Physiol. Res. 53: 109-113, 2004

Fluorescence Competition Assay for the Assessment of ATP Binding to an Isolated Domain of Na^+, K^+-ATPase

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Received October 11, 2002
Accepted May 14, 2003

Summary

An equation allowing estimation of the dissociation constant for binding of a non-fluorescent ligand to the enzyme is presented that is based on the competitive replacement of the ligand by its fluorescent analog. We derived an explicit formula for the probe fluorescence intensity, which is suitable for nonlinear least-squares analysis. We used this formula to evaluate the binding of ATP to the large cytoplasmic loop of Na^+,K^+-ATPase. The estimated value of K_D (6.2 ± 0.7 mM) is comparable with the results from other laboratories for similar constructs obtained by a different method.

Key words

Competition • Dissociation constant • Fluorescence • Enzyme-ligand interaction • TNP-ATP

Introduction

Fluorescence analysis represents a powerful tool for investigating the interaction of biopolymers with various ligands. Unfortunately, the ligands of interest do not usually emit strong fluorescence, and/or the biopolymers themselves exhibit fluorescence that may exceed considerably the weak ligand emission (e.g. when proteins that contain multiple aromatic acids are studied). To overcome this problem, fluorescent analogs of many important ligands have been synthesized that are capable of emitting bright fluorescence in a spectral range that is far from that of the biopolymer autofluorescence. However, even a small fluorochrome attached to the ligand may obviously influence the dissociation constant that characterizes the binding of the ligand to its specific binding site. Therefore, the results of such binding assays must be interpreted very carefully.

In many studies, an accurate quantitative evaluation of dissociation constants for ligand binding is essential. To obtain the correct value of a dissociation constant that is free of fluorochrome-related artifacts, the binding assays must be performed in the presence of a pure ligand whose binding to the binding site competes with the fluorescent analog. The evaluation of experimental data from such competition assays still relies on a few approximate formulas, although this method was introduced many years ago (Moczydlowski and Fortes 1981, Horovitz and Levitzki 1987, Corin *et al.* 1991, Kakkar *et al.* 1999). In this paper, we present an explicit formula that is suitable for fitting experimental titration data from competition binding assays, and thus for determining a dissociation constant of the pure ligand.

As an example of the use of this formula we studied the binding of ATP to the H_4-H_5 loop of Na^+/K^+-ATPase, in order to understand how this process is

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influenced by various loop mutations. Na^+/K^+-ATPase, a membrane enzyme transporting sodium ions outside and potassium ions inside the cell at the expense of energy from ATP hydrolysis, consists of two subunits. The α-subunit is a protein with 10 transmembrane helices that is responsible for ATP binding and hydrolysis as well as transport of ions. The β-subunit is a glycoprotein that is important for proper maturation of the enzyme in a membrane (for review see Kotyk and Amler 1995). Recent reports also suggest its role in K^+-transport (Abriel *et al.* 1999) and possible oligomerization (Ivanov *et al.* 2002). The ATP-binding site is localized on the α-subunit within the H_4-H_5 loop, which is a large cytoplasmic loop between transmembrane helices 4 and 5 (Lingrel and Kuntzweiler 1994). It has been shown that this loop retains its tertiary structure even when separated from the rest of the protein, and can thus still bind ATP (Tran and Farley 1999).

Recently, we have shown that the H_4-H_5 loop is able to bind 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP). We found that this fluorescent probe is suitable for investigating the ATP binding to this loop (Kubala *et al.* 2003). TNP-ATP is a well-known fluorescent analog of ATP that is only moderately fluorescent in aqueous solutions, while its fluorescence quantum yield increases about seven-fold upon the probe binding to ATPase (Hiratsuka and Uchida 1973, Hiratsuka 1976). However, when interpreting the results of assays performed with TNP-ATP, we have to take into account the fact that its interaction with proteins may not mimic perfectly the behavior of pure ATP, because i) it is more bulky, and ii) it can interact with ATPase through its trinitrophenyl moiety.

Methods

Protein preparation

The GST-fusion protein of the H_4-H_5 loop (sequence Leu354-Ile604) was expressed in *E. coli* and purified on Sephadex column as described earlier (Kubala *et al.* 2002). The stock solution of this protein was kept at –20 °C in 50 mM Tris-HCl (pH 7.5).

