Chapter 2

Measurement of GTP-Binding and GTPase Activity of Heterotrimeric Gα Proteins

Swarup Roy Choudhury, Corey S. Westfall, Dieter Hackenberg, and Sona Pandey

Abstract

Heterotrimeric G-proteins are important signaling intermediates in all eukaryotes. These proteins link signal perception by a cell surface localized receptor to the downstream effectors of a given signaling pathways. The minimal core of the heterotrimeric G-protein complex consists of Gα, Gβ, and Gγ subunits, the G protein coupled receptor (GPCR) and the regulator of G-protein signaling (RGS) proteins. Signal transduction by heterotrimeric G-proteins is controlled by the distinct biochemical activities of Gα protein, which binds and hydrolyses GTP. Evaluation of the rate of GTP binding, the rate of GTP hydrolysis, and the rate of GTP/GDP exchange on Gα protein are required to better understand the mechanistic aspects of heterotrimeric G-protein signaling, which remains significantly limited for the plant G-proteins. Here we describe the optimized methods for measurement of the distinct biochemical activities of the Arabidopsis Gα protein.

Key words Heterotrimeric G-protein, Gα protein, GTP-binding, GTP hydrolysis, GTPase activity, GDP/GTP exchange, Arabidopsis GPA1

1 Introduction

G-proteins, localized at the inner surface of the plasma membrane, are key signaling intermediates in eukaryotes [1, 2]. In mammals, the importance of G-proteins in regulating fundamental signaling pathways involved in sensory perception (vision, olfaction, and taste), neurotransmission, hormone perception, and immunity-related cues has prompted in-depth characterization [1–3]. Such studies have revealed an elegant signaling mechanism, where the Gα subunit acts as a bimodal molecular switch, alternating between signal-dependent Gα·GDP and Gα·GTP conformations. In the inactive state, Gα·GDP associates with the Gβγ dimer, which represents the "off" signaling status. Signal perception by a G-protein

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coupled receptor (GPCR) causes the exchange of GTP for GDP on Gα. The GPCR thus acts as a guanine nucleotide exchange factor (GEF) causing the dissociation of the Gαβγ trimer. The active Gα·GTP and the freed Gβγ dimer then transduce the signal downstream by interaction with different effectors. The intrinsic GTPase-activity of Gα causes hydrolysis of the bound GTP, regenerating Gα·GDP, which re-associates with the Gβγ dimer [1, 2]. The GTPase activity of the Gα proteins is regulated by a group of accessory proteins, e.g., the regulators of G-protein signaling (RGS) proteins. The RGS proteins, by enhancing the GTPase activity of Gα, accelerate the rate of G-protein cycle [4, 5]. This classic signaling mechanism thus entails three biochemically distinct reactions of the Gα of the heterotrimer: *the rate of GTP binding*, *the rate of GTP hydrolysis*, *and the rate of GTP/GDP exchange,* which control all physiological responses regulated by G-proteins.

The biochemical activities of mammalian Gα proteins have been characterized in great detail, and it has been established that the rate of GTP-binding on Gα proteins is the rate limiting step for mammalian G-protein signaling. In contrast, only few plant Gα proteins have been characterized at the biochemical level, and many of them exhibit relatively higher rates of GTP-binding but slower GTPase activities [6–11]. This suggests that the GTPase activity of Gα could be the rate limiting step of the G-protein cycle in higher plants, although detailed characterization of multiple Gα proteins from different plant lineages is needed to make a generalized statement. In the following sections, we describe optimized methods for purification of plant Gα proteins and their biochemical characterization using radiolabeled or fluorescently labeled tags.

