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

Richard R. Neubig*, Mark P. Connolly, Ann E. Remmers

Department of Pharmacology, University of Michigan, 1301 M.S.R.B. III, Ann Arbor, MI 48109-0632, USA

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Abstract. G protein subunit association and dissociation are thought to play an important role in signal transduction. We measured subunit-heterocomplex formation using resonance energy transfer. Fluorescein-labelled αF-α emission was quenched ~10% on mixing with eosin-labelled βγ(E-βγ). Unlabelled βγ did not quench αF-α fluorescence. Stopped-flow kinetics showed a τ_eq ranging from 2.5 s to 0.25 s for 50 nM to 1200 nM E-βγ. The rate saturated at high E-βγ 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 βγ combine by a two-step process with a maximal rate of 4.1 ± 0.4 s⁻¹.

Key words: Resonance energy transfer; Rapid kinetics; Fluorescence

1. Introduction

G proteins transmit signals to many effectors including adenyl cyclase, K⁺ and Ca²⁺ 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 βγ 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 βγ 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 ~4 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). [³²SGTPγS was obtained from New England Nuclear (Boston, MA). Other reagents were from standard suppliers.

2.2. Fluorescent subunit preparation

Fluorescein- and eosin-labelled Gα were prepared essentially as described [10]. Singly labelled F-Gα was isolated by Mono-Q chromatography. Free eosin was removed from eosin-labelled-Gα by gel filtration on Sephadex G-50 and Centriprep 30 ultrafiltration. Individual labelled subunits F-α and E-βγ were resolved by activation in AMF (10 mM MgCl₂, 10 mM NaF, 20 µM AlCl₃) in TEDN buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) containing 0.5% 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. [³²SGTPγS 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 f 10 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 MgCl₂ at 20°C. Similar results were seen with both 1.5 and 10 mM MgCl₂ 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 heterotrimeric G protein association experiments were determined from the averaged data at each F-βγ concentration fit to the simple exponential function:

\[ F(t) = A F \times \exp (-k \times t) + F_\infty \]  

(Equation 1)

where \( F(t) \) represents the fluorescence over time (t), \( A F \) represents the change in fluorescence, \( F_\infty \) 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-βγ. Mixing of fluorescently labelled subunits F-α (60 nM) and E-βγ (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...
Resonance Energy transfer between F-α and E-βγ

Fig. 1. Resonance energy transfer between G-alpha and G-gamma 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 βγ 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 βγ shows that the reduction in fluorescence is not due to a βγ-induced conformational change of the α subunit rather it depends on energy transfer to the eosin on βγ.

3.2. Mechanism of F-α and E-βγ association

The rate and extent of F-α quenching clearly increased with the concentration of E-βγ (Fig. 3). The magnitude of fluorescence quenching saturated with an apparent Kd 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-βγ is of very high affinity and the E-βγ 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 βγ-subunits. At E-βγ concentrations ranging from 50-1200 nM, we found half times for quenching ranging from 2.5 to 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 βγ we would have expected a linear dependence of rate constants with increasing E-βγ 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:

\[
\alpha + \beta\gamma \stackrel{k_1}{\rightarrow} \alpha\beta\gamma \stackrel{k_2}{\rightarrow} \alpha\beta\gamma^* \quad (\text{Equation 2})
\]

If the first step is fast compared to the second, the Kd for the amplitude would represent the overall affinity of both steps of
binding \((k_1/k_2, k_{-2}/k_2)\) and the \(K_d\) for the rates would represent the affinity of the first (pre-equilibrium) step alone \((k_1/k_2)\) [11]. With this assumption the pre-equilibrium \(K_d\) estimated from the average of three experiments was \(253 \pm 70 \text{ nM}\) and the equilibrium \(K_d \approx 88 \pm 44 \text{ nM}\). The equilibrium \(K_d\) probably underestimates the overall affinity as \(60-100 \text{ nM} F-\alpha\) was present and the \(88 \text{ nM}\) probably represents a titration of available \(F-\alpha\) subunits. Thus our value is consistent with effects of \(\beta\gamma\) at subnanomolar concentrations on \(\alpha\), which were shown by Ueda et al. [12] and the \(10 \text{ nM} K_d\) estimated by Helmreich et al. [13] from equilibrium energy transfer studies.

We have shown for the first time that the association of fluorescently labelled \(\alpha\) 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 \(\alpha\) subunit. 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 \(\alpha-\text{GDP}\) after hydrolysis from \(\alpha-\text{GTP}\). While hydrolysis of GTP is often slow (~0.1 \(s^{-1}\)) 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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References