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The linkage between adenosine nucleotide binding and amidase activity in human α-thrombin

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The amidase activity of human α-thrombin has been studied in the presence of the adenosine nucleotides AMP, ADP and ATP. At low concentrations, adenosine nucleotides increase thrombin activity up to 30%, while at high concentrations (> 5 mM) inhibition takes place up to 20%. Inhibition is progressively reduced by increasing substrate concentration. A simple, phenomenological description of the linkage between adenosine nucleotide binding and amidase activity of human α-thrombin is proposed and the free energy changes for the underlying reactions involved in the linkage scheme are resolved by global analysis of the experimental data. The linkage scheme assumes that thrombin activation is determined by a conformational transition due to binding of adenosine nucleotides to a regulatory site. Inhibition, on the other hand, would be a consequence of competitive binding to the catalytic site.

1. Introduction

In the past few years, substantial experimental effort has been devoted to the study of the modulation of α-thrombin amidase activity by several effector molecules that modify the rate of catalysis and/or the affinity for different substrates [1–6]. These studies provide a quantitative basis for assessing the molecular mechanisms underlying the functional properties of thrombin in its reaction with substrates. In general terms, they represent a suitable experimental strategy for probing the energetics of the macromolecule. Small molecules such as indole are able to activate thrombin esterase activity with respect to the synthetic substrate Tos-Arg-OMe [5], presumably through conformational changes induced upon binding to a regulatory site. Other molecules, such as ATP, can either activate or inhibit thrombin amidase activity, this effect being concentration dependent [6]. The coexistence of activation and inhibition cannot be reconciled with the existence of a single regulatory site, but rather it demands explanation in terms of a more elaborate scheme. This fact has prompted us to investigate quantitatively thrombin amidase activity in the presence of adenosine nucleotides that are potentially important effectors of thrombin activity in vivo [5,7]. In this paper, we wish to propose a possible linkage scheme for the modulation of thrombin amidase activity by adenosine nucleotides. The thermodynamic scheme is investigated by means of experi-

Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-disphosphate; AMP, adenosine 5′-monophosphate; Chromozym TH, tosyl-Gly-Pro-Arg-pNA-AcOH; PEG, poly(ethylene glycol) 6000; k_{cat}, catalytic constant; K_m, Michaelis-Menten constant; HK, hexokinase; G-6PDH, glucose-6-phosphate dehydrogenase; $NADP^+$, β-nicotinamide adenosine dinucleotide phosphate; NADPH, β-nicotinamide adenosine dinucleotide phosphate, reduced form.

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mental strategies usually employed in the study of systems involving a biological macromolecule and several ligands [8–10]. Global analysis of the experimental data collected along different 'coordinates', each one corresponding to a particular ligand, yields the free energy levels associated with the underlying reactions.

2. Materials and methods

Human α-thrombin (3310 NIH units/mg protein) was obtained from Sigma and further purified as described elsewhere [11]. The purity was assessed by SDS-polyacrylamide gel electrophoresis on 10% gels and found to be 99%. Thrombin concentration was measured using an extinction coefficient of E_{280} (mg ml^{-1} cm^{-1}) = 1.83 and a molecular weight of 36 000 [1]. Vanadium-free ATP and ADP (disodium salts) from equine muscle and AMP (sodium salt) were from Sigma. The synthetic chromogenic peptide Chromozym TH was purchased from Boehringer Mannheim HK (150–175 U/mg protein), G-6PDH (75–90 U/mg protein), β-$NADP^+$ and proflavin (hemisulphate salt) were from Sigma.