Fluorescence measurements

Fluorescence intensity of TNP-ATP stained protein solutions was measured using FluoroMax-2 (Jobin Yvon/Spex), in 0.4x1 cm quarz cuvettes at room temperature (22 °C). The excitation and emission wavelengths were chosen at 462 nm and 527 nm, respectively. To improve the signal-to-noise ratio, both the excitation and the emission band pass were set at 10 nm and the integration time to 5 s. Measured fluorescence intensities were corrected for both blank signal due to a protein emission and scattered excitation light.

Protein staining and titration experiments

TNP-ATP (Molecular Probes, Oregon, USA) was kept as 100 μM aqueous stock solution. Aliquots of this stock solution were added to protein samples, and the stained samples stirred gently before fluorescence measurements started about 2 min after adding the dye to the protein. In titration experiments, sequential additions of TNP-ATP were made into the identical cuvettes in a total volume of 1 ml. Protein, TNP-ATP and ATP concentrations were corrected for the corresponding diluting. For simple titration experiments with TNP-ATP, GST-fusion protein was diluted to a concentration of 1.6 μM with 50 mM Tris-HCl, pH 7.5. The competitive assays in the presence of ATP were performed with 1.6 μM GST-fusion protein in 50 mM Tris-HCl and with 20 mM ATP. In these solutions, pH was adjusted to 7.5 after the addition of ATP.

Results

Explicit formula describing probe fluorescence intensity in the presence of a competing non-fluorescent ligand

For one binding site per enzyme, the equilibrium binding of fluorescence probe to an enzyme obeys the equation

$$(1) \qquad K_P = \frac{[P][E]}{[PE]}$$

where K_P is the dissociation constant, $[P]$ is free probe concentration, $[E]$ is free protein concentration, and $[PE]$ is the concentration of protein-bound probe. The total probe concentration, $[P]_T$, and the total concentration of protein, $[E]_T$, can be expressed as

$$(2) \qquad [P]_T = [P] + [PE]$$

$$(3) \qquad [E]_T = [E] + [PE]$$

Thus the total intensity of fluorescence from stained protein solutions, F_T, consists of two components, a signal due to the free probe, F_f, and a signal due to the bound probe, F_b,

$$(4) \qquad F_T = F_f + F_b$$

Under experimental conditions typical of usual binding experiment we can write for the emission from the free probe

(5) $F_f = [P]\, Q$

where Q is a multiplicative constant that reflects both the photophysical properties of free probe and the sensitivity of fluorimeter. For F_b we can write

(6) $F_b = \gamma\, [PE]\, Q$

where γ is the ratio of respective fluorescence intensities of bound and free probe (fluorescence enhancement). The value of Q can be readily assessed by measuring the fluorescence intensity from probe solutions in pure buffer. For practical reasons we will present the following formulas using a normalized fluorescence intensity F_T/Q, which we will denote F. In our preceding paper (Kubala *et al.* 2003) we derived an explicit formula describing the dependence of the normalized fluorescence intensity on the dissociation constant K_P and experimental parameters $[P]_T$, $[E]_T$, and γ:

(7) $$F = [P]_T + \frac{\gamma - 1}{2}\left([P]_T + [E]_T + K_P - \sqrt{([P]_T + [E]_T + K_P)^2 - 4[P]_T[E]_T}\right)$$

We have also shown that the use of this explicit formula for fitting experimental titration data is superior to the use of linearized plots, such as Scatchard plot, because any linearization inevitably affects also the noise distribution in experimental data. This noise transformation may result in erroneous estimations of dissociation constants.

