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GDP–GTP Exchange Processes of $G\alpha_{i1}$ Protein are Accelerated/Decelerated Depending on the Type and the Concentration of Added Detergents

Makoto Kubota[1], Takeshi Tanaka[2], Toshiyuki Kohno[2] and Kaori Wakamatsu[1,*]

[1]Department of Chemistry and Chemical Biology, Graduate School of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma 376-8511; and [2]Mitsubishi Kagaku Institute of Life Science (MITILS), Machida, Tokyo 194-8511, Japan

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Although detergents have been widely used in G-protein studies to increase solubility and stability of the protein, we noticed that detergents modulate the nucleotide-binding properties of G-proteins. Hence, we analysed the effects of detergents on guanine nucleotide exchange reactions of $G\alpha_{i1}$. Lubrol PX, a non-ionic detergent, which has been widely used in nucleotide dissociation/binding assays, was found to accelerate both GDP dissociation and GTPγS binding from/to Gα in parallel at above its critical micelle concentration (cmc). Sodium cholate, an anionic detergent, which have been used to extract G-proteins from animal tissues, decelerated and accelerated GDP dissociation below and above its cmc, respectively. Surprisingly, micellar cholate decelerated GTPγS binding, and the binding rate constant was decreased by three orders of magnitude in the presence of 2% cholate. These results demonstrate that the guanine nucleotide exchange reactions of $G\alpha_{i1}$ are drastically modulated by detergents differently depending on the type and the state (monomeric or micellar) of the detergents and that dissociation of GDP from $G\alpha_{i1}$ does not necessarily lead to immediate binding of GTP to $G\alpha_{i1}$ in some cases. These effects of detergents on G-proteins must be taken into account in G-protein experiments.

Key words: detergent, dissociation/binding, guanine nucleotide exchange, rate-limiting step.

Abbreviations: G protein, guanine nucleotide-binding protein; cmc, critical micelle concentration; GST, glutathion-*S*-transferase.

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) transduce extracellular signals from G-protein coupled receptors (GPCRs) to intracellular effectors; thus they play essential roles in cell functions (*1–5*). Because G-proteins are membrane proteins, detergents are usually needed in experiments on G-proteins, such as extraction from membranes, solubilization, stabilization and re-constitution with GPCRs or effectors.

Proper choice of detergents is not, however, a trivial task. Previously, we found that 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxypropanesulfonate (CHAPSO) has destabilizing effects on $G\alpha_q$ although this detergent was effective in extracting $G\alpha_q$ from bovine brain membranes (*6*). Although Lubrol PX had stabilizing effects on $G\alpha_q$, the activation of $G\alpha_q$ by a peptide fragment of a C-terminal portion of the third intracellular loop of m3 muscarinic receptor, m3i3c, was inhibited by the addition of 0.1% Lubrol PX. Other researchers also reported various effects of detergents on G-proteins: cholate and 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate (CHAPS) reduced the affinity between $G\alpha_i$- and βγ-subunits (*7*); mastoparan failed to activate G_o in the presence of cholate or deoxycholate (*8*); and Lubrol PX strongly inhibited the GTPase activity of $G\alpha_s$ (*9*). It is thus critical to choose an appropriate detergent (and its concentration) to set up proper experimental conditions. The effects of detergents on G-protein α-subunits, however, have been poorly understood due to the lack of systematic studies.

In the present study, we analysed the effects of Lubrol PX and cholate, the detergents frequently used in G-protein studies, on the guanine nucleotide exchange of G-proteins. Since the Gα in the GDP-bound form are inactive whereas those in the GTP-bound form are active, activities of G-proteins are regulated by guanine nucleotide exchange reactions. In addition, the dissociation of GDP is generally regarded as the rate-limiting step in the activation process (*2*, *3*, *10*), and GTP molecules are expected to bind to empty α-subunits as soon as GDP molecules are dissociated from the α-subunits. Here, we demonstrate that the two commonly used detergents exert profound effects both on the dissociation rate of GDP and the binding rate of GTPγS, an unhydrolyzable analogue of GTP. Most importantly, we found that the dissociation of GDP is not always the rate-limiting step in the activation of G-proteins.

