, so these parameters seem to be scattered around a mean.

The value of k_2 increases by enhancing the concentration of the fragment, and plateaus at concentrations above $2 \mu\text{M}$. The total effect on k_2 is small in all cases (the value is increased approx. 1.2-fold), but the variance of best-fit k_2 values showed that the variations were significant and allowed analysis of the results in terms of linkage effects, as shown for proton binding to α -thrombin [14,16].

Table 4 Effect of C-terminal hir-(54–65) fragment on the equilibrium and kinetic constants calculated using eqn (7) and involved in thrombin interaction with CH-Gly-Ala-Arg-pNA under experimental conditions reported in the text σ , S.E.M. of the fit.

Hir-(54–65) (μ M)	K_s (μ M)	k_2 (s^{-1})	k_3 (s^{-1})	σ (s^{-1})
0.031	5.11 ± 2.73	180.1 ± 7.6	949.5 ± 146.0	2.64
0.062	4.69 ± 2.10	180.4 ± 6.8	942.7 ± 128.0	2.66
0.125	5.78 ± 3.06	183.5 ± 7.6	992.3 ± 153.4	2.68
0.250	4.61 ± 2.03	185.5 ± 6.9	984.1 ± 134.1	2.70
0.500	5.58 ± 2.95	193.0 ± 8.0	1028.0 ± 157.1	2.81
1.000	4.99 ± 1.15	198.2 ± 5.3	1051.5 ± 102.2	2.90
2.000	6.08 ± 3.27	204.7 ± 8.6	1082.7 ± 166.7	3.01
4.000	4.69 ± 1.09	206.7 ± 5.6	1083.2 ± 105.1	3.03

The only problem we had to deal with was the necessary condition that binding of C-terminal hir-(54–65) fragment reached equilibrium before the Michaelis complex underwent catalysis. A series of experimental results supported the hypothesis that this is the case. It is known that the rate of association in the thrombin–hirudin interaction is approx. equal to $1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. This very high rate is due to the extraordinary complementarity between hir-(54–65) and FRS [27]. Since the value of the equilibrium constant for hir-(54–65) is approx. $0.6 \mu\text{M}$, as derived from fibrinogen experiments, it follows that processes involving binding and dissociation of C-terminal hirudin fragment occur in a shorter time scale than synthetic substrates (see Table 1). This means that binding of hir-(54–65) reaches equilibrium before the synthetic substrates are hydrolysed. Under these conditions a suitable kinetic scheme may be given in terms of linkage effects [14].

The reaction shown in Scheme 1 can be expanded as shown in Scheme 2:

where α is the equilibrium association constant of hir-(54–65)-binding to E and ES, k_2^0 and k_2^1 are the acylation rate constants in the absence and presence of hir-(54–65) fragment, H, respectively.

Values of apparent k_2 measured at different concentrations of H, were fitted to the following linkage equation [14]:

$$k_2^* = \frac{k_2^0 + k_2^1 \alpha H}{1 + \alpha H} \quad (12)$$

The results with the best-fit parameter values for CH-Gly-Ala-Arg-pNA are shown in Figure 3. In all cases, results were accurate to allow for the resolution of all parameters involved in

Figure 3 Results of the dependence of k_2 values of CH-Gly-Ala-Arg-pNA hydrolysis on the concentration of hir-(54–65)

Points are best-fit values of k_2 as listed in Table 4 for CH-Gly-Ala-Arg-pNA. Experimental conditions are reported in the text. Continuous lines are drawn from eqn. (12) by using the best-fit parameter values (k_2 , k_3 and σ are given in s^{-1}): $k_2^0 = 177.9 \pm 0.96$, $k_2^1 = 213.0 \pm 1.96$, $\alpha = 1.38 \pm 0.27 \times 10^6 \text{ (M}^{-1}\text{)}$, $\sigma = 2.55 \times 10^{-3}$. The values of the same best-fit parameters were found to be for p-Phe-Ppc-Arg-pNA: $k_2^0 = 124.2 \pm 1.83$, $k_2^1 = 148.4 \pm 2.71$, $\alpha = 2.02 \pm 0.92 \times 10^6 \text{ (M}^{-1}\text{)}$, $\sigma = 6.2 \times 10^{-3}$; and Tos-Gly-Pro-Arg-pNA for: $k_2^0 = 159.9 \pm 0.8$, $k_2^1 = 180.0 \pm 1.44$, $\alpha = 1.45 \pm 0.37 \times 10^6 \text{ (M}^{-1}\text{)}$, $\sigma = 2.24 \times 10^{-3}$.