In case of the simultaneous binding of TNP-ATP and pure ATP that compete for the same binding site (in general terms, of the simultaneous binding of any non-fluorescent ligand competing with its fluorescent analog), an additional equilibrium equation that describes the binding of pure ATP to the enzyme must be combined with Eq.1. For the ATP binding to the enzyme we can write

(8) $$K_A = \frac{[E][A]}{[AE]}$$

where $[A]$ is the concentration of free ATP and $[AE]$ is the concentration of ATP bound to the enzyme. Then the total ATP concentration is obviously

(9) $[A]_T = [A] + [AE]$

and Eq. (3) must be replaced by

(10) $[E]_T = [E] + [PE] + [AE]$

With some algebra, one can combine Eq.8 with equations (1), (2), (4) - (6), (9) and (10) to obtain an explicit equation for K_A

(11) $$K_A = \frac{K_P(F - [P]_T)}{\gamma[P]_T - F}\left(\frac{[A]_T}{[E]_T - \frac{K_P(F-[P]_T)}{\gamma[P]_T - F} - \frac{F-[P]_T}{\gamma - 1}} - 1\right)$$

where the denominator of the fraction shown in parentheses stands for $[AE]$. We also tried to derive an explicit formula from Eq.11 analogous to Eq.7. This has resulted in a cubic equation for F. A way to solve similar problems in radioactive labeling assays was presented by Wang (1995), but we have not found their approach suitable for practical use in fluorescence competitive assays.

Fortunately, the binding of pure ATP to the H_4-H_5 loop is much weaker than the binding of its fluorescent analog, i.e. it holds that $K_A >> K_P$. Therefore, a reasonable extent of competition between these two ligands will only be observed if $[A]_T >> [P]_T$. Optimum titration experiments with TNP-ATP have been performed with the maximal value of $[P]_T$ close to $[E]_T$. Under these conditions $[A]_T >> [E]_T > [AE]$, and finally $[A]_T/[AE] >> 1$. Then the number 1 in Eq. (11) can be neglected and transform this equation into a relatively simple quadratic equation:

(12) $(F - [P]_T)^2 - (F - [P]_T)(\gamma\text{-}1)([P]_T + [E]_T + K_P + [A]_T K_P/K_A) + (\gamma\text{-}1)^2 [P]_T [E]_T = 0$

that yields finally:

(13) $$F = [P]_T + \frac{1}{2}(\gamma - 1)\left([P]_T + [E]_T + K_P + [A]_T\frac{K_P}{K_A} - \sqrt{\left([P]_T + [E]_T + K_P + [A]_T\frac{K_P}{K_A}\right)^2 - 4[P]_T[E]_T}\right)$$

Competitive binding of TNP-ATP and ATP to H_4-H_5 loop of the GST-fusion protein

As shown in our previous study of TNP-ATP binding to the H_4-H_5 loop of the GST fusion protein, the dissociation constant for TNP-ATP is $K_P = 3.0 \pm 0.3$ μM,

and the fluorescence enhancement factor is $\gamma = 7.0 \pm 0.7$ (Kubala *et al.* 2003). We found also that fusion with the GST protein has no influence on TNP-ATP or ATP binding to the H_4-H_5 loop.

To assess the dissociation constant for pure ATP, titration experiments with TNP-ATP were performed in the presence of ATP. Since the binding of ATP to the H_4-H_5 loop appeared to be much weaker than the binding of TNP-ATP, we had to use very high concentrations of ATP ($\geq$ 5 mM) to see some significant competition between TNP-ATP and ATP binding. At this concentration, ATP added to GST-fusion protein stained with TNP-ATP will inevitably cause a substantial shift in the pH of the solution. Therefore, we had to carry out the competition assays at a constant ATP concentration.

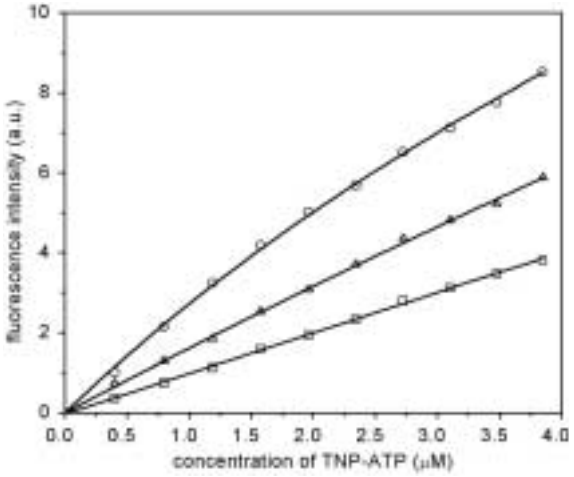

Fig. 1. Dependence of relative fluorescence intensity on TNP-ATP concentration. H_4-H_5 loop-GST-fusion protein (1.6 μM) was incubated with increasing concentration of TNP-ATP in 50 mM Tris-HCl, pH 7.5 (circles, data taken from Kubala et al. 2003) or in 50 mM Tris-HCl + 20 mM ATP, pH 7.5 (triangles). For comparison, titration in 50 mM Tris-HCl in the absence of any protein is shown (squares). Binding of TNP-ATP to the protein results in an increase of fluorescence intensity. The presence of ATP in the solution inhibited this binding.