*To whom correspondence should be addressed. Tel/Fax: +81 277 30 1439, E-mail: wakamats@chem-bio.gunma-u.ac.jp

MATERIALS AND METHODS

Preparation of $G\alpha_{i1}$—$G\alpha_{i1}$ was prepared from GST-$G\alpha_{i1}$ (a fusion protein of GST and $G\alpha_{i1}$ connected by a PreScission protease-cleavage sequence) as reported (*11*) with slight modifications. *Escherichia coli* cells that expressed GST-$G\alpha_{i1}$ were lysed with buffer A [50 mM Tris–HCl (pH 8.0), 2 mM DTT and 100 mM NaCl] containing 2 mM EDTA and 0.1 mM Pefablock SC (serine protease inhibitor), and GST-$G\alpha_{i1}$ was purified with a glutathione sepharose CL-4B column and digested with PreScission protease in-column. $G\alpha_{i1}$ was eluted with buffer A containing 1 mM EDTA and 0.1 mM PMSF, and the protein was exchanged into buffer B [50 mM Tris–HCl (pH 8.0), 0.02 mM EDTA, 1 mM DTT and 0.1 mM PMSF] and purified by MonoQ anion-exchange chromatography with NaCl gradient (5–300 mM) in buffer B. Purified $G\alpha_{i1}$ was pooled and exchanged into buffer C [20 mM Tris–HCl (pH 7.4), 0.1 mM EGTA and 0.1 mM DTT] and stored at –80°C until use. Both GST-$G\alpha_{i1}$ and $G\alpha_{i1}$ were highly soluble even in the absence of detergents, and we did not use any detergents during the preparation. Thus, there was no contamination of detergents in the purified $G\alpha_{i1}$.

[^{35}S]GTPγS-binding Assay—GTPγS-binding assay was performed, according to Linder *et al*. (*12*) with slight modifications, in 100 μl of buffer D [100 mM Hepes-KOH (pH 8.0), 5 mM EDTA and 10 mM $MgSO_4$] containing 2 μM [^{35}S] GTPγS (6,000 cpm/pmol, PerkinElmer, Waltham, MA, USA) and 0.2 μM $G\alpha_{i1}$ supplemented with detergents (Lubrol PX: 0.0001–0.1%, sodium cholate: 0.0005–2%) at 30°C. The binding reaction was stopped at 5, 10, 20, 30, 45, 60, 90, 120 and 150 min by the addition of 1 ml of ice-cold buffer E [25 mM Tris–HCl (pH 8.0), 25 mM $MgCl_2$ and 100 mM NaCl]. The concentration of detergents in the stopped reaction mixture was adjusted to be the same (Lubrol PX: 0.009%, sodium cholate: 0.18%) by supplementing appropriate concentration of detergents to the buffer E, to ensure the same degree of protein binding to the nitrocellulose filters irrespective of the concentration of detergents during the binding reaction. To determine the amount of the GTPγS bound, reaction mixture was filtered through BA85 nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) and the filters were washed eight times with 3 ml of buffer E (without detergents). After wash, the filters were dried and put into 4 ml of scintillation cocktail [5 g 2,5-diphenyloxazole (DPO) and 0.1 g 1,4-*bis*(2-methylstyryl)benzene (Bis-MSB) per litre of toluene] and counted by a liquid scintillation counter. GTPγS-binding rate (k_{on}) was determined by assuming a pseudo-first-order reaction (*10*) with the following equation:

$$B(t) = B_{max}(1 - e^{-k_{on} \cdot t}) \quad (1)$$

in which $B(t)$ is the bound fraction at time t and B_{max} is the bound fraction at infinite time. Recovery of protein binding to the filter was greater than 90% for samples containing Lubrol PX (0.009% during filtration) and approximately 50% for samples containing cholate (0.18% during filtration) when calculated from the B_{max} value.