The release of *p*-nitroaniline that resulted from the hydrolysis of Chromozym TH was followed by measuring the increase in absorbance at 405 nm in a Perkin-Elmer Lambda 5 spectrophotometer. The spectral bandwidth was 2 nm. Assays were performed in polystyrene cuvettes at 37°C under experimental conditions of 0.1 M Tris-HCl buffer, 0.15 M NaCl, 0.01% PEG and pH 8.00. The concentration of Chromozym TH was measured spectrophotometrically at the isosbestic wavelength (342 nm) using an extinction coefficient of 8270 M^{-1} cm^{-1} [12]. Substrate concentrations ranged from 0.5 to 400 μM, while α-thrombin was used at concentrations ranging from 0.2 to 0.8 nM. The concentration of released *p*-nitroaniline was evaluated by using an extinction coefficient of E_{405} = 9920 M^{-1} cm^{-1} [13]. The kinetic parameters, K_m and k_{cat}, were obtained by fitting the experimental data in the form of a Michaelis-Menten plot. The results yielded best-fit values of $K_m = (5.12 \pm 1.11) \times 10^{-6}$ M, and $k_{cat} = 226 \pm 6$ s^{-1}. Stock solutions of ATP, ADP, and AMP were made in buffer from fresh powder and dilute NaOH was used to adjust the pH. Solutions were then mixed with buffered solutions of α-thrombin (3.6×10^{-10} M, final concentration). Chromozym TH (5–500 μM, final concentration) was added in a 1:9 volume ratio after incubation for 15 min at 37°C, and the release of *p*-nitroaniline was monitored as described above. Final concentrations of nucleotides ranged from 0 to 25 mM. Measurements of the release of *p*-nitroaniline in the absence of adenosine nucleotides were used as reference. Measurements made in the presence of adenosine nucleotides, at different concentrations, were then expressed relative to the reference values by taking the ratio of velocities at a given substrate concentration.

The possibility of a catalytic action of thrombin on adenosine nucleotides due to nucleophilic attack on the 5′-esteric bond was carefully considered and ruled out as follows. Sample solutions of ATP at concentrations ranging from 66 to 333 μM were incubated with 0.5 μM α-thrombin for 1 h at 37°C in 0.1 M Tris-HCl buffer, 0.1 M NaCl (pH 8.00). Solutions of ATP to be used as controls were incubated in the same buffer without thrombin. The concentration of ATP was then measured in both the sample and control solutions using the HK and G-6PDH assay [14]. Equimolar ATP, glucose, and $NADP^+$ solutions were used in stoichiometric amounts. The overall reaction was followed by monitoring NADPH formation at 340 nm. No significant difference was observed in ATP concentration between sample and control solutions.

The standard free energy levels involved in the linkage scheme (see below) were evaluated by global analysis of the experimental data using nonlinear least-squares procedures. Absolute convergency of the fitting procedure was tested by extensive grid search in the parameter space, and approximate confidence intervals for the free energy levels were estimated by *F*-testing at the cut-off of one standard deviation.

Proflavin difference spectra were determined according to the method described by Sonder and Fenton [15] with minor modifications. Difference spectra were recorded on a double-beam Perkin Elmer Lambda 5 spectrophotometer at 25°C.

Solutions of 2 ml of α-thrombin at concentrations ranging from 2 to 3 μM in 50 mM Tris-HCl, 0.15 M NaCl (pH 8.00) were titrated with 5-μl droplets of proflavin solution in the same buffer. The final proflavin concentration ranged from 5 to 100 μM. The difference spectrum was taken from 400 to 500 nm. The absorbance difference, δA, between 468 nm (the absorbance peak of the thrombin-proflavin complex) and 444 nm (the absorbance peak of free proflavin), was used to determine the amount of proflavin bound at each step as follows:

$$\Delta A = \Delta A_T \theta \quad (1)$$

Here ΔA_T is the total absorbance change observed upon complete saturation and θ is the fractional saturation of proflavin. From the value of ΔA_T the difference molar extinction coefficient, ΔE, for the proflavin-thrombin complex can be derived. A value of $20\,000 \pm 5000$ M^{-1} cm^{-1} was obtained, in good agreement with previous determinations [4]. The same procedure was followed in replacement experiments, except that 1.5 ml of α-thrombin solutions (2–3 μM) containing a fixed concentration of proflavin (50 μM) were titrated with 5-μl droplets of buffered ATP solution at final concentrations ranging from 5 to 400 μM. The effect of ATP on proflavin binding to thrombin was evaluated by measuring the proflavin fractional saturation as a function of ATP concentration.

