Exchange Kinetics and Activity analysis of GTP-Binding Proteins with Fluorescent GTP-Analogs

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ABSTRACT

Guanine Tri-phosphate (GTP) Binding proteins function as mediators in important intracellular pathways and are mainly present in cell membranes and the cytoplasm. They function as molecular switches that initiate several intracellular signaling pathways by GDP and GTP exchange. Ras is an important member from the family of GTP-binding proteins, which is present in the cell membrane. It plays an important role in crucial cell mechanisms such as gene expression, cell proliferation and cell differentiation. Mutated Ras can lead to abnormal signaling and uncontrolled cell proliferation resulting in cancer. Studying the association and dissociation kinetics of the fluorescent GTP analogs with Ras can be helpful in obtaining information about the binding kinetics of GTP molecules under different activation states. In this study, we have investigated the exchange kinetics of fluorescent GTP-analogs with Ras and the structural changes that occur upon binding. We have used fluorescence spectroscopy and biochemical techniques with fluorescent GTP-analogs such as BODIPY FL GTP-γ-S and MANT-GMPPNP. The absorption cross-sections of these analogs are reduced systematically upon Ras titration with concurrent fluorescence enhancement. Our results indicate an equilibrium constant of 1.1 for BODIPY FL GTP-γ-S with Ras. The fluorescence enhancement is correlated with structural restrictions of the protein environment on the quenching mechanism of the fluorescently tagged GTP. Our long term goal is to investigate the in vivo activities of Ras protein in live cancer cells.

1. INTRODUCTION

Small GTP binding proteins (also known as G-proteins) are monomeric proteins that comprise of a large family of more than 50 GTPases (1) with molecular masses ranging from 20-40 kDa (2,3). This family includes Ras, Rho, Cdc42, Rac, Rap,Ral, Sar1/Arf1, and Ran (1). These proteins act as molecular switches that regulate a number of intracellular functions such as cell growth, cell differentiation, apoptosis, cell adhesion, migration, vesicular trafficking, endocytosis, exocytosis, gene expression, cell transformation, cell shape and motility (1). Most importantly, these GTP-binding proteins regulate several downstream signal transducing effectors in different signaling pathways (1). The activity of these GTP binding proteins mainly depends upon the exchange of GTP and GDP molecules at the GTP binding sites. GTP-Binding proteins switch between the active state (i.e., GTP bound form) and the inactive state (i.e., GDP bound). During normal cell function, these switches exchange between active and inactive state in the presence of biochemical and physical cues.

Due to varying conditions such as mutations in the GTP binding protein expressing genes, these proteins can loose their function causing a continuous activation or inactivation of
these switches (1, 3). There are several other molecules such as glucose present in the cell environment that can possibly affect and support the abnormal cell growth. Glucose is an important reactant in the glycolysis process, which the main energy metabolic pathway in cancer cells.

Fluorescence-based techniques are a non-invasive approach that allows the monitoring of cellular processes without interfering with the cell machinery. However, most of native biomolecules (such as G proteins) are not intrinsically fluorescent and researchers have to rely on use of fluorescent tags to monitor their biological role in cell functions. However, one question remains concerning, which is the affects of using fluorescent labels on bimolecular activities of cells. Also, the quantitative analysis of role of Ras in glycolysis requires basic knowledge of the correlation between the fluorescence properties of the tag and the biological function of interest. At this preliminary stage of this project, we investigated these questions in Ras and the exchange kinetics of GTP molecules with fluorescent analogs.

2. MATERIALS AND METHODS

2.1 Protein: Human Ras (Sigma, R9894) was chosen for its importance in numerous biological systems. The fluorescent GTP analogs used to tag Ras were BODIPY FL GTP-γ-S (Sigma, G22183) and MANT-GMPPNP (Sigma, M22353). These fluorophores were purchased from molecular probes and were used as received without further purifications. The excitation/emission wavelengths are 504/520 nm for BODIPY FL GTP-γ-S and 357/447 for MANT-GMPPNP. The structures of these GTP analogs are shown in Figure 1.

![A. Structure of MANT-GMPPNP](image1.png) ![B. Structure of BODIPY FL GTP-γ-S](image2.png)

Figure 1: Molecular structures of fluorescent GTP analogs to be used in this study.

2.2 Buffer Preparation:

Hepes Buffer used as a media for the experimental procedures was prepared using 2.383g of Hepes salt and 5.8g of NaCl. These salts were added to 1000 ml of double-distilled water and mixed well using a magnetic stir bar, filtered and stored at -20°C. The molarity of this buffer obtained was 100mM and was at a pH 7.5, which was supplemented with 100mM in NaCl. NaCl was added to the buffer to avoid degradation of the protein during the experiment.
2.3 Absorption Spectrum BODIPY FL GTP-γ-S and MANT-GMPPNP

A stock solution of 50 mM BODIPY and MANT-GMPPNP were diluted using Hepes buffer to obtain a final concentration of 0.15 µM BOBIPY and MANT respectively. These samples were used to obtain the absorption spectrum of both fluorophores using a spectrophotometer (DU 800; Beckman Coulter). A 3 mm X 3 mm cuvette made from fused silica was used to make absorption measurements.