[α-^{33}P]GDP Dissociation Assay—GDP dissociation assay was performed, according to Linder *et al*. (*12*) with slight modifications, with buffer D containing 1 μM [α-^{33}P] GTP (3,000 cpm/pmol, PerkinElmer) and detergents. $G\alpha_{i1}$ (0.2 μM) was preincubated with [α-^{33}P] GTP for 60 min at 30°C, and the dissociation reaction was started by the addition of cold 100 μM GDP at 30°C. Aliquots (100 μl) were withdrawn at 5, 10, 30, 60, 120 and 180 min from the reaction mixture and diluted with 1 ml of ice-cold buffer F [20 mM Tris–HCl (pH 8.0), 15 mM $MgCl_2$, 0.01 mM $AlCl_3$, 5 mM NaF and 100 mM NaCl] for termination of the dissociation reaction. Bound GDP was determined as in the case of GTPγS except that buffer F was used instead of buffer E to prevent the dissociation of the bound GDP. GDP dissociation rate (k_{off}) was determined by a simple exponential decay of the form

$$B(t) = B_0 e^{-k_{off} \cdot t} \quad (2)$$

in which $B(t)$ is the bound fraction at time t and B_0 is the bound fraction at time zero.

Purification of Detergents—Lubrol PX (Nakalai Tesque, Kyoto, Japan) was purified essentially as described (*13*). Lubrol PX solution (10%, v/v) was passed successively through a column of DEAE-Sephacel (Cl^--form, GE Healthcare, Buckinghamshire, England) and a column of the mixture of Dowex 50W (H^+-form, Bio-Rad) and AG1 (OH^--form, Bio-Rad, Hercules, CA, USA), and the purified Lubrol PX solution was stored at 4°C under dark until use. Although unpurified Lubrol PX solution looked slightly turbid and yellow, purified one was transparent and colourless. Sodium cholate (Wako Pure Chemicals, Osaka, Japan) was purified as described (*14*). Sodium cholate solution (2.5%, w/v) was passed through a column of DEAE-Sephacel (Cl^--form), and the eluate was acidified with HCl to precipitate cholic acid. The precipitate was collected by filtration, washed with milli-Q water to remove salts, washed with anhydrous ether to remove fat, and then dried for several days in a desiccator. Dried cholic acid was dissolved in milli-Q by the addition of NaOH, and finally adjusted to pH 8.02 and 20% w/v. Thesit (Sigma, St. Louis, MO, USA), $C_{12}E_{10}$ (Sigma) and CHAPS (Wako Pure Chemicals) were used without further purification.

RESULTS

Lubrol PX Increased both Rates of GDP Dissociation and GTPγS Binding—Lubrol PX accelerated both reactions of GDP dissociation and GTPγS binding in a concentration-dependent manner (Fig. 1). Both rates in the presence of 0.1% Lubrol PX were larger by ~35% than those in the presence of 0.0001% Lubrol PX. The EC_{50} values of the enhancements were 0.003%. Because this concentration does not differ so much from the reported cmc value of Lubrol PX (0.0024%) (*15*), the increase in the rates are considered to be caused by detergent micelles rather than by monomers. Interestingly, the GTPγS binding rate was very close to the GDP dissociation rate at all Lubrol PX concentrations. These results indicate that the binding of GTPγS occurs immediately after the dissociation of GDP and that the rate-limiting

step in the GTPγS binding reaction is the dissociation of GDP from G-proteins under these conditions.

GDP Dissociation Rate was Increased in the Presence of Cholate at the Concentration Above its cmc but Decreased Below its cmc—Cholate exhibited complex effects on the GDP dissociation as shown by the non-monotonous concentration dependence (Fig. 2A). Although the increase in cholate concentration up to 0.1% resulted in the deceleration of the dissociation reaction, further increase in the cholate concentration beyond 0.1% conversely resulted in its acceleration. The dissociation rate at 0.1 and 2% cholate was 50 and 95%, respectively, in the presence of 0.0005% cholate. Since the reported cmc and aggregation number of cholate are 0.3% and 2, respectively (*16*), and those detergents that form small micelles with an aggregation number less than 90 are known to exhibit broad cmc values depending on the experimental conditions (*17*), these results suggest that monomeric cholate decelerates, while micellar cholate accelerates the dissociation reactions.