3. Results

The effect of adenosine nucleotides on the catalytic activity of α-thrombin was studied at different concentrations of Chromozym TH and at 37 °C. The results are shown in fig. 1a–c. In the presence of adenosine nucleotides, we consistently observe a significant activation of thrombin catalysis followed by inhibition. This observation is in agreement with previous results obtained with ATP at 25 °C [6]. Inhibition progressively disappears on increasing the concentration of Chromozym TH, so that at high substrate concentrations only activation can be seen.

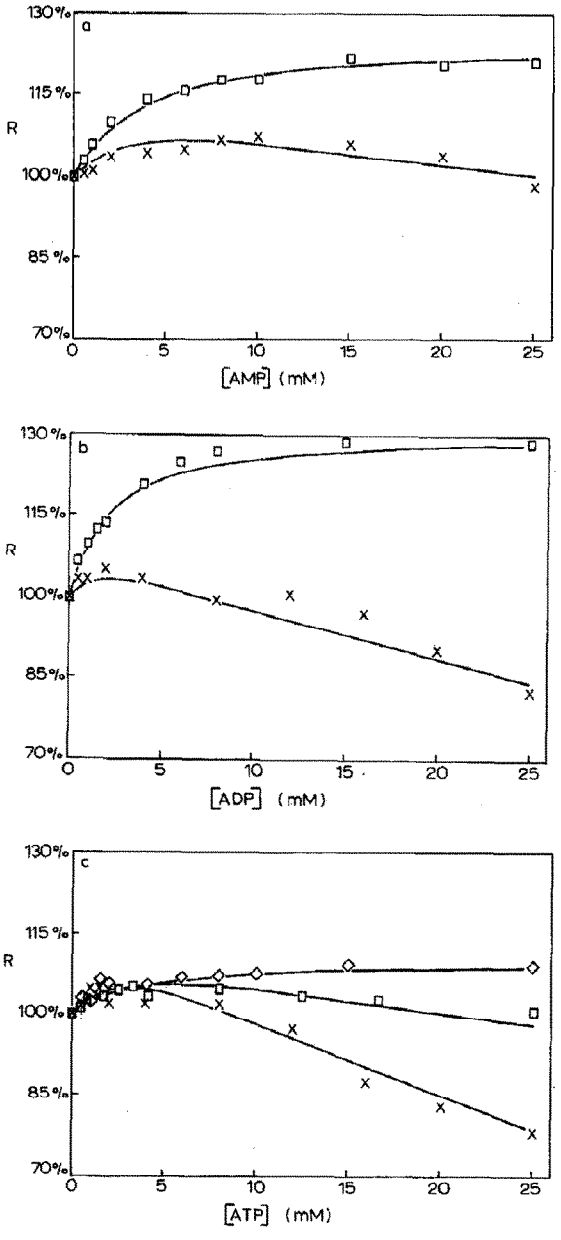

Fig. 1. (a–c) Experimental data for the linkage between binding of adenosine nucleotides and amidase activity of human α-thrombin. The data are expressed as $R = v(x, y)/v(x, 0)$, i.e., relative velocity with respect to the presence of substrate alone (see eq. 7). Measurements were taken at different substrate concentrations as follows: (a) (*) 66 μM, (□) 1 mM; (b) (*) 5 μM, (□) 400 μM; (c) (*) 5 μM, (□) 25 μM, (◇) 500 μM. Continuous plots are best-fit lines obtained by using eq. 7 with the parameter values reported in table 1. Standard error of the fit: 2.17 (a); 2.5 (b); 1.82 (c).

