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Probing Protein Kinase-ATP Interactions Using a Fluorescent ATP Analog

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Abstract

Eukaryotic protein kinases are an intensely investigated class of enzymes which have garnered attention due to their usefulness as drug targets. Determining the regulation of ATP binding to a protein kinase is not only critical for understanding function in a cellular context but also for designing kinase-specific molecular inhibitors. Here we provide a general procedure for characterizing ATP binding to eukaryotic protein kinases. The protocol can be adapted to identify the conditions under which a particular kinase is activated. The approach is simple, requiring only a fluorescent ATP analog such as TNP-ATP or MANT-ATP and an instrument to monitor changes in fluorescence. Although the interaction kinetics between a kinase and a given ATP analog may differ from that of native ATP, this disadvantage is offset by the ease of performing and interpreting this assay. Importantly, it can be optimized to probe a large variety of conditions under which the kinase-nucleotide binding might be affected.

Keywords

kinase; nucleotide; ATP binding; CASK; pseudokinase; TNP-ATP; fluorescence

1. INTRODUCTION

Eukaryotic protein kinases are one of the largest gene families and constitute 2 % of the proteome [1]. Protein kinases function as versatile molecular switches which play a critical role in the fundamental biology of the cell. Dysregulation of kinase activity is the cause of various diseases including certain forms of cancer [2,3]. The enzymatic functions of these phosphotransferases are tightly regulated by various molecular strategies, a prominent one being obstruction of nucleotide binding [4]. The regulation of nucleotide binding to protein kinases therefore is a field of intense investigation both in basic science and drug development [5,6]. In fact kinases represent the most druggable component of the human

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⁷Proteins that are purified especially from eukaryotic sources like insect cells may be post-translationally modified, which can affect the nucleotide binding activity.

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proteome [7]. A number of screening technologies for kinase profiling have been reported. Many of the kinase assays use radioactive reagents such as $\gamma^{32}\text{P}$ -ATP [8] or are based on luminescence from the luciferase system [9]. Both systems require some knowledge of the kinase's substrate, which may not always be available. An alternative approach to testing kinase activity is to examine the affinity of nucleotide binding to its hydrophobic pocket. Indeed most of the kinase inhibitors discovered to date inhibit ATP binding to kinases either by directly binding to the ATP binding pocket (type I inhibitors) or to adjacent sites (type II inhibitors) [10]. A high throughput and rapid method for testing nucleotide binding to kinases may therefore be a very useful and versatile tool for the study of kinases and for drug discovery. Here we describe the use of fluorescent ATP analogs like 2'-(or-3')-O-(trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) or (2'-(or-3')-O-(N-Methylanthraniloyl) adenosine 5'-triphosphate (MANT-ATP) to measure the ATP interaction with kinases. This method has been effectively used for a number of different purposes: to test specific binding conditions of ATP to proteins initially described as pseudokinases such as CASK and JAK2 [11,12], to test how specific mutations affect ATP binding properties [13], to test nucleotide binding in known eukaryotic protein kinases [14], and as a high throughput assay to screen for inhibitors for bacterial kinases [15]. The advantages of the method include ease of experimental design, no requirement for radioactive reagents, and ease of interpretation. Additionally, there is no need for large quantities of protein, and detection is not dependent upon the protein undergoing a large conformational change, as is required by some assays but does not happen with all kinases.

In the described assay, we use TNP-ATP as a fluorescent probe to detect the ATP interaction with eukaryotic protein kinases. Upon addition of a protein which can bind TNP-ATP, there is a 3- to 5-fold increase in fluorescence, and a clear blue shift in emission maxima is observed (Fig. 1a) [16]. In the example data provided from the unusual kinase, CASK, a distinct leftward shift and increase in peak fluorescence intensity is observed between the spectrum of TNP-ATP alone shown in blue and the spectrum of TNP-ATP bound by CASK shown in yellow. The increase in fluorescence is instantaneous and does not require incubation time. To ensure specificity, ATP can be used to compete with TNP-ATP binding to the protein of interest, resulting in a measurable reversal of the change in fluorescence intensity and emission maximum (Fig. 1a; note difference between green and yellow curves). This assay is also useful for identifying and investigating kinase inhibitors; for example, the surprising finding that the high-affinity interaction of the CaMK domain of CASK with TNP-ATP is inhibited by magnesium (Fig. 1a,d) laid the groundwork for establishing that the kinase activity of the intact CASK can be also inhibited by magnesium [11,13,17].

