

Rapid kinetics of G protein subunit association: a rate-limiting conformational change?

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Abstract G protein subunit association and dissociation are thought to play an important role in signal transduction. We measured $\alpha\beta\gamma$ heterocomplex formation using resonance energy transfer. Fluorescein-labelled α (F- α) emission was quenched ~10% on mixing with eosin-labelled $\beta\gamma$ (E- $\beta\gamma$). Unlabelled $\beta\gamma$ did not quench F- α fluorescence. Stopped-flow kinetics showed a $t_{1/2}$ ranging from 2.5 s to 0.25 s for 50 nM to 1200 nM E- $\beta\gamma$. The rate saturated at high E- $\beta\gamma$ concentrations consistent with a two-step mechanism. We report the first rapid-mix studies of G protein subunit association kinetics which suggest that α and $\beta\gamma$ combine by a two-step process with a maximal rate of $4.1 \pm 0.4 \text{ s}^{-1}$.

Key words: Resonance energy transfer; Rapid kinetics; Fluorescence

1. Introduction

G proteins transmit signals to many effectors including adenylyl cyclase, K^+ and Ca^{2+} channels, and phosphodiesterases [1–3]. Heterotrimeric G proteins consist of α , β , and γ subunits [4] and are activated by seven transmembrane spanning receptors [5,6]. Binding of ligand to receptor causes exchange of GDP for GTP on the α subunit which induces a conformational change in the heterocomplex and subsequent activation of the subunits [2,5]. The heterocomplex is thought to dissociate into α and $\beta\gamma$ subunits each capable of mediating cellular responses [2,7–9]. While dissociation is well documented in detergent solutions it has been hard to examine in natural or synthetic membranes.

To better understand the physical interactions of G protein subunits during activation and deactivation we used fluorescent G protein subunits. With different fluorophores on the α and $\beta\gamma$ subunits, subunit interactions can be followed in real time by resonance energy transfer (RET). We report the first rapid kinetic analysis of G protein subunit association and propose a two-step model for association. An initial pre-equilibrium interaction occurs with an affinity ~300 nM, followed by the appearance of a high affinity interaction occurring with a maximum rate of $\sim 4 \text{ s}^{-1}$. This conformational change may limit the rate of deactivation of G protein α subunits after GTP hydrolysis in the physiological setting.

2. Materials and methods

2.1. Materials

Fluorescein 5-isothiocyanate (FITC) and eosin-5-isothiocyanate (EITC) were purchased from Molecular Probes, Inc. (Eugene, OR). [^{35}S]GTP γS was obtained from New England Nuclear (Boston, MA). Other reagents were from standard suppliers.

2.2. Fluorescent subunit preparation

Fluorescein- and eosin-labelled G_o were prepared essentially as de-

scribed [10]. Singly labelled F- G_o was isolated by Mono-Q chromatography. Free eosin was removed from eosin-labelled- G_o by gel filtration on Sephadex G-50 and Centricon 30 ultrafiltration. Individual labelled subunits F- α and E- $\beta\gamma$ were resolved by activation in AMF (10 mM MgCl_2 , 10 mM NaF, 20 μM AlCl_3) in TEDN buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) containing 0.3% sodium cholate followed by heptylamine Sepharose chromatography in a sodium chloride and cholate gradient and storage in aliquots at -70°C for several weeks prior to use. [^{35}S]GTP γS binding activity from two F- α preparations were 1.2 nmol/mg and 3.4 nmol/mg.

2.3. Rapid-mix subunit association kinetics

G protein association and dissociation kinetics were studied at 20°C using an Applied Photophysics model DX-17MV stopped-flow fluorimeter. The use of resonance energy transfer to detect proximity of subunits is illustrated in Fig. 1. Quenching of F- α was measured by exciting at 460 nm, with slit widths of 14 nm and emission was detected using a $510 \pm 10 \text{ nm}$ (500–520 nm range) band pass filter. Data were collected with a 2 ms time constant and a 999.5 V photomultiplier tube setting. Purified subunits were diluted at least 20-fold in HEDNML buffer (50 mM HEPES, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.1% Lubrol PX, pH 8.0) with either 1.5 mM or 10 mM MgCl_2 at 20°C . Similar results were seen with both 1.5 and 10 mM MgCl_2 in the buffer so results were pooled. Samples were kept on ice prior to use, then loaded into the rapid-mix syringe chamber at 20°C . Fifty μl of each reactant were used per shot and all data are averages of 2 to 9 shots.

2.4. Rapid-mix data analysis and presentation

Rate constants and amplitudes for heterocomplex association experiments were determined from the averaged data at each E- $\beta\gamma$ concentration fit to the simple exponential function:

$$F(t) = \Delta F \times \exp(-k \times t) + F_\infty \quad (\text{Equation 1})$$

where $F(t)$ represents the fluorescence over time (t), ΔF represents the change in fluorescence, F_∞ is fluorescence at long times and k is the rate constant.