2.4 Fluorescence emission spectra measurements

Sample containing 2 µM BODIPY FL GTP-γ-S in 10mM Hepes buffer was titrated with increasing concentrations of Ras ranging between 0 µM to 8 µM. Each sample was excited at two wavelengths: 340 and 470nm. The fluorescence of each sample was collected between 350-700 nm when excited at 340 nm and 480-700 nm when excited at 470 nm. Fluorescence emission of these samples was plotted against the concentration range for each sample to obtain the peak fluorescence. The spectrofluorometer was set at RT for right angle measurements of the sample.

2.5 Determination of Dissociation Constant

The 2 µM of BODIPY was added to 10mM Hepes buffer and 8 samples, 200 µL each were prepared. Eight different concentrations of Ras (ranging between 0.5 µM to 8 µM) were added to eight separate Hepes-BODIPY solution samples. Fluorescence measurements of these eight samples were obtained using spectrofluorometer (FL3-21; Fluorolog). The fluorescence of BODIPY in the absence of Ras was also measured. Blank solution containing 10 mM Hepes was used to measure fluorescence of Hepes buffer if any. Water was also used to verify the absorption spectrum of blank by comparing the differences between the fluorescence data of Hepes and water.

![Figure 2: Illustration of the setup inside of a spectrofluorometer](image)

The fluorescence emitted by the sample and collected by the detector is different for different molecules and also varies with varying excitation wavelengths as shown in the setup in Figure 2.
2.6 Fluorescence Lifetime Imaging:

Fluorescence lifetime imaging is a technique that can be used to exploit the sensitivity of excited state dynamics of a protein to its surrounding environment containing the fluorophore. The technique is based upon emission of fluorescence photons emitted by excited electrons while returning back to the ground state converting the absorbed energy internally to fluorescence. The time resolved anisotropy also provides valuable information about the structure of the molecule being studied, its surrounding environmental factors such as ionic strength, hydrophobicity, viscosity, oxygen concentration, concentrations of glucose and other such molecules. When a large number of similar molecules within a local environment are excited using a short laser pulse, the fluorescence decay function for these molecules is a single exponential [Becker-Hickl]. The fluorescence decay lifetimes of commonly used fluorophores in microscopy are of the order of few nano-seconds [Becker-Hickl]. Fluorescence lifetime imaging requires a pulsed excitation source with a definite repetition rate ranging between 76 MHz and a pulse width in the range of femto-second to pico-second.

2.7 Laser Scanning Microscopy:

Figure 3: Jablonski Diagram (Principles of fluorescence spectroscopy, Joseph R. Lakowicz, 1983) illustrating the different energy states and the associated fluorescence transitions.

Figure 4: Setup of a two photon laser scanning microscope.
The laser beam is focused into the optical setup using a series of lenses and dichroic mirrors prior to being focused into the sample by the objective lens of the microscope. The focused beam excites the sample and it emits light which is collected and focused by the objective lens, passes through the scanner and is reflected by the dichroic mirror into the detector. Since the two photon microscopy causes less scattering, hence the laser beam can penetrate deeper into the tissue and also causes less photo damage to the sample. The light detected by the detector is processed by Single Photon Counting Module-SPCM [Becker-Hickl] and plotted using non-linear least squares fitting algorithm.

A combination of Fluorescence lifetime imaging and Laser scanning microscopy will be used for imaging the lifetime decay of Ras at different concentrations of the analog in its environment.

3. RESULTS:

3.1 Absorption Spectrum: Absorption spectrum of BODIPY FLGTP-γ-S shows three absorption peaks at 256 nm, 365 nm and 505 nm whereas the absorption spectrum of MANT-GMPPNP shows two absorption peaks at 255 and 363 nm respectively.

![Absorption spectra BODIPY and MANT fluorophores](image)

The absorption of both the dyes gets reduced upon binding to Ras. Absorbance of BODIPY got reduced from 0.19 to 0.09 upon addition on 15µM Ras. Similarly absorption of MANT-GMPPNP was reduced from 0.01 to about 0.005. Absorption for both dyes was quenched by an average of upto 50% upon addition of 15 µM Ras.
3.2 Fluorescence Emission Spectrum BODIPY:

Hepes buffer (10mM) containing 2 µM BIODIPY was titrated with Ras ranging between concentrations of 0-8 µM. Upon excitation of BODIPY at 340 and 470 nm in the presence of different concentrations of Ras the following fluorescence curves were obtained.