In the Presence of High Concentrations of Cholate GTPγS-binding Reaction is Decelerated and does not Obey the Pseudo-first-order Kinetics—Figure 2B shows the time course of GTPγS binding in the presence of cholate or Lubrol PX. The kinetics in the presence of 0.1% Lubrol PX exhibited the time course that is characteristic of the pseudo-first-order kinetics as indicated by the convex curve at all time points. In the presence of 0.1% cholate, the binding reaction became slow, and further increase in the cholate concentration (1 and 2%) resulted in much slower binding. Moreover, the kinetics in the presence of 1 and 2% cholate exhibited complex curve, which is concave at early time points (0–20 min in the presence of 1% cholate) but convex at later time points. Such kinetics cannot be described by the simple pseudo-first-order formulation, because this kinetics would exhibit convex curves irrespective of the dissociation rate (Fig. 3A).

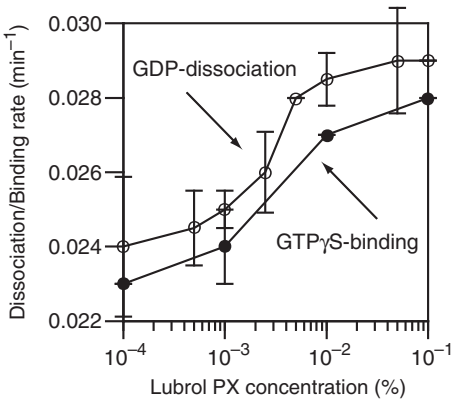

Fig. 1. **Effects of Lubrol PX on guanine nucleotide exchange reactions of $G\alpha_{i1}$.** Empty and filled circles indicate GDP-dissociation and GTPγS-binding rates, respectively. Data shown are average ± SEM of three independent experiments.

The Binding Rate Constant was Decreased in the Presence of High Concentrations of Cholate—In the absence of the pseudo-first-order approximation, both steps of GDP dissociation and GTPγS binding must be taken into account explicitly (*10*).

$$\alpha \cdot \mathrm{GDP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha(\mathrm{empty}) + \mathrm{GDP} + \mathrm{GTP}\gamma\mathrm{S} \xrightarrow{k_2} \alpha \cdot \mathrm{GTP}\gamma\mathrm{S}$$

When the concentration of total GTPγS is much larger than that of the total α-subunits, the rebinding of GDP to the empty α-subunits is neglected, the simultaneous differential equations of the concentrations of GTPγS-bound, empty and GDP-bound α-subunits would be

$$\begin{aligned} \frac{\mathrm{d}[\alpha \cdot GTP\gamma S](t)}{\mathrm{d}t} &= k_2 \times [GTP\gamma S]_0 \times [\alpha(\mathrm{empty})](t) \\ \frac{\mathrm{d}[\alpha(\mathrm{empty})](t)}{\mathrm{d}t} &= -k_2 \times [GTP\gamma S]_0 \times [\alpha(\mathrm{empty})](t) \\ &\quad + k_1 \times [\alpha \cdot \mathrm{GDP}](t) \\ [\alpha \cdot GTP\gamma S](t) + [\alpha(\mathrm{empty})](t) + [\alpha \cdot GDP](t) &= [\alpha]_0 = \text{constant} \end{aligned} \tag{3}$$