3. Results and discussion

3.1. Detection of fluorescent G protein subunit association

Because the quenching of F- α fluorescence was too fast to study by manual methods (not shown) we utilized stopped-flow analysis to monitor association of F- α with E- $\beta\gamma$. Mixing of fluorescently labelled subunits F- α (60 nM) and E- $\beta\gamma$ (150 nM) in the rapid mix apparatus at 20°C results in the time-dependent quenching of F- α emission (Fig. 2). Mixing F- α with buffer

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Abbreviations: GTP γS , guanosine 5'-3-O-(thio)triphosphate; E- $\beta\gamma$, eosin- $\beta\gamma$; F- α , fluorescein- α ; RET, resonance energy transfer, FITC, fluorescein-5-isothiocyanate; EITC, eosin-5-isothiocyanate.

Resonance Energy transfer between F- α and E- $\beta\gamma$

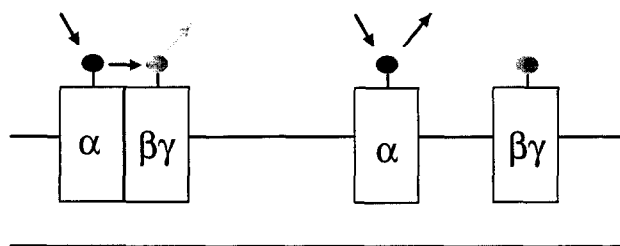


Fig. 1. Resonance energy transfer between G- α and G- γ to study association. G protein subunits are labelled with fluorescein and eosin. When the two fluorescent groups are in close proximity (ca. 50 Å) there is transfer of energy from the donor (fluorescein) to the acceptor (eosin). The fluorescence of the donor is quenched and the fluorescence of the acceptor is enhanced.

alone or with unlabelled $\beta\gamma$ does not cause significant quenching of the fluorescein signal. The amount of quenching is approximately 10% of the total fluorescein signal. The lack of effect of unlabelled $\beta\gamma$ shows that the reduction in fluorescence is not due to a $\beta\gamma$ -induced conformational change of the α subunit rather it depends on energy transfer to the eosin on $\beta\gamma$.

3.2. Mechanism of F- α and E- $\beta\gamma$ association

The rate and extent of F- α quenching clearly increased with the concentration of E- $\beta\gamma$ (Fig. 3). The magnitude of fluorescence quenching saturated with an apparent K_d of 27 nM (Fig. 4) which is exactly half of the concentration of F- α . This indicates that the binding of F- α to E- $\beta\gamma$ is of very high affinity and the E- $\beta\gamma$ is titrating the amount of F- α in the sample. This is expected since the F- α has stoichiometric amounts of GDP bound which should result in tight binding of α -GDP and $\beta\gamma$ -subunits. At E- $\beta\gamma$ concentrations ranging from 50–1200 nM, we found half times for quenching ranging from 2.5 to

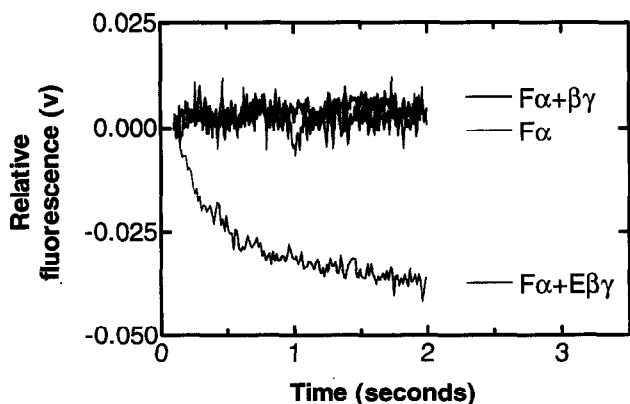


Fig. 2. Kinetics and specificity of F- α fluorescence quenching by E- $\beta\gamma$. F- α in HEDNML was incubated at 20°C in one syringe of the rapid-mix apparatus for 10 min. Fifty μ l of 120 nM F- α and 50 μ l of either buffer, 300 nM unlabelled $\beta\gamma$, or 300 nM E- $\beta\gamma$ were mixed and the change in fluorescence was measured at 20°C as described in section 2. Data were collected every 2 ms and are averages of 2–9 traces. For presentation the first 100 ms were excluded because of a mixing artifact and then every 4 time points were averaged to smooth the curve.