2 Materials

2.1 Reagents

1. Purified Gα protein (at least 95 % pure).
2. Purified recombinant RGS domain (for performing GTPase assay in the presence of RGS protein).
3. 5 mM GTP (Guanosine triphosphate).
4. 5 mM GDP (Guanosine diphosphate).
5. 5 mM ATP (Adenosine triphosphate).
6. 5 mM ADP (Adenosine diphosphate).
7. [^{35}S]GTPγS (Perkin-Elmer, 1,250 Ci/mmol).
8. [^{35}S]GTPγS-binding reaction buffer (Tris pH 8.0, 50 mM; $MgCl_2$, 10 mM; DTT, 1 mM).
9. [^{35}S]GTPγS-binding wash buffer (Tris pH 8.0, 20 mM; $MgCl_2$, 25 mM; NaCl,100 mM).
10. GF/B filter disks (Millipore).
11. Scintillation cocktail (OptiPhase HiSafe 2, Perkin-Elmer).

12. [α^{32}P]GTP (Perkin-Elmer, 3,000 Ci/mmol).
13. PEI cellulose TLC plates (Sigma-Aldrich).
14. TLC assay reaction buffer (Tris pH 8.0, 50 mM; $MgCl_2$, 10 mM; EDTA, 1 mM; DTT, 1 mM).
15. TLC assay stop solution (EDTA, pH 8.0, 0.5 M).
16. TLC assay developer solution (KH_2PO_4, pH 3.4, 0.5 M).
17. BODIPY-assay reaction buffer (Tris pH 8.0, 10 mM; $MgCl_2$, 10 mM).
18. 5 mM BODIPY-GTPγS (Invitrogen).
19. 5 mM BODIPY-GTP FL (Invitrogen).
20. Bovine serum albumin (fraction V powder, Sigma-Aldrich).
21. 5 mM Mant-GTP (Invitrogen).
22. 5 mM Mant-GDP (Invitrogen).
23. Mant reaction Buffer (Tris pH 8.0, 20 mM; NaCl, 100 mM; $MgCl_2$, 10 mM).

2.2 Equipment

1. Water bath (30 °C).
2. Vacuum-filtration device.
3. Scintillation vials.
4. Scintillation counter.
5. Cassette and phosphor screen.
6. Phosphorimager.
7. 96 Well Polystyrene Microplates (Greiner Bio One).
8. Fluorescence microplate reader (FLUOstar Optima, BMG Lab Technologies or equivalent).
9. Amicon© Centrifugal Filters (10 KDa cut off).
10. Olis© DM45 spectrofluorimeter with a 150-W xenon lamp, stopped-flow accessory, and water bath.
11. 420 nm cutoff-filter.
12. Fitting software such as KinTek Global Kinetic Explorer [12].

3 Methods

3.1 Radiolabeled GTP-Binding Assay

1. Adjust purified, recombinant Gα protein concentration to 100 nM in 200 µL of [^{35}S]GTPγS-binding reaction buffer.
2. Add 0.2 µM [^{35}S]GTPγS and incubate samples at 30 °C in a water bath for up to 2 h (*see* **Note 1**).
3. Take a small reaction aliquot (5–10 µL) in a separate tube kept chilled on ice at desired time points (*see* **Note 2**).
4. Add 1 mL of ice-cold wash buffer to the aliquot immediately to stop the reaction. Keep tubes on ice.

5. Wash a GF/B filter with ice cold wash buffer using a vacuum filtration device.
6. Filter the reaction mixture through the GF/B filter, followed by washing with 3–5 mL of ice cold wash buffer.
7. Dry GF/B filters (air dry or oven dry) and place them in a scintillation vial.
8. Add 5 mL of scintillation cocktail to each vial and measure the incorporated radioactivity using a scintillation counter.
9. Analyze and fit data using appropriate software of your choice.