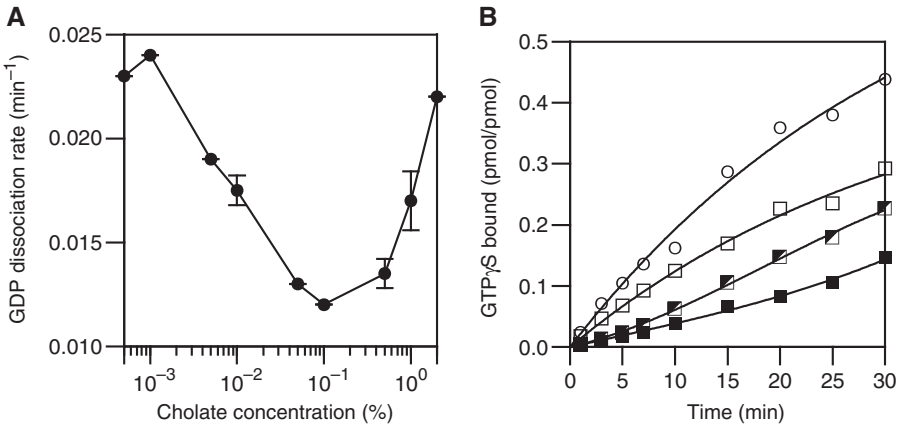

Fig. 2. **The effect of cholate on guanine nucleotide exchange of $G\alpha_{i1}$.** (A) GDP-dissociation rate. Data shown are average ± SEM of three independent experiments. (B) The kinetics of GTPγS binding in the presence of 0.1% Lubrol PX (empty circles), 0.01% cholate (empty squares), 1% cholate (half-filled squares) and 2% cholate (filled squares). Data shown are the representative of three with similar results.

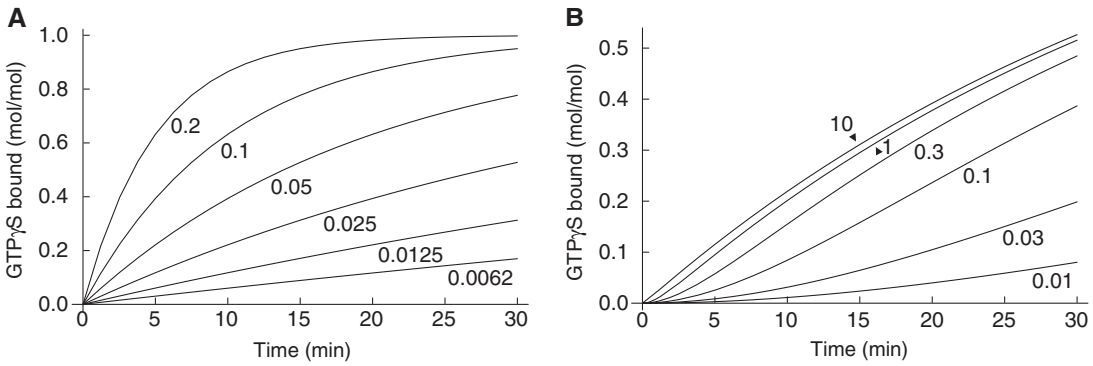

Fig. 3. **Simulation of GTPγS-binding time course.** (A) With pseudo-first-order approximation. The number below each trace indicates k_{on} value in Eq. (1). (B) Without pseudo-first-order approximation. The number indicates $k_2[GTP\gamma S]_0$ value in Eq. (3). The k_1 value was fixed at $0.02\,min^{-1}$. Simulation was carried out by using Mathematica.

in which $[GTP\gamma S]_0$ corresponds to the total concentration of GTPγS. These equations were solved by using Mathematica software to give

$$[\alpha \cdot GTP\gamma S](t) = [\alpha]_0 \cdot \left\{1 + \frac{k_2[GTP\gamma S]_0 e^{-k_1 \cdot t} - k_1 e^{-k_2[\mathrm{GTP}\gamma S]_0 \cdot t}}{k_1 - k_2[GTP\gamma S]_0}\right\} \quad (4)$$

or in a form like that of Eq. (1),

$$B(t) = B_{\max}\left\{1 + \frac{k_2[GTP\gamma S]_0 e^{-k_1 \cdot t} - k_1 e^{-k_2[GTP\gamma S]_0 \cdot t}}{k_1 - k_2[GTP\gamma S]_0}\right\} \quad (5)$$

With the pseudo-first-order approximation of $k_1 \ll k_2[GTP\gamma S]_0$, Eq. (5) boils down to Eq. (1) ($k_{on} = k_1$).