Although the binding kinetics and binding affinity of TNP-ATP are different from that of ATP, competition assays and K_i determination can be of tremendous value when comparing other nucleotides to establish binding-site specificity of kinases (Fig. 1b,c). This is especially important because some kinases (e.g., casein kinase and CaMKII) can utilize GTP as a substrate [18,19]. In the case of CASK, ATP, but not GTP, can compete with TNP-ATP, indicating that CASK's nucleotide binding pocket is specific for adenine nucleotides (Fig. 1b). Furthermore, ATP is more effective in competing with TNP-ATP for binding to CASK

than other adenine nucleotides, suggesting that CASK has a higher affinity for and binds preferentially to ATP.

The TNP-ATP binding assay can be easily converted to a screen for identifying conditions under which a kinase can bind to TNP-ATP [15]. These assays can be done reliably either in a cuvette or in a plate if a suitable instrument is available. We have used these assays to examine competition using several nucleotides (Fig. 1c) and to determine the effect of divalent ions (Fig. 1d). We have also used this assay to effectively screen for mutant CASK CaMK domains capable of coordinating magnesium (Fig. 2). Our results were subsequently validated by X-ray crystallography and enzymology studies [13,11].

2. MATERIALS

Prepare all solutions and dilutions fresh, just prior to the assay, using ultrapure water and analytical grade reagents (*see* Note 1). Follow steps specific for performing either a cuvette-based binding assay, microplate-based binding assay, or microplate-based saturation assay (*see* Note 2).

2.1 Solutions and Sample Preparation for All Assays

- 1 Tris-HCl buffer: 50 mM Tris-HCl, 50 mM KCl, pH 7.2 (*see* Note 3). Dissolve appropriate amounts of Tris base and KCl in water nearly to desired volume with stirring. Use a pH meter to monitor pH during gradual drop-wise addition with stirring of a solution of hydrochloric acid until pH is 7.2. Bring Tris-HCl buffer to final volume with water. The buffer can be stored at room temperature for several months.
- 2 Lysozyme control stock solution: 420 μ M (*see* Note 4). Prepare the protein control sample by dissolving 6 mg of lyophilized lysozyme in 1 mL of Tris-HCl buffer (molecular weight of lysozyme is 14.3 kDa).
- 3 Lysozyme control assay solution: 4 μ M. Add 10 μ L of the 420 μ M lysozyme control stock solution to 990 μ L of Tris-HCl buffer.
- 3 Protein sample: 1–4 μ M protein sample (*see* Note 5). Prepare the kinase sample of interest by determining the concentration of the purified (*see* Notes 6–8) protein (using the Bradford assay or similar method of protein concentration determination). For best results, initial concentration should be at least 50–100

¹TNP fluoresces brightly in a hydrophobic environment, therefore it is critical to avoid hydrophobic contaminants in the assay buffer.

²Use of a microplate-based assay allows the investigator to set up relatively high-throughput assays to test a wide array of conditions (nucleotide variants, cofactors, protein binding partners) under which a protein binds the nucleotide. The microplate also reduces the amount of protein sample needed for an assay, even when done in triplicate.

³We use 50 mM KCl in our experiments but different proteins may have different salt preferences; buffer composition should therefore be optimized for the protein of interest. We do not recommend the use of 150 mM NaCl because complete dissociation of chloride (not an intracellular anion) produces higher electroconductivity, which may interfere with the interactions under study. If higher salt concentrations are used, we recommend the use of potassium glutamate or potassium acetate at 150 mM, which more closely resembles the intracellular ionic composition.

⁴The control protein has to be carefully selected. Proteins such as albumin bind to ATP, confronting investigators with what they presume to be high background. In our hands, lysozyme works well.

⁵It may be prudent to express and purify the full-length protein since other domains may affect ATP binding. In the case where only the kinase domain is expressed, careful selection of domain demarcation has to be performed, and may include several trials since unstructured regions of a protein fragment may affect ATP binding.

μM. The protein sample should be diluted from the stock solution in Tris-HCl buffer to achieve a final concentration of 1–4 μM. A final volume of 2 mL is needed for the cuvette assay. A total volume of 600 μL (for triplicates) is needed for the microplate assay.

- 4 TNP-ATP stock solution: 6.4 mM of 2',3'-*O*-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate, trisodium salt (TNP-ATP; Invitrogen) (*see* Note 9).

2.2 Microplate Binding Assay

1. TNP-ATP solution, dilution 1 (0.64 mM). Add 10 μL TNP-ATP stock solution (6.4 mM) to 90 μL Tris-HCl buffer.

2.3 Microplate Saturation Assay

1. TNP-ATP solution, dilution 2 (2 mM). Add 62.5 μL stock TNP-ATP solution to 137.5 μL Tris-HCl buffer.
2. TNP-ATP solution, dilution 3: 0.2 mM. Add 20 μL of 2 mM TNP-ATP solution to 180 μL Tris-HCl buffer.