The possible influence of aspecific effects due to the relatively high ionic strength was investigated by experiments carried out in the presence of NaCl up to 0.2 M. However, no difference in the catalytic activity of α-thrombin on Chromozym TH was observed as compared to that measured with the standard buffer solution containing 0.15 M NaCl.

The results described above provide details about the linkage between thrombin amidase activity and the binding of effector molecules such as adenosine nucleotides. The linkage scheme has been explored along two coordinates, namely, the effector and substrate concentrations, following the experimental strategy usually employed in the study of linkage effects [8–10]. The information collected in this way allows for some qualitative conclusions, that however apply to all effectors considered here. Activation of thrombin activity is a common feature of these effector molecules and seems to be independent of substrate concentration. Inhibition, on the other hand, eventually disappears at relatively high concentrations of substrate. These findings can be rationalized within the framework of a simple thermodynamic treatment so that the linkage scheme can also be given a quantitative description.

4. The linkage scheme

The thermodynamic treatment of the effect of adenosine nucleotides on the amidase activity of α-thrombin is based on the formulation of a partition function, Z, which takes into account all the ligated species involved in the linkage scheme. The basic effect to be described is the coexistence of activation and inhibition of thrombin activity induced by adenosine nucleotides at low concentrations of substrate, and the persistence of activation alone at high concentrations of substrate. The simplest scheme consistent with these effects is as follows. The substrate, denoted by X, is assumed to bind to a single, catalytic site of thrombin, M. This reaction can be modulated by an effector molecule, Y, which is assumed to have two binding sites. The first effector binding site is the catalytic site itself, or part of it. The second effector binding site is a regulatory site different from the catalytic one. When ligand Y binds to the regulatory site the catalytic activity of thrombin is enhanced, say, by a conformational transition. When ligand Y binds to the catalytic site it competes with the substrate and the catalytic efficiency of thrombin decreases. Such a competition would explain the presence of inhibition by adenosine nucleotides observed experimentally at low substrate concentrations and its disappearance at high substrate concentrations.

The scheme proposed here incorporates two basic linkages. The former is of allosteric nature, the latter is competitive, or 'identical' [8,10], as ligand Y binds to the same site as ligand X, and the binding of either ligand to the site excludes the binding of the other. The partition function Z can then be written as [16]

$$Z = \sum_{i=0}^{1} \sum_{j=0}^{2-i} \exp\left[\left(i\mu_X + j\mu_Y - \Delta G_{ij}\right)/RT\right] \qquad (2)$$

where R is the gas constant, and T the absolute temperature, while μ_X and μ_Y are the chemical potentials of substrate X (Chromozym TH) and effector Y (adenosine nucleotides). The (standard) overall free energy change ΔG_{ij} is associated with the reaction $M + iX + jY = MX_iY_j$. There are five different such reactions. One gives rise to the identity M = M and sets a reference value of zero for the free energy change ΔG_{00}. The remaining ones describe the equilibria between the reference species, M, and the four possible ligated species MX, MY, MXY and MY_2. The free energy changes associated with these reactions give the energetic levels of each ligated species with respect to the reference state. They include the intrinsic free energy change due to binding of X and Y, and the cooperative free energy between the regulatory and catalytic sites. The number of moles of ligand X bound per mole of thrombin is given by

$$X = RT(\partial \ln Z/\partial\mu_X)_{\mu_y} = (\partial \ln Z/\partial \ln x)_y \qquad (3)$$

and likewise for ligand Y one has

$$Y = RT(\partial \ln Z/\partial\mu_Y)_{\mu_x} = (\partial \ln Z/\partial \ln y)_x \qquad (4)$$