Figure 4 Effect of hir-(54–65) on thrombin–fibrinogen interaction

K_m of thrombin–fibrinogen interaction under experimental conditions reported in the text, are plotted as a function of hir-(54–65) fragment. The continuous line was derived from eqn. (11) using the best-fit parameter values $K_m^0 = 2.21 \pm 0.19 \mu\text{M}$, $K_i = 0.68 \pm 0.07 \mu\text{M}$, $\sigma = 4.19 \times 10^{-7} \text{ (M)}$.

eqn. (12). Analysis of results reported in Tables 2–4 yielded similar values for the equilibrium dissociation constant of hirudin fragment, that ranged from $0.5 \mu\text{M}$ to $0.7 \mu\text{M}$, as reported in Figure 3. This result is of particular interest, since it was obtained by using three different substrates and supports the validity of the linkage effect (Scheme 2).

Since C-terminal hirudin fragment is a competitive inhibitor of the thrombin–fibrinogen interaction, an increase in the apparent K_m of this reaction is expected to occur as a function of hirudin fragment concentration, according to eqn. (11). This was indeed the case, as clearly shown in Figure 4. The best-fit value of the equilibrium dissociation constant of hirudin fragment was found

to be equal to $0.68 \pm 0.08 \mu\text{M}$, which is in excellent agreement with the values derived from viscosity experiments. The value of the binding constant of hirudin fragment derived from fibrinogen experiments should refer to the free form of the enzyme, while the value of the same parameter derived from viscosity experiments should refer to the Michaelis complex [14]. The convergence of results corroborated the finding derived from viscosity experiments, that binding of substrates does not affect binding of hirudin fragment and vice versa.

DISCUSSION

In this study we have attempted to characterize the effects of C-terminal hirudin fragment on the amidase activity of human α -thrombin in terms of linkage effects. The experimental and computational procedure described in the present study seems to be a suitable method by which to dissect the values of K_m and k_{cat} of amide substrates into individual rate constants of binding and catalysis. Determination of kinetic mechanisms is a necessary step in providing a detailed and rigorous analysis of the allosteric effects on macromolecular systems working at steady state.

Previous studies on the effect of C-terminal fragment on thrombin catalysis have reported contrasting results. Two studies reported that binding of C-terminal hirudin domain caused a reduction of K_m for amide substrates, without any significant variation of k_{cat} [9,28]. Other studies reported that both K_m and k_{cat} were reduced by the same factor by hir-(45–65) fragment [8] or else that k_{cat} was increased [11]. Another study reported that desulpho-hirudin (residues 45–65) is a non-competitive inhibitor of the amidase activity of thrombin [12]. These conflicting reports are difficult to rationalize.

The results obtained in this study, by means of an extensive investigation using different substrates, showed consistently that binding of C-terminal hir-(54–65) fragment results only in a small but significant increase of the acylation rate in the hydrolysis reaction.

It seems, indeed, that binding of hirudin fragment brings about a small conformational transition in the catalytic site of the enzyme, responsible for a moderate increase of the acylation rate constant.

Recent crystallographic data showed that binding to human α -thrombin of *N*-acetylhirudin (residues 53–64) with sulphated Tyr-63 (hirugen) does not induce a large change in the active site of thrombin [29]. The only significant variation reported in that study was a minor displacement by 0.09 nm (0.9 Å) of the hydroxyl group of Ser-195 out of the plane of the imidazole ring of His-57, which moves closer to the Cys-42–Cys-58 disulphide bridge in the active site [28]. Although the molecular dynamics of the catalytic activity of serine proteases is still an unanswered question [30], it might be hypothesized that the results reported in the present study could be consistent with the crystallographic data on the thrombin–hirugen complex. It is conceivable that the subtle structural changes of the active site, occurring upon binding of hir-(54–65), might underlie the small increase of the acylation rate in the catalytic reaction without significantly changing the affinity of the substrates.

The information derived from this study raises a series of further questions about the role played by the FRS in the modulation of thrombin function. Important points arise here as to whether the ligation of FRS by macromolecular ligands such

as fibrinogen, thrombomodulin or the platelet receptor is just a method for increasing the free-energy of binding of these macromolecules; or whether ligation of FRS plays a positive role of modulation in the kinetic mechanisms of hydrolysis of fibrinogen, protein C or the platelet receptor. Present results suggest that ligation of FRS does not induce extensive modifications of functional properties of the catalytic site of the enzyme.

Application of the theoretical and experimental strategy described in this paper to thrombin–fibrinogen interaction is underway in our laboratory. The possibility of determining the equilibrium dissociation constant of this interaction allows thorough investigation of the role played by the FRS in the modulation of the energetics and enzymic mechanisms of thrombin interaction with fibrinogen.

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