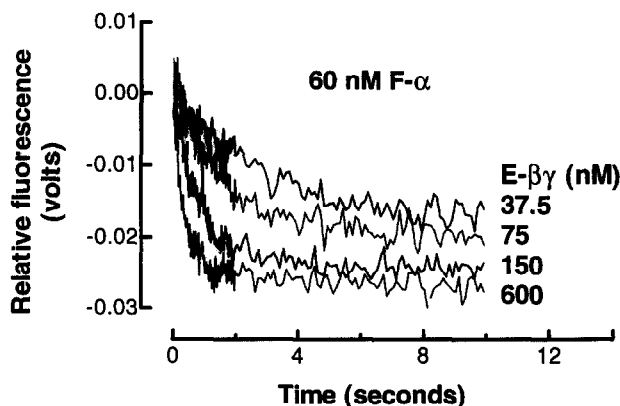


Fig. 3. Kinetics of F- α quenching with increasing concentrations of E- $\beta\gamma$. Sixty nM (final) F- α was mixed with the indicated concentrations of E- $\beta\gamma$ as described for Fig. 2. Data were collected every 2 ms for 2 s then every 10 ms after that. The data are from a single experiment representative of three similar experiments in which each trace is the average of 4 to 7 shots. The data were smoothed by averaging 10 time points.

0.25 s. The data were fit to single and double exponential functions and a single exponential adequately accounted for the data.

Surprisingly, secondary plots of the rate constants also showed saturation (Fig. 4, squares) suggesting that a more complex mechanism is occurring than a simple bimolecular reaction. For a simple bimolecular association of α and $\beta\gamma$ we would have expected a linear dependence of rate constants with increasing E- $\beta\gamma$ concentrations. In all three experiments saturation was observed. We thus considered that G protein subunit association might involve a two-step process described by the following model:



If the first step is fast compared to the second, the K_d for the amplitude would represent the overall affinity of both steps of

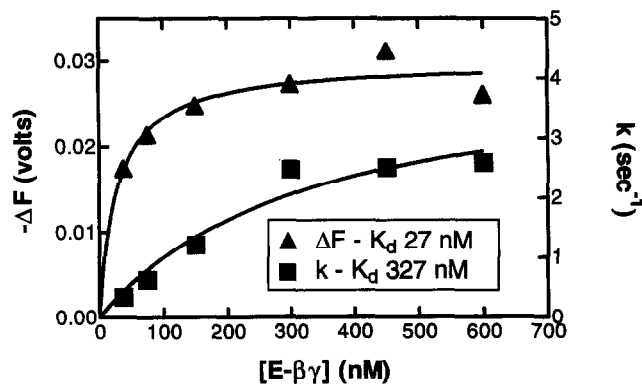


Fig. 4. Secondary plot of amplitude and rate constants with increasing E- $\beta\gamma$ concentration. The F- α quenching data in Fig. 3 were fit to a single exponential as described in section 2. Both amplitudes (ΔF , filled circles) and rate constants (k , filled triangles) are plotted against concentration of E- $\beta\gamma$. The amplitudes and rate constants saturate at different concentrations of E- $\beta\gamma$. Hyperbolic fits determined apparent K_d 's and maximum values for amplitudes and rate constants (see section 3). The K_d values are shown in the box.

binding ($k_{-1}/k_1 \cdot k_{-2}/k_2$) and the K_d for the rates would represent the affinity of the first (pre-equilibrium) step alone (k_{-1}/k_1 ; [11]). With this assumption the pre-equilibrium K_d estimated from the average of three experiments was 253 ± 70 nM and the equilibrium K_d 88 ± 44 nM. The equilibrium K_d probably underestimates the overall affinity as 60–100 nM F- α was present and the 88 nM probably represents a titration of available F- α subunits. Thus our value is consistent with effects of $\beta\gamma$ at subnanomolar concentrations on α_0 which were shown by Ueda et al. [12] and the 10 nM K_d estimated by Helmreich et al. [13] from equilibrium energy transfer studies.

We have shown for the first time that the association of fluorescently labelled α and $\beta\gamma$ subunits can be measured by stopped-flow kinetics. Furthermore, we have found that our subunit association data are not consistent with a single bimolecular interaction but suggest a two-step model for association. We can not specify the functional significance of $\alpha\beta\gamma$ and $\alpha\beta\gamma^*$, but it is tempting to speculate that $\alpha\beta\gamma$ may be an active but non-dissociated form of the G protein. Alternatively, the slow step in the high affinity binding of subunits could be due to a rearrangement of the detergent micelles. The maximal rate of subunit association observed in 0.1% lubrol at 20°C is 4.1 s^{-1} . If the conversion from $\alpha\beta\gamma$ to $\alpha\beta\gamma^*$ does represent deactivation then this rate of 4.1 s^{-1} would represent the maximal rate of deactivation of α -GDP after hydrolysis from α -GTP. While hydrolysis of GTP is often slow ($\sim 0.1 \text{ s}^{-1}$) and is usually rate-limiting for deactivation it is greatly accelerated by effector enzymes acting as GTPase activating proteins. Thus the rate of deactivation of G protein could be limited by a conformational change such as the one postulated here ($\alpha\beta\gamma \rightarrow \alpha\beta\gamma^*$). This value may be faster within the cell due to higher temperatures. It would also depend on the local concentration of $\beta\gamma$ subunits.

Additional studies will be necessary to identify the functional state of the different G protein conformations postulated in this work. Also, the role of receptors and nucleotides to modify the rates and affinities of G protein subunit interactions must be determined.

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