Here x and y denote the ligand activities of X and

Y. The catalytic activity of thrombin, v, is expressed by the sum

$$v(x,y) = \left[v_{10}\exp(-\Delta G_{10}/RT)x + v_{11}\exp(-\Delta G_{11}/RT)xy\right]/Z(x,y) \quad (5)$$

with

$$Z(x,y) = 1 + \exp(-\Delta G_{10}/RT)x + \exp(-\Delta G_{01}/RT)y + \exp(-\Delta G_{02}/RT)y^2 + \exp(-\Delta G_{11}/RT)xy \quad (6)$$

The quantities v_{10} and v_{11} represent the maximum velocities of the two ligated species MX and MXY. In this respect, one recognizes that v_{10} corresponds exactly to k_{cat}, times the concentration of thrombin. Differences in these values reflect the linkage between the regulatory and catalytic sites due to binding of ligand Y.

The data taken experimentally were expressed as relative reaction velocity with respect to the absence of adenosine nucleotides. The appropriate fitting equation is derived from eqs 6 and 7 as follows:

$$\frac{v(x,y)}{v(x,0)} = \frac{\exp(-\Delta G_{10}/RT) + r\exp(-\Delta G_{11}/RT)y}{\exp(-\Delta G_{10}/RT)} \times \frac{Z(x,0)}{Z(x,y)} \quad (7)$$

where $r = v_{11}/v_{10}$ designates the ratio between the

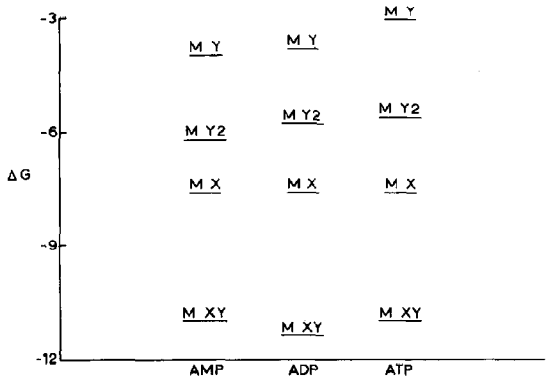

Fig. 2. Standard free energy levels of the ligated species involved in the linkage scheme discussed in the text.

catalytic constants of the two ligation intermediates, and

$$Z(x,0) = 1 + \exp(-\Delta G_{10}/RT)x \quad (8)$$

is the partition function in the absence of adenosine nucleotides. In the treatment given above it is assumed that binding equilibria are faster as compared to the catalytic rate.

Experimental data obtained at different concentrations of Chromozym TH were globally analysed using eq. 7. The results are shown by continuous lines in fig. 1a–c, and the best-fit values of the free energy changes associated with the relevant binding reactions are listed in table 1. The standard free energy levels of the linkage scheme are shown in fig. 2, which reveals the peculiar features of the reactions involved. The value of ΔG_{10} gives the free energy change associated with Chromozym TH binding to the catalytic site, which should be compared with the value of

Table 1

Standard overall free energy changes (in kcal/mol) of the linkage scheme obtained by fitting the experimental data according to eq. 7 in the text

Errors are calculated by F-testing at the cut-off of one standard deviation.

	ΔG_{10}	ΔG_{01}	ΔG_{02}	ΔG_{11}	r	ΔG_{in} [a]	ΔG_I [b]
AMP	−7.68 ± 0.40	−4.09 ± 0.40	−6.32 ± 0.34	−11.05 ± 0.36	1.28 ± 0.04	4.73 ± 0.50	−0.91 ± 0.52
ADP	−7.68 ± 0.40	−3.95 ± 0.20	−5.91 ± 0.22	−11.41 ± 0.20	1.32 ± 0.02	5.50 ± 0.30	−1.04 ± 0.30
ATP	−7.68 ± 0.40	−3.24 ± 0.50	−5.74 ± 0.30	−11.03 ± 0.44	1.11 ± 0.02	5.29 ± 0.54	0.21 ± 0.58

[a] Regulatory free energy of inhibition: $\Delta G_{02} - \Delta G_{11}$.