2.4 Equipment for Cuvette and Microplate Assay

1. pH meter and electrode compatible with Tris buffers.
2. Spectrofluorometer equipped with magnetic stirrer (for the cuvette assay). The instrument to be used should be set up in advance to excite at 410 nm and to detect emission at 540 nm, as well as for wavelength scan from 500 nm to 600 nm. The excitation and emission slits should be set at 3 nm and 5 nm respectively.
3. 10 mm X 10 mm cuvette suitable for fluorescence.
4. Magnetic stir bar for cuvette.
5. Spectrofluorometer with plate-reading capability (for the microplate assay). The instrument to be used should be set up in advance to excite at 410 nm and to detect emission at 540 nm, as well as for wavelength scan from 500 nm to 600 nm. The excitation and emission slits should be set at 3 nm and 5 nm respectively. The instrument should be set up to read a 96-well plate and the readings should be taken from the top.
6. 96-well microplate with a black bottom to minimize background fluorescence.

⁶Although in an enzymatic assay, such as a phosphotransferase assay, the purity of protein preparation is of highest priority due to turnover rates, in ATP binding assays such as the one described, which have a finite and easily saturable endpoint, it is more important to obtain a well-folded protein. Multiple steps of protein purification which may affect the conformation of the protein should therefore be avoided. We typically use single-step affinity purification of the sample protein under physiological concentrations of electrolytes and pH.

⁸Activated kinases rapidly toggle between a closed and an open conformation [21]. Kinases operate in a cellular milieu replete with nucleotides including ATP. Purification of kinase domains without nucleotide may trap a conformation with low affinity for nucleotides, so it may be useful to include non-hydrolyzable analogs of ATP such as AMPPNP in the protein purification buffer. Using ATP itself should be avoided since this may cause autophosphorylation in vitro.

⁹Freeze TNP-ATP immediately upon arrival and protect from long-term light exposure.

7. Scientific graphing program (e.g., GraphPad Prism® for the microplate assay).

3. Methods (see Note 10 and 11)

3.1 Standard Cuvette-based Binding Assay

1. Place magnetic stir bar in cuvette.
2. Add 2 mL of Tris-HCl buffer to cuvette.
3. Add 1.6 µL of 6.4 mM stock TNP-ATP solution to cuvette to make final TNP-ATP concentration equal to 5 µM.
4. Place cuvette in spectrofluorometer with magnetic stirring turned on.
5. Excite sample at 410 nm.
6. Perform scan from 500 nm to 600 nm, and confirm peak fluorescence at 561 nm (see Note 12).
7. Remove and rinse cuvette.
8. Add protein sample solution of interest (1–4 µM) to cuvette. Total volume should equal 2 mL.
9. Add 1.6 µL of 6.4 mM stock TNP-ATP solution to the cuvette to make final TNP-ATP concentration equal to 5 µM.
10. Place cuvette in spectrofluorometer with magnetic stirring turned on.
11. Excite sample at 410 nm.
12. Read and record emission maximum at 540 nm or perform scan from 500 nm to 600 nm. Examine the emission scan for peak fluorescence at 540 nm (blue-shifted from peak observed in step 6) (see Note 13).
13. Remove and rinse cuvette.
14. Add 2 mL of lysozyme control assay solution (4 µM) to cuvette.
15. Add 1.6 µL of 6.4 mM stock TNP-ATP solution to the cuvette to make final TNP-ATP concentration equal to 5 µM.
16. Place cuvette in spectrofluorometer with magnetic stirring turned on.
17. Excite sample at 410 nm.
18. Read and record emission maximum at 540 nm or perform scan from 500 nm to 600 nm.

¹⁰Rapidly purified proteins with high nucleotide affinity may have nucleotide bound to its pocket, which will interfere with ATP binding assays. For example, we found that the CASK CaM-kinase domain binds to nucleotides such as 3' AMP present in bacterial lysates. Occupation of the nucleotide binding pocket after purification can be ruled out by acquiring an absorbance spectra prior to beginning the assay; the presence of a peak at 260 nm indicates a bound nucleotide.

¹¹TNP-ATP may display much higher affinity than ATP itself, so when screening for inhibitors, false negatives are more likely than false positives using this assay.

¹²If peak fluorescence is not at 561 nm, contaminants may be present and must be eliminated before continuing with experiment.

¹³We have observed that a mere increase in fluorescence may be an artifact; it is essential to determine that in the presence of the protein sample of interest, there is a blue shift and the emission maximum is at 540 nm.

