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Determination of the Kinetics and Thermodynamics of Ligand Binding to a Specific Inactive Conformation in Protein Kinases

Sanjay B. Hari¹, Pratistha Ranjitkar, and Dustin J. Maly

Department of Chemistry, University of Washington, Seattle, WA 98195

Summary

Recent interest in inactive kinase conformations has generated the need to develop new biophysical tools to study them. Here, we describe the use of a fluorescent probe that selectively and potently binds to kinases in inactive, “DFG-out” conformations to obtain valuable thermodynamic and kinetic information.

Keywords

Kinase; Inactive conformation; DFG-out; Activation loop; Fluorescent probe

1. Introduction

Protein kinases are known to represent approximately 1.7% of all human genes (1). This number is a testament to the vast array of kinase-mediated signal transduction pathways that regulate cellular processes such as immunity, division, and morphogenesis (2). Due in part to the success of second-generation kinase inhibitors such as Gleevec (imatinib) (3), kinase research has expanded into studying how inactive conformations can be used to study regulation and develop new, targeted therapeutics. One commonly observed inactive kinase conformation, known as the “DFG-out” conformation, is characterized by a striking activation loop translocation and revealed allosteric binding site (4).

Structure-activity relationship (SAR) studies using known inactive, DFG-out inhibitors have been the primary method to determine which kinases can adopt inactive conformations. One caveat to SAR studies is that they require kinases to be enzymatically active, rendering mutants with limited activity incompatible with this approach. To this end, we have developed a small-molecule fluorescent probe that relies on binding rather than activity to assess conformation (5). Fluorescent probes have been used for decades to study protein conformation (6). These small molecules rely on increased quantum yield (or reduced fluorescence quenching) to signify a binding event. Our probe is comprised of a potent DFG-out inhibitor (7) conjugated to a BODIPY fluorophore and exhibits an increase in fluorescence upon active site binding.

¹Correspondence: Sanjay B. Hari, Department of Chemistry, Box 351700, Seattle, WA 98195, USA. Tel: (206) 616-4269; hari1@u.washington.edu.

Despite the clear utility of fluorescent probes that target the ATP-binding sites of protein kinases, few have been reported. For example, the bicyclic imidazole SK&F 86002 fluoresces in complex with the MAP kinase p38 (8), and a fluorescent analog of BIRB 796 has been used in p38 binding studies (9). However, these probes lack the target generality conferred by our inhibitor, which has been shown to inhibit kinases from multiple families.

Here we use this probe in a kinase titration assay to assess if a kinase is able to adopt the DFG-out inactive conformation. The experiment takes approximately one hour from start to finish and yields direct binding data that would be otherwise difficult to obtain. Additionally, we describe a kinetic competition assay which can be used to determine how quickly the probe conjugate dissociates from the kinase active site, providing kinetic insight into activation loop movement.

2. Materials

Prepare all buffers and solutions using ultrapure water with a resistivity of 18 MΩ at 25 °C. The fluorescent probe is light sensitive and should always be handled in the dark (see Note 1).

2.1. Assay components

1. Assay buffer (2×): 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM MgCl₂, 10% glycerol. Make 250 mL and sterilize using a 0.22 μm filter. Store at room temperature.
2. Pluronic F-68 10% (Sigma).
3. Dithiothreitol (DTT) (1M): Dissolve 1.54 g in 10 mL water. Thaw frozen aliquots as needed.

2.2. Reagents

1. Kinase of interest: purified to >90% homogeneity as determined by SDS-PAGE (see Note 2).
2. Competitor C1 (Fig. 1, left): Prepare according to Seeliger et al (7). Freeze 2 mM aliquots in DMSO.
3. Fluorescent probeF1 (Fig. 1, right): Prepare BODIPY-conjugated C1 according to Ranjitkar et al (5). Cover and freeze 1 mM aliquots in DMSO.

2.3. Special equipment and consumables

1. Black, 96-well microplate (see Note 3).

¹Ambient light should be kept to a minimum whenever handling fluorescent probe stocks or solutions containing probe.

²For best results, the kinase should be in a buffer free of imidazole and ionic detergents. Either desalting columns or dialysis membranes can be used for this purpose.

³Presumably, any black microplate exhibiting low autofluorescence can be used. We use PerkinElmer OptiPlate-96 F plates (#6005270) exclusively.

2. Microplate reader capable of fluorescence excitation at 485 nm and emission detection at 535 nm (see Note 4).
3. 96-well PCR microplates (see Note 5).