[b] Regulatory free energy of interaction: $RT \ln 4 + 2\Delta G_{01} - \Delta G_{02}$.

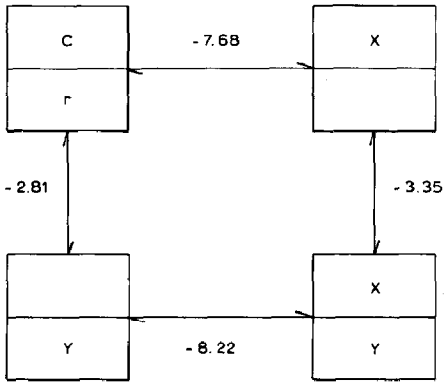

Fig. 3. Linkage scheme for the effect of ATP (Y) on Chromozym TH (X) hydrolysis by human α-thrombin. The regulatory (r) and catalytic (c) sites are schematically shown in the diagram in their possible ligation states. The scheme takes into account only the activatory modulation of ATP, in order to show the change in free energy levels due to ATP binding to the regulatory site. The binding affinity of Chromozym TH increases, with a free energy change of −0.5 kcal/mol, when ATP is bound to the regulatory site, thus indicating the presence of interactions between the two sites. It is worth mentioning that no interaction is present between the two sites in ATP binding (see table 1).

-7.48 ± 0.10 kcal/mol associated with K_m (see section 2). This result is consistent with the assumption that binding equilibria are faster than catalysis. The values of ΔG_{11} obtained for different effectors cluster around −11 kcal/mol, which suggests that the ligated species MXY can be assigned an almost unique energetic level, regardless of the effector. On the other hand, the catalytic enhancement, r, observed by adding the effector Y to the ligated species MX depends strongly on the particular effector used, and ranges from 11% in the case of ATP to 32% for ADP.

A quantitative measure of inhibition can be obtained from the difference $\Delta G_{02} - \Delta G_{11}$, which yields the free energy change associated with the replacement of the effector by the substrate at the catalytic site, when the regulatory site is bound. The values of the regulatory free energy of inhibition, ΔG_{in}, for adenosine nucleotides are listed in table 1, from which one can see that ADP is the most effective inhibitor of thrombin amidase activity.

The presence of two binding sites for adenosine nucleotides naturally raises the question of whether there is cooperativity in the binding of these effector molecules to thrombin. The corresponding regulatory free energies of interaction [8] are derived from the overall free energies corrected for statistical factors, and are listed in table 1. The values obtained for ADP and AMP are significantly negative, which indicates a possible binding heterogeneity between the two sites. The value found for ATP is practically zero, thus indicating that the two sites behave independently and have similar binding affinities. In all cases, no positive cooperativity is present in the binding reaction of adenosine nucleotides to thrombin. This allows one to express the overall free energy changes in terms of contributions arising from the regulatory and catalytic sites separately, i.e.,

$$\Delta G_{10} = \Delta G_X \tag{9a}$$

$$\Delta G_{01} = -RT \ln\left[\exp\left(-\Delta G_Y^r/RT\right) + \exp\left(-\Delta G_Y^c/RT\right)\right] \tag{9b}$$

$$\Delta G_{02} = \Delta G_Y^r + \Delta G_Y^c \tag{9c}$$

$$\Delta G_{11} = \Delta G_Y^r + \Delta G_X + \Delta G_{int} \tag{9d}$$

Here ΔG_X denotes the free energy change for binding the substrate to the catalytic site, ΔG_Y^r and ΔG_Y^c the free energy changes for binding the effector to the regulatory and the catalytic sites, and ΔG_{int} the interaction free energy between the two sites with both substrate and effector bound. However, the equations above are equally satisfied by interchanging ΔG_Y^r and ΔG_Y^c and adjusting ΔG_{int} accordingly. Only in the case of ATP does the similarity between ΔG_Y^r and ΔG_Y^c allow for a unique solution. The results are shown in fig. 3 as a thermodynamic cycle for the binding reactions of Chromozym TH and ATP to thrombin. From the diagram one can readily derive a value for the regulatory free energy of activation as the difference between the free energies of binding the substrate to the catalytic site in the presence and absence of effector. This value is about −0.5 kcal/mol.