The GST fusion protein was dissolved in 50 mM Tris buffer containing 20 mM ATP (pH 7.5). After adding purified protein to this buffer, the concentration of ATP decreased to 19.36 mM. This solution was titrated with aliquots of TNP-ATP and corrections for ATP and protein dilution were calculated. Figure 1 shows the titration plots. These experimental curves were fitted to Eq. (13) using least-squares analysis, with K_A left as a fit parameter and γ and K_P fixed to values found in preceding pure TNP-ATP titration experiments. Three independent series of titration yielded a K_A value of 6.2 ± 0.7 mM.

Discussion

Fluorimetry can easily be used as a powerful tool in quantitative studies of enzyme-ligand interaction, provided that one of the interacting species exhibits an intrinsic fluorescence whose intensity varies upon ligand binding. Eq.7 describes this situation adequately, as shown in our preceding paper.

In the present paper we analyze the case of competitive binding of a non-fluorescent ligand and its fluorescent analog to a non-fluorescent protein, and present an explicit formula (Eq.13) that makes it possible to assess fluorimetrically the dissociation constant for the binding of non-fluorescent ligand to a non-fluorescent protein. This formula is applicable to the ligands and their fluorescent analogs that satisfy the following requirements: (i) the non-fluorescent ligand and the fluorescent probe compete for the same binding site, (ii) the pure ligand is not fluorescent and neither is the macromolecule, (iii) the fluorescent analog of the ligand exhibits a considerable increase (or decrease, $\gamma < 1$) of fluorescence intensity upon its binding to the protein, and (iv) the dissociation constant of the fluorescent analog is much lower than that of the pure ligand.

If the above requirements are fulfilled, Eq.13 can be used for fitting experimental titration curves by the least-squares analysis. Even in a situation when condition (iv) is not fulfilled, the value of the dissociation constant can still be assessed using Eq.11 that is free of approximations. In this case we have to calculate the K_A value for each particular set of ligand, probe and protein concentrations and the corresponding intensity of probe fluorescence, and then to determine a mean value.

We employed Eq.13 to evaluate experimental data from titration experiments that had been designed to determine the dissociation constant for ATP binding to the H_4-H_5 loop in GST fusion protein, with TNP-ATP used as the fluorescent analog of ATP. As demonstrated in Figure 1, a reasonable difference between respective titration curves for TNP-ATP only and for TNP-ATP in the presence of ATP was found for 1.6 μM GST-fusion protein when the concentration of TNP-ATP ranged from 0.4 to 4.0 μM and the ATP concentration was 20 mM. This suggests that the actual values of $[A]_T$, $[E]_T$ and [AE] as used in our experiments justify the approximation described above in the Results, and thus the fitting of experimental data to Eq.13.

The observed value of the dissociation constant for ATP binding to the H_4-H_5 loop of Na^+,K^+-ATPase was 6.2 ± 0.7 mM. This value is comparable with dissociation constants that Tran and Farley (1999) found for Na_2-ATP binding to several different GST-fusion proteins containing the cytoplasmic domain of α_1-subunit of Na^+,K^+-ATPase. The published data ranged from 2 to 11 mM (Tran and Farley 1999). These authors interpreted this fact in terms of a possibility that the cytoplasmic domains of Na^+,K^+-ATPase, when expressed as fusion proteins, are folded in an E_2-like conformation to which ATP binds with relatively low affinity. The fact that the dissociation constant for pure ATP binding is about three orders of magnitude higher than that for TNP-ATP suggests a certain stabilizing role of the trinitrophenyl moiety in the interaction of this ATP analog with the H4-H5 loop of the fusion protein.

Acknowledgements

This work was supported by MSMT113200001 grant from The Ministry of Education, Youth and Sports of the Czech Republic and Research Projects AVOZ 5011922 and CZE00/033.

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