The effects of the product $k_2[GTP\gamma S]_0$ on the binding kinetics are simulated in Fig. 3B. When the product value is 10 (min^{-1}, top trace) and much larger than k_1($0.02\,min^{-1}$ throughout this simulation), the binding kinetics resembles that of pseudo-first-order reaction (Fig. 3A). As the product value becomes smaller, the binding reaction becomes slower and exhibits longer concave curves at early time points. The curve for $k_2[GTP\gamma S]_0 = 0.1$ (min^{-1}) resembles the binding time course in the presence of 2% cholate (Fig. 2B). When the GTPγS-binding data in the presence of 2% cholate were fitted to Eq. (3), $k_2[GTP\gamma S]_0$ was found to be $0.194\,min^{-1}$ and k_2 was calculated as $1.6 \times 10^3\,M^{-1}{\cdot}s^{-1}$. This value is smaller, by three orders of magnitude, than the binding rate constant of $G\alpha_{i1}$ ($2 \times 10^6\,M^{-1}{\cdot}s^{-1}$), which was determined under conditions similar to the present study (*10*). Figure 4 shows the fraction of α-subunits bound to [α-^{33}P]GDP/GTP after incubation with [α-^{33}P]GTP for 60 min in the presence of different concentrations of cholate. As expected from the deceleration of GTP-binding reactions in the presence of high concentrations of cholate, the radioactivity bound to $G\alpha_{i1}$ was found to decrease with increasing cholate concentrations. If the dissociation of GDP were the rate-limiting step in the [α-^{33}P]GTP-binding reaction, the radioactivity bound to $G\alpha_{i1}$ would have increased with increasing concentrations of cholate because the dissociation rate increased with increasing cholate concentration for the concentration range of 0.1–2% (Fig. 4).

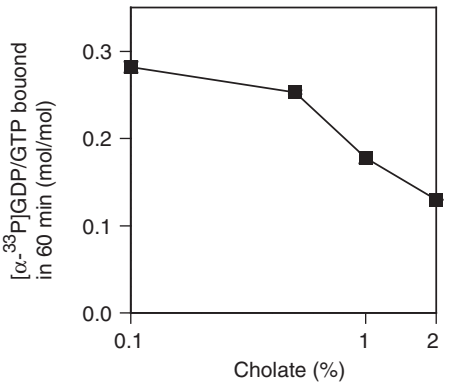

Fig. 4. **The fraction of $G\alpha_{i1}$ bound to [α-^{33}P]GTP or its hydrolyzed product [α-^{33}P]GDP after incubation with [α-^{33}P]GTP for 60 min in the presence of different concentrations of cholate.** Data shown are representative of two with similar results.

DISCUSSION

Guanine Nucleotide Exchange Reactions are Profoundly Affected by Detergents—Figure 5 summarizes the findings obtained in the present study. Lubrol PX accelerated the GDP dissociation reaction, and the GTP-binding was indirectly accelerated because the dissociation of bound GDP is the rate-limiting step in the guanine nucleotide exchange reactions as long as Lubrol PX is used. Cholate micelles accelerated the GDP dissociation reaction but severely interfered with the GTP-binding reaction. Thus, it was found that detergents strongly affect the guanine nucleotide exchange reactions of $G\alpha_{i1}$ and that the detailed effects vary depending on both the type and the concentration of the detergent.

Effects of Lubrol PX—Since both rates of GDP dissociation and GTPγS binding were increased by the addition of Lubrol PX with the EC_{50} value as low as 0.003% and since Lubrol PX is usually added in reaction buffers at 0.1%, these α-subunits are not always in

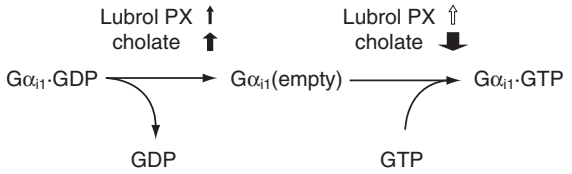

Fig. 5. **The effects of Lubrol PX and cholate on guanine nucleotide exchange reactions at the concentration above their cmc.** Filled up- and down-arrow indicate acceleration and deceleration, respectively, as a consequence of the direct effect of the detergent on the steps. Empty up-arrow indicates the indirect acceleration of the step as a result of the acceleration of the preceding rate-limiting step.

a basal (non-activated) state. Mastoparan, a tetradecapeptide from wasp venom, has been known to activate G_o and G_i (*18*). The potency and efficacy of mastoparan to activate G-proteins were, however, found to decrease by the addition of Lubrol (*8*). These observations can now be explained by the elevated basal activity of G-proteins in the presence of Lubrol. Moreover, some structural changes seem to be induced in $G\alpha_q$ by Lubrol, because the protection of $G\alpha_q$ from extensive digestion by trypsin requires the inclusion of Lubrol to the reaction medium in addition to the GTPγS binding to the $G\alpha_q$ (*6*).