![Fluorescence vs. wavelength spectra for BODIPY at varying Ras concentrations](image)

(i) Excitation 340nm  
(ii) Excitation 470nm

Figure 6: Fluorescence vs. wavelength spectra for BODIPY at varying Ras concentrations

At 340 nm excitation the net fluorescence of the mixture of BODIPY increases with addition of higher concentrations of Ras. Similar pattern of increasing fluorescence for observed when the titration samples were excited at 470 nm.

The net areas under each curve corresponding to different concentrations of Ras were calculated. The area under each fluorescence curve signifies average fluorescence of the sample at that particular concentration. The average fluorescence of each sample, at different concentrations of Ras was plotted against their corresponding concentrations.

![Area vs. Ras concentrations for calculating the saturating fluorescence levels at equilibrium](image)

(i) Excitation at 340 nm  
(ii) Excitation at 470 nm

Figure 7: Area vs. Ras concentrations for calculating the saturating fluorescence levels at equilibrium
Using the average fluorescence vs. Ras concentration curve, the dissociation constant Kd for Ras-BODIPY system was calculated separately for excitations at 340 and 470. The Kd for both excitation curves were calculated and were found to be very similar, 0.89 consequent to excitation at 340 and 1.2203 for excitation at 470. Hence the average Kd for the system was estimated to be an average of approximately 1.1.

4. DISCUSSION

Absorption peaks BIODIPY and MANT: Peaks obtained in the absorption spectrum of BODIPY FLGTP-γ-S at 365nm and 505nm correspond to the absorption of the fluorophore and the base respectively. A similar pattern was observed in the absorption spectrum of the MANT-GMPPNP where it showed two absorption peaks at 255 and 363nm. Both the fluorophores have one common absorption band in the range of 360-365nm which corresponds to the common Guanine Di-phosphate base that both the dyes have in common, whereas the 505nm for BODIPY and 255nm for MANT correspond to the absorption bands of the fluorophore linked to the base through the linker as can be seen in Figure1.

The absorbance quenching for BODIPY is due to the binding of Ras to BODIPY which restricts its free movement hence restricting its capability to absorb the incident light. This argument can be further supported by analyzing the quenched absorption peaks separately for the fluorophore and the base, at 365 and 505 nm.

Absorption of the common Guanine base (at 365 nm) decreased from 0.025 to 0.0012 and also the absorption of the fluorophore (at 505 nm) decreased from 0.19 to 0.08. Similarly for MANT-GMPPNP, a net quenching of absorption was observed at both the peaks. The absorption decreased from 0.0829 to 0.029 for the band corresponding to the fluorophore at 255 nm and from 0.0079 to 0.0028 corresponding to the Guanine base at 363 nm. This shows that the net fluorescence of the common base gets quenched by 95% for BODIPY and 64% for MANT. The fluorescence of fluorophores gets quenched by 57% for BODIPY and 65% for MANTGMPPNP. The quenching interaction is weaker in compounds with long linkers between bases and fluorophores (7) which is what was observed from the fluorescence quenching behavior of BODIPY and MANT fluorophores but not the bases. The BODIPY fluorophore which is attached to the base via a longer linker exhibited a net lower fluorescence quenching as compared to MANT which is linked to the base with a smaller linker molecule. A completely opposite result was observed for the BODIPY and MANT Guanine base fluorescence quenching. The possible reason behind higher fluorescence quenching in the case of BODIPY base can be because of greater hindrance of the long linker molecule with the aromatic base chain, as it is closer to base in case of BODIPY as compared to MANT. In the case of MANT the linker molecule being smaller and on the opposite end from the aromatic Guanine base ring, has lower interaction with the linker and hence a lower reduction of absorbance. Fluorescence on the other hand increases with increasing Ras concentrations which shows that the fluorescence of Ras bound BODIPY is higher than BODIPY itself.
5. FUTURE DIRECTIONS

The main interest in determining the dissociation constant of Ras-BODIPY system in solution is to understand the kinetics of the binding and unbinding of the GTP analog (BODIPY) with Ras. Knowing the dissociation constant, time resolved anisotropy measurements are currently being conducted in order to have a better understanding of the conformational changes in Ras upon activation. The main goal is to correlate the molecular dynamics of Ras-GTP analog complex with Ras activity in normal and cancer cells under different physiological conditions. However specific labeling of different G-proteins in live cells with GTP analogs is quite challenging. GTP-analog microinjection and expression of GFP-cDNA plasmid are the two different protein labeling strategies that we are further planning to investigate in both cancer and normal cells.

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