3. Methods

3.1. Affinity measurements with fluorescent probe

1. Mix 2.25 mL water, 2.25 mL assay buffer, and 4.5 μ L DTT in a 15-mL conical tube to yield a 1 $\times$ solution with 1 mM DTT.
2. Add 25 μ L Pluronic F-68 to the solution and set aside.
3. Mix 1 μ L of thawed F1 with 19 μ L DMSO to make a 50 μ M stock.
4. Mix 1.1 μ L of the 50 μ M stock with 53.9 μ L DMSO to yield a 1 μ M working stock. The remaining 50 μ M stock can be stored frozen for later use (see Note 6). Cover the working stock and set aside.
5. Perform a two-fold serial dilution of the kinase across one full row of a PCR microplate. Maintain the buffer composition of the kinase stock solution. Start with 80 μ L of 12 μ M stock to yield 40 μ L in each well.
6. Transfer 10 μ L of the kinase dilution in tandem to each of three rows of a black microplate.
7. Thoroughly mix 49.9 μ L of the 1 μ M F1 working stock with the assay buffer mixture. Dispense into a plastic reservoir.
8. Working quickly, add 110 μ L of the probe mixture in tandem to each row of the black microplate (see Note 7). Incubate without agitation in the dark for 30 min at room temperature.
9. Read the plate at Ex:485/Em:535 nm.
10. Use data analysis software (e.g. GraphPad Prism “Binding – Saturation, One site – Total”) to determine the probe dissociation constant (Fig. 2) (see Note 8).

⁴We use a PerkinElmer VICTOR3 Multilabel Plate Reader.

⁵Any microplate can be used for serial dilutions, but we have found low-volume, V-bottom plates such as those used for PCR to be optimal for this task. We use those sold by Phenix Research (#MPS-499)

⁶The quantum yield of the fluorescent probe diminishes significantly after multiple freeze-thaw cycles. Include a positive control in your plate to confirm probe viability.

⁷We have found that aiming the pipet tips directly at the wells and rapidly dispensing obviates the need to mix the solutions.

⁸Other data fitting programs such as QtiPlot can be used, but the equation used to fit the data must be entered manually. The binding equation used by GraphPad Prism is $Y = (B_{\max} * X) / (K_d + X) + NS * X + \text{“Background,”}$ where X is protein concentration, Y is binding (in this case fluorescence units), $B_{\max}$ is maximum binding, K_d is the dissociation constant, NS is the slope of nonspecific

3.2. Kinetic measurements of the off rate (k_{off}) for the fluorescent probe

1. Mix 1 mL water, 1 mL assay buffer, and 2 μL DTT in a 15-mL conical tube to yield a 1 $\times$ solution with 1 mM DTT.
2. Mix 109.8 μL 1 $\times$ solution with 0.6 μL Pluronic F-68 and set aside the remaining 1 $\times$ solution.
3. Prepare 10 μL of kinase solution at 10 μM and add 8.4 μL to the reaction mixture.
4. Mix 1 μL of thawed fluorescent probe with 19 μL DMSO to make a 50 μM stock (see Note 1).
5. Add 1.2 μL of the 50 μM stock to the reaction mixture and mix thoroughly by pipetting. The remaining 50 μM stock can be stored frozen for later use (see Note 6).
6. Incubate without agitation in the dark for 4 h at room temperature.
7. Shortly before the end of the incubation time, mix 886.5 μL 1 $\times$ solution with 4.5 μL Pluronic F-68 in a microcentrifuge tube.
8. Mix 12.5 μL of thawed C1 with 37.5 μL DMSO to make a 500 μM stock.
9. Add 9 μL of the 500 μM stock to the solution in the microcentrifuge tube and mix thoroughly by pipetting.
10. At the end of the incubation period, dispense 10 μL of the reaction mixture to each of three wells of a black microplate.
11. Add 290 μL of the competition solution to each well and read immediately at Ex:485/Em:535, taking readings every 10 min for 8 h (see Note 9).
12. Use data analysis software (e.g. GraphPad Prism “Binding – Kinetics, Dissociation – One phase exponential decay”) to determine the probe off rate (k_{off}) and dissociative half-life ($t_{1/2}$) (Fig. 3) (see Note 10).

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binding (assumed to be linear), and “Background” is binding in the absence of ligand. GraphPad Prism fits B_{max} , K_d , NS, and “Background” given a data set of X and Y. The “Background” variable can be constrained if its value is known: omit the twelfth well during the titration in step 3.1.5 to empirically determine background fluorescence.

¹⁰Other data fitting programs such as QtiPlot can be used, but the equation used to fit the data must be entered manually. The kinetic equation used by GraphPad Prism is $Y = (Y_0 - NS) * \exp(-K * X) + NS$, where X is time, Y is binding (in this case fluorescence units), Y_0 is Y at time zero, NS is binding after a long time, and K is the rate constant. GraphPad Prism fits Y_0 , NS, and K given a data set of X and Y. The variable Y_0 can be constrained if its value is known (see Note 9).

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Fig. 1.
Competitor C1 (left) and fluorescence conjugate F1 (right)

Fig. 2. Binding titration plots of p38α (open circles) and p38δ (closed circles), representative of kinases that can (p38α) and cannot (p38δ) adopt the DFG-out inactive conformation. Data were fit using GraphPad Prism software to determine a dissociation constant (K_d) of 14 nM for p38α.

Fig. 3. Dissociation of fluorescent probe conjugate from p38α. Data were fit using GraphPad Prism software to determine an off rate (k_{off}) of $161 \mu\text{s}^{-1}$ and a dissociative half-life ($t_{1/2}$) of 72 min.