19. Export data for analysis.
20. Correct experimental values for kinase of interest by subtracting emission values obtained with lysozyme control readings (representing background fluorescence).

3.2 Microplate-based Binding Assay

1. Prepare buffer control wells by pipetting 200 μ L of Tris-HCl buffer into three wells (triplicates).
2. Add 1.6 μ L of 0.64 mM TNP-ATP solution into these three wells to make final TNP-ATP concentration equal to 5 μ M.
3. Place plate in microplate reader.
4. Set microplate reader to shake plate prior to acquiring data.
5. Set up reader for excitation at 410 nm and emission scan from 500 nm to 600 nm.
6. Prepare protein control wells by pipetting 200 μ L of lysozyme control assay solution (4 μ M) into three wells.
7. Add 1.6 μ L of 0.64 mM TNP-ATP solution into these three wells to make final TNP-ATP concentration equal to 5 μ M.
8. Add 200 μ L of protein sample solution (1–4 μ M) to three wells.
9. Add 1.6 μ L of 0.64 mM TNP-ATP solution into these three wells to make final TNP-ATP concentration equal to 5 μ M.
10. Place plate in microplate reader.
11. Set microplate reader to shake plate prior to acquiring data.
12. Set up reader for excitation at 410 nm and either single wavelength emission reading at 540 nm or emission wavelength scans from 500 nm to 600 nm (*see* Notes 12 and 13).
13. Export data for analysis.
14. Correct experimental values for kinase of interest by subtracting emission values obtained with lysozyme control readings (representing background fluorescence).

3.3 Microplate-based TNP-ATP Saturation Assay (20 min) (see Note 14)

1. Prepare buffer control wells by pipetting 190 μ L of Tris-HCl buffer into three wells (triplicates).

¹⁴High affinity for the adenine nucleotide may not always translate into a low Michaelis constant for ATP in an enzymatic reaction, since rapid phosphotransfer may be limited by the displacement of ADP by the incoming ATP molecule. We observe a very high K_m (~1mM) for ATP in the case of CASK.

2. Add 10 μ L of 2 mM TNP-ATP solution into these three wells to make final TNP-ATP concentration equal to 100 μ M.
3. Place plate in microplate reader.
4. Set microplate reader to shake plate prior to acquiring data.
5. Set up reader for excitation at 410 nm and emission scan from 500 nm to 600 nm. (*see* Notes 12 and 13)
6. Prepare protein control wells by pipetting 190 μ L of lysozyme solution (4 μ M) into three wells.
7. Add 10 μ L of 2 mM TNP-ATP solution into these three wells to make final TNP-ATP concentration equal to 100 μ M.
8. Prepare triplicates of protein sample wells for each concentration of TNP-ATP to be tested (1 μ M, 5 μ M, 10 μ M, 25 μ M, 50 μ M and 100 μ M) for a total of 18 wells. If target concentration is, for example, 2 μ M, add 100 μ L of a 4 μ M solution to each well. Final well volume will equal 200 μ L, yielding a final protein concentration of 2 μ M.
9. Add TNP-ATP and Tris-HCl buffer into the 18 protein sample wells according to Table 1 (Table 1 near here):
10. Place plate in microplate reader.
11. Set microplate reader to shake plate prior to acquiring data.
12. Set up reader for excitation at 410 nm and obtain single wavelength emission reading at 540 nm (*see* Notes 12 and 13).

3.4 Data Analysis

1. Correct experimental values for kinase of interest by subtracting emission values obtained with lysozyme control readings (representing background fluorescence).
2. For the high-throughput assays or single-wavelength emission competition assays, a simple bar graph analysis can be used to display differences between controls and experimental values.
3. For the 500–600 nm scan measurements, ASCII files are opened in Microsoft Excel as data files. The reading at 500 nm is considered to be zero and blanked from the data set. The scan is plotted as XY scatter plot between the wavelength (X-axis) and the reading (Y-axis).

3.5 Data Analysis for TNP-ATP Saturation Assay in GraphPad Prism®

1. Nonlinear curve fitting can be performed on data from the saturation assay using a program such as GraphPad Prism® to determine $K_d^{\text{TNP-ATP}}$ (Fig. 3) (*see* Note 15). GraphPad Prism® contains a built-in model for saturation binding using the equation: $Y = B_{\text{max}} * X / (K_d + X) + NS * X + \text{background}$, in which B_{max} is the maximum specific binding, K_d is the equilibrium binding constant, NS is the

slope of nonspecific binding and background is the amount of nonspecific binding [20].