Effects of Cholate—Cholate had complex effects on the guanine nucleotide exchange reactions. Monomeric cholate molecules decelerated the dissociation reactions, whereas cholate micelles accelerated the dissociation reaction. Quite interestingly, a high concentration of cholate (2%) decreased the binding rate constant of GTPγS by three orders of magnitude. Although a high concentration of cholate (usually 1%) is used for the extraction of G-proteins from membranes (*19*), G-proteins have been known to be unstable in the presence of cholate (*20*). This observation can now be fully explained by our present findings: severe deceleration of GTPγS/GTP binding to α-subunits (with apparently normal GDP dissociation from the α-subunits) in the presence of a high concentration of cholate leads to the accumulation of guanine-free α-subunits, which are known to be unstable (*10*). It should also be noted, however, that the slower GTPγS binding in the presence of high concentrations of cholate is not due to simple denaturation of α-subunits. If this were the case, the binding time course would have obeyed the simple pseudo-first-order kinetics.

Effects of Other Detergents—Thesit and $C_{12}E_{10}$ are polyethyleneglycol dodecyl ether [with the degree of ethyleneglycol polymerization of 9 (average value) and 10, respectively] and structurally similar to Lubrol PX [equivalent to the mixture of $C_{12}E_9$ and $C_{12}E_{10}$ (*21*)]. In good agreement with this structural similarity, both detergents at 0.1% concentration accelerated GTPγS binding to $G\alpha_{i1}$ by 35% as did Lubrol PX. Although CHAPS has a steroid skeleton as cholate, this detergent did not affect the GTPγS-binding reaction both in the monomeric and micellar states because the GTPγS-binding rate was constant at 0.023 min^{-1} in the presence of 0.1–1% CHAPS whose cmc is 0.5% (*22*). Strong effects of cholate on the nucleotide binding to $G\alpha_{i1}$ may be partly due to its anionic charge (CHAPS is a zwitterionic detergents).

GDP Dissociation is not Always the Rate-limiting Step in the GTP-binding Reaction—Although it is generally accepted that the dissociation of GDP from α-subunits is the rate-limiting step in the binding of GTP to the subunits (*10*), several observations are incompatible with this notion. Brandt and Ross (*23*) found that the dissociation rate of GDP from $G\alpha_s$ is significantly faster than the binding rate of GTPγS to the $G\alpha_s$, and argued that the dissociation is not the rate-limiting step. Carty *et al*. (*24*) found that G_{i3} releases GDP faster than G_{i1}, although G_{i3} and G_{i1} bound GTPγS at very similar rates. The common view that the dissociation of GDP is the rate-limiting step in the activation of G-protein may have come from the routine use of Lubrol PX in the buffers for guanine nucleotide exchange experiments.

Implication of Possible Differential Regulation of Guanine Nucleotide Exchange Processes—Because the dissociation of GDP is not always the rate-limiting step in the GTP-binding reaction of G-proteins, there is a possibility that the two steps (GDP dissociation and GTP binding) are regulated independently under some conditions. In other words, there may exist activators that accelerate the GTP binding to empty α-subunits as well as inhibitors that prevent empty α-subunits from association with GTP. One candidate for such an activator is GPCRs (*23*). Some lipids in cell membranes or in lipid rafts (*25*) may modulate the GTP-binding reaction. In fact, GTPγS binding to $G\alpha_o$ was recently found to be inhibited specifically by phosphatidic acid, but not by other anionic phospholipids (phosphatidylserine and phosphatidylglycerol) or zwitterionic phospholipids (*26*). From a technical viewpoint, cholate may be useful for preparing stable G-protein–GPCR complexes for X-ray crystallographic analysis because the complex is stable when the G-protein α-subunit is in the guanine nucleotide-free state. We hope that our finding are helpful for choosing appropriate detergents for experiments of G-proteins and for stimulating new experiments on the regulation of G-proteins.

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CONFLICT OF INTEREST

None declared.

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