3.2 GTPase Assay Using Thin Layer Chromatography

1. Adjust purified, recombinant Gα protein concentration to 100 nM in 200 μL of TLC assay reaction buffer.
2. Add 20 pmol [α^{32}P]GTP and incubate samples at 30 °C in a water bath for up to 2 h.
3. Take a 10 μL aliquot in a separate tube kept chilled on ice at desired time points (*see* **Notes 1** and **3**).
4. Add 10 μL of 0.5 M EDTA to the tubes immediately to stop the reaction. Keep tubes on ice.
5. Spot 1 μL of reaction on to PEI-cellulose TLC plates and air dry. Repeat this step two more times, each time spotting on top of the original spot.
6. Develop plate in 0.5 M KH_2PO_4 (pH 3.4) solution and dry.
7. Expose plate to a phosphorimager screen for 6–12 h. Scan using a phosphorimager.

3.3 GTP Binding Assay Using BODIPY-GTP or BODIPY GTPγS

1. Use 50 mL BODIPY assay buffer to make 10 nM BODIPY-GTPγS-FL or BODIPY-GTP-FL solutions. BODIPY-GTPγS-FL is used for detection of GTP-binding only while BODIPY-GTP-FL can be used for estimation of both GTP-binding and GTP-hydrolysis.
2. Adjust Gα protein concentration to 200 nM in BODIPY assay buffer.
3. Add 100 μL of 200 nM Gα protein in 100 μL assay buffer in 96 well, flat bottom polystyrene microplates and mix properly. A total three to five replicates for each reaction are highly recommended. Reaction mixes containing 100 nM BSA and without any protein should be included as negative controls with each run.
4. Wash the injection tubes getting in and out of the fluorescent plate reader with sterile water, followed by BODIPY-assay buffer.
5. Add 100 μL of 10 nM BODIPY-GTPγS or 10 nM BODIPY-GTP FL solutions to the protein solution to initiate the reaction using the automatic injector of the plate reader. The final protein concentration is 100 nM.

6. Record fluorescent reading using following set up for FLUOstar Optima, BMG Lab Technologies fluorescent plate reader: Positioning delay-0.1 s, number of kinetic windows-1, number of cycles-100, number of flashes per cycle-10, filter and integration-fluorescence intensity, number of multichromatics-1, excitation filter-485 nm, emission filter-520, gain-1,500, pump speed-420 μL/s, pump prime volume-500 μL, temperature-25 °C, data recording-100 cycles every 16 s (total time per read: ~30 min). These parameters can be adjusted to desired specifications for any available fluorescent plate reader.
7. For competition experiments, 5 μM of non-labeled nucleotides (GTP/GDP/ATP/ADP) can be added to the assay buffer before starting the reaction. The additives should also be included in the negative controls.
8. For the effect of RGS protein on the rate of GTP hydrolysis, recombinant RGS domain can be added to the reaction mix at a stoichiometric ratio of RGS to Gα at 2:1. A reaction mix containing only RGS protein should be included as a negative control for these assays.
9. Subtract fluorescent readings for the negative controls at each time point and plot relative fluorescence of the proteins as a factor of time using any software of your choice. When using BODIPY GTP-FL, the increase in fluorescent with time denotes GTP-binding and subsequent decrease in fluorescent with time denotes GTP hydrolysis by Gα protein.

3.4 GTP-Binding Using Mant-GTP

1. Using the Amicon©, exchange purified protein into Mant-GTP reaction buffer and concentrate to 2 μM, keep on ice (*see* **Note 4**).
2. 5 mM Mant-GTP should be diluted into reaction buffer to form 20, 30, 40, 50, 100, 160 μM, keep on ice (*see* **Note 5**).
3. Wash fluorometer injection tubes (Olis© DM45 spectrofluorimeter or equivalent) 3 times with reaction buffer.
4. Fill stopped-flow syringes with Gα protein solution and Mant-GTP solution.
5. Run 2 injections to wash machine.
6. Let solutions come up to 20 °C for 10 min.
7. Set excitation wavelength to 280 nm and use the >420 nm cut-off for emission. This actually tracks the FRET between a tryptophan and the Mant group. If there is no FRET, an excitation of 350 nm can be used to directly measure Mant fluorescence. FRET is usually less noisy, so it is recommended if possible.
8. Run 1 injection, adjust PMT voltage as needed.
9. Run 9–11 injections, collecting around 1–2 s per injection.
10. Repeat **steps 3**–**7** for each Mant-GTP concentration.