2. In Prism, create an XY data table, with TNP-ATP concentration in the X column and fluorescence units in the Y column.
3. Choose nonlinear regression from the Analyze menu.
4. Choose saturation binding equations.
5. Choose *One site—Total*.
6. Constrain the “background” parameter to zero since the data has already been corrected for background fluorescence.
7. Perform curve fitting to determine $K_d^{\text{TNP-ATP}}$.

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Figure 1. The TNP-ATP kinase binding assay can be used to compare affinities of different nucleotides to the ATP binding pocket as well as testing for inhibitors

a) Fluorescence emission spectra (excitation = 410 nm) of TNP-ATP (1 μM) in Tris-HCl buffer containing 2 mM $MgCl_2$ in the absence (blue) or presence (magenta) of the CASK CaM-kinase domain (1 μM), after subsequent addition of EDTA (4 mM, to remove the Mg^{2+} ; yellow) and of Na^+ -ATP (500 μM ; green) to the same cuvette. **b.)** Titration of the TNP-ATP (1 μM) fluorescence as a function of the GTP (blue) and ATP (magenta) concentration in two parallel cuvettes containing CASK CaM-kinase domain (1 μM) in Tris-HCl buffer supplemented with 4 mM EDTA (excitation = 410 nm; emission = 540 nm). Data shown represents fluorescence units after subtraction of the TNP-ATP background fluorescence followed by normalization to the initial reading (relative units). **c.)** Inhibition of the TNP-ATP interaction with the CASK CaM-kinase domain by adenine nucleotides. The fluorescence (excitation = 410 nm; emission = 540 nm) of the CASK CaM-kinase domain/TNP-ATP complex (1 μM each) was measured in EDTA (4 mM) before and after addition of the indicated nucleotides (500 μM each). Fluorescence units were normalized to the respective initial readings following background subtraction (relative units). **d.)** Inhibition of TNP-ATP binding to the CASK CaM-kinase domain by divalent ions. The fluorescence (excitation = 410 nm; emission = 540 nm) of the CASK CaM-kinase domain/TNP-ATP complex (1 μM each) was measured in Tris-HCl buffer supplemented with EDTA (4 mM) or

2 mM of the indicated divalent cations. The data represent normalized fluorescent units following background subtraction (relative units) [11].

Figure 2. The TNP-ATP binding assay provides a robust method to test the effects of point mutations in a kinase domain

Fluorescence emission spectra of TNP-ATP binding in the presence of wildtype (WT) and mutant CASK. The protein (WT or mutant) is indicated in the upper right corner. Amino acid mutations at specific locations are shown. Control spectrum of TNP-ATP (1 μ M) in Tris-HCl buffer (pH 7.0) with EDTA (4 mM) (blue). Spectra of samples containing 1 μ M of the indicated recombinant CASK CaM-kinase domain, TNP-ATP (1 μ M) and EDTA (4 mM) in Tris-HCl buffer (pH 7.0) (green). Spectra of samples containing 1 μ M of the indicated recombinant CASK CaM-kinase domain, TNP-ATP (1 μ M) and 100 μ M $MgCl_2$ in Tris-HCl buffer (pH 7.0) (magenta). Samples were excited at 410 nm and spectra were recorded between 500 nm and 600 nm. The spectra are representatives of experiments repeated three times with essentially identical results [13].

Figure 3. The TNP-ATP binding assay can be used to compare nucleotide binding affinities under different conditions

Increasing amounts of TNP-ATP were added to cuvettes containing 10 mM Tris-HCl pH 7.0, 1 μM quadruple-mutant CASK (CASK^{4M}, a mutant engineered to bind nucleotide in the presence of magnesium), and either 4 mM EDTA (magenta symbols) or 200 μM Mg²⁺ (green symbols). The TNP-ATP fluorescence of the samples (excitation: 410 nm; emission: 540 nm) is plotted after subtracting background TNP-ATP fluorescence obtained with control samples, which contained the same TNP-ATP, Tris-HCl, EDTA or Mg²⁺ concentrations but lysozyme instead of CASK. The plot is representative of three independent experiments [13].

Table 1

Sample well preparation for microplate-based TNP-ATP saturation assay.

[TNP-ATP] in well	TNP-ATP solution	Volume of TNP-ATP solution	Volume of Tris-HCl buffer
1 μ M	0.2 mM	2.5 μ L	97.5 μ L
5 μ M	0.2 mM	5 μ L	95 μ L
10 μ M	0.2 mM	10 μ L	90 μ L
25 μ M	2 mM	2.5 μ L	97.5 μ L
50 μ M	2 mM	5 μ L	95 μ L
100 μ M	2 mM	10 μ L	90 μ L