5. Discussion

The results obtained in this study provide details about the linkage between thrombin amidase activity and the binding of adenosine nucleotides. The biphasic effect observed in the presence of AMP, ADP and ATP rules out the possibility that modulation of thrombin amidase activity by adenosine nucleotides is accomplished by a single regulatory site. This is in agreement with a previous study where the presence of at least two binding sites was implicated [6]. The overall free energy levels of the linkage scheme, as resolved by global analysis of the experimental data, demonstrate that no positive cooperativity is present between effector molecules bound to the regulatory and the catalytic sites. This conclusion applies to all adenosine nucleotides under the experimental conditions employed in this study.

The linkage scheme proposed here also seems to be supported by the effect of adenosine nucleotides on the binding of proflavin to α-thrombin. In the presence of low ATP concentrations proflavin binds with higher affinity than in the absence of the nucleotide. This indicates a specific enhancement of proflavin binding affinity due to binding of ATP to the regulatory site. However, proflavin is displaced from the catalytic pocket of thrombin at high ATP concentrations, as shown in fig. 4. This evidence is supportive of competitive binding of adenosine nucleotides near the catalytic moiety of the enzyme.

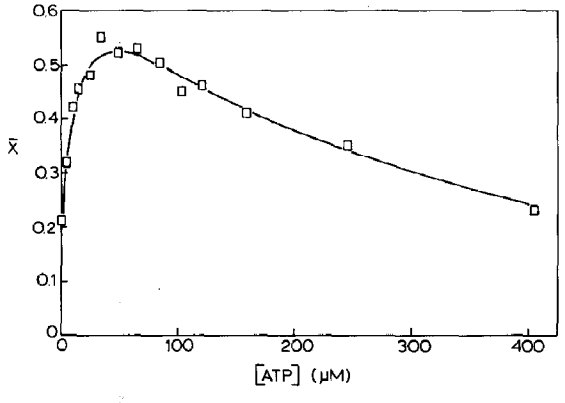

Fig. 4. Experimental data for the linkage between ATP and proflavin binding to human α-thrombin. The data are expressed as proflavin fractional saturation, X, as a function of ATP concentration, y. Measurements were taken under solution conditions as reported in the text. The continuous line was drawn according to eq. 3 with best-fit parameter values: $\Delta G_{10} = -5.20 \pm 0.12$ kcal/mol; $\Delta G_{01} = -5.68 \pm 0.52$ kcal/mol; $\Delta G_{11} = -12.44 \pm 0.18$ kcal/mol; and $\Delta G_{02} = -11.61 \pm 0.14$ kcal/mol. The standard error of the fit is 0.018.

The absence of cooperativity in effector binding to thrombin, along with the change in catalytic rate, as well as proflavin affinity, observed upon binding of effectors to the regulatory site, together indicate that thrombin activity is modulated by conformational transitions that seem to be effector-specific. This draws attention to the existence of specific nearest-neighbor interactions between the regulatory and catalytic sites, that translate into the enhanced catalytic activity observed experimentally. The role of an 'anionic site' [17–19] in the modulation of thrombin activity can be brought out in this connection. This site has been proposed to play a key role in the recognition of fibrinogen by thrombin and in the binding of thrombin to fibrin [19]. A critical involvement of the phosphate residues is also supported by the observation that Co[III](NH_3)-ATP, which has its phosphate residues blocked by the coordination complex, can only inhibit Chromozym TH hydrolysis by human α-thrombin [6].

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