3.5 Analysis of Mant-GTP Binding Data

1. Normalize all sample runs.
2. Average normalized samples.
3. Repeat for each Mant-GTP concentrations.
4. Depending on the protein, fit the data to either a single or double exponential curve.
5. A graph of Mant-GTP versus observed rate constants should be a line with a slope equal to the binding constant and an *x*-intercept of the dissociation constant. The Mant-GTP concentration used should be half of starting concentration due to the 1:1 mixing with the protein solution in the stopped-flow.
6. For data that is fit to double exponential curves, one set of observed rate constants will behave linearly with a slope equal to the binding constant and an *x*-intercept of the dissociation constant. The second observed rate constant should be constant for all Mant-GTP concentrations and corresponds to a conformational change upon binding [13] (*see* **Note 6**). The equation for the linear fit is $k_{obs} = k_{-1} + k_1[\text{Ligand}]$.

3.6 Measurement of GDP/GTP Exchange

1. Using the Amicon©, exchange purified protein into reaction buffer and concentrate to 2 μM, keep on ice as in Subheading 3.4 **step 1** (*see* **Note 4**).
2. Add Mant-GDP directly to the protein solution to a final concentration of 5 μM (*see* **Note 7**).
3. Make solutions of GTP (no Mant group) of 40, 80, 160, 320 μM in Mant reaction buffer.
4. Fill stopped-flow syringes with Gα protein plus Mant-GDP solution and GTP solution.
5. Run 2 injections to wash machine.
6. Let solutions come up to 20 °C for 10 min.
7. Set excitation wavelength to 280 nm and use the >420 nm cutoff for emission.
8. Run 1 injection, adjust PMT voltage.
9. Run 9–11 injections collecting 30–45 s per injection.
10. Repeat **steps 4–9** for each GTP concentration.

3.7 Analysis of Nucleotide Exchange Data

1. Normalize all 9–11 sample runs and average normalized samples.
2. Repeat for each GTP concentrations.
3. Data for each GTP concentration should be able to fit to a single-exponential curve. If not, removal of beginning points or end points might be necessary.
4. A graph of the observed rate constants should be hyperbolic. If the rate constants do not reach a plateau, higher GTP concentrations might need to be used.

5. The observed rate constant for saturated GTP corresponds to the dissociation rate of the Mant-GDP [14] (*see* **Note 8**).

4 Notes

1. Strictly follow all safety procedures for handling and disposal of radioisotopes and liquid and solid waste containing radiochemicals. Survey the usage area and equipment before and after the use of radioisotopes. Follow your institutional guidelines for recording of radioisotope usage and disposal.
2. The aliquots should be collected every 30 s to 1 min, if possible to achieve better resolution of binding kinetics at the initial time points. Three to five aliquots should be collected for each time point. For strong GTP-binding proteins, 30–60 min incubation is sufficient.
3. The aliquots should be collected every 30 s to 1 min at the initial time points and should be added directly to ice cold tubes containing chilled EDTA. Three to five aliquots should be collected for each time point. Maximum 18 samples can be spotted on a standard TLC plate for each run.
4. Protein concentration needs to be calculated using accurate techniques, such as absorbance at 280 nm.
5. Exact concentrations of protein, Mant-GTP, and Mant-GDP will depend on the protein being used. To aid in analysis, always maintain pseudo-first order kinetics by keeping the nucleotide 10× higher than the protein.
6. This analysis only works if you always maintain pseudo-first order kinetics. If you are not pseudo-first order, you must either fit computationally, using a program like KinTek©, or use a more complicated fitting equation [13].
7. Mant-GDP concentration needs to be high enough to saturate binding sites.
8. Some proteins might have a negative hyperbolic curve for the exchange reaction. *See* ref. 14 for this circumstance.

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