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Membrane association and conformational change of palmitoylated $G_o\alpha$

Shengyu Yang, Ling Zhang, Youguo Huang*

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

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Abstract Bovine brain $G_o\alpha$ was specifically palmitoylated in vitro. The apparent dissociation constant for depalmitoylated $G_o\alpha$ ($dG_o\alpha$) was 0.273 μM, while that for palmitoylated $G_o\alpha$ ($pG_o\alpha$) was 5.77 nM. The dissociation rate constant (K_{21}) and dissociation half-life for $dG_o\alpha$ were 8.4×10^{-4} min and 825 min respectively, while no significant dissociation of $pG_o\alpha$ was detected. The limiting membrane insertion pressures for $pG_o\alpha$ and $dG_o\alpha$ were 44.4 mN/m and 41.3 mN/m respectively. These data suggested that palmitoylation facilitated the membrane association of $G_o\alpha$. Conformational changes of $dG_o\alpha$ and $pG_o\alpha$ detected by monitoring fluorescence spectra and fluorescence quenching were significantly different after they were associated with the membrane. It was suggested that conformational changes of $G_o\alpha$ upon membrane association might be related to regulation of $G_o\alpha$ signaling by palmitoylation. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: G protein; Palmitoylation; Membrane association; Conformational change

1. Introduction

A family of heterotrimeric guanine nucleotide-binding proteins (G proteins) transduce signals across the plasma membrane by coupling between receptors and effectors [1]. G proteins are bound to the inner surface of plasma membranes. An efficient coupling function of G proteins requires proper membrane targeting [2,3]. It is well known that three kinds of lipid modifications, i.e. palmitoylation [4–6] and myristoylation [7–9] of Gα and prenylation of Gγ [10], mediate the interaction between the G protein and the membrane. However, only palmitoylation is readily reversible and likely to be regulated [2]. Activation of G protein by mutations, agonist-bound receptors or toxins promotes the turnover of associated palmitate [11–13]. Accompanying activation, some groups also observed the release of Gα subunits from membranes [14,15], and the release was supposed to be due to depalmitoylation [11]. The idea that depalmitoylation of Gα causes the consequent release of Gα from the plasma membrane and thus down-regulates G protein signaling is attractive, however, site-directed mutation data addressing the role that palmitoylation plays in the membrane association of Gα are still controversial. Some data indicate that palmitoylation may be involved in mediating and regulating the membrane attachment [6,16,17], while others suggest that it may not [4,13]. Recently, some papers reported that activated G protein α subunits stably associate with the membrane [18]. More recently, depalmitoylated Gα was found to be associated persistently with the membrane [19]. Instead of releasing from the plasma membrane, activated Gα was found to be concentrated in subdomains [19,20]. It should be noticed that most of the experiments on palmitoylation of G protein were carried out with intact cells, which made them difficult to explain in some aspects. To gain an appreciation of the role that palmitoylation plays in the membrane association of Gα, in this paper $G_o\alpha$ was purified from bovine brain and palmitoylated in vitro, and then the characteristics of the association of palmitoylated $G_o\alpha$ ($pG_o\alpha$) and depalmitoylated $G_o\alpha$ ($dG_o\alpha$) with asolectin large unilamellar vesicles (LUVs) and the conformational changes of the membrane-bound $pG_o\alpha$ and $dG_o\alpha$ were examined.

2. Materials and methods

2.1. Materials

Guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) and GDP were purchased from Boehringer-Mannheim. Coenzyme A and palmitoyl coenzyme A were from Sigma. [^{3}H]Palmitate and [^{35}S]GTPγS were from DuPont NEN. Asolectin was the product of Fluka. Hypocrellin B (HB) was a generous gift from Dr. Yue [21]. All other reagents were of the highest purity available.

2.2. Methods

2.2.1. Non-enzymatic palmitoylation of bovine brain $G_o\alpha$. $G_o\alpha$ was purified from bovine brain cortex as described by Sternweis et al. [22] with modifications [23]. Non-enzymatic palmitoylation of $G_o\alpha$ was conducted exactly as described by Duncan and Gilman [24] except that the palmitoylation buffer always contains 5 μM GDP. After palmitoylation $G_o\alpha$ was dialyzed to buffer A (20 mM HEPES, 2 mM $MgCl_2$ and 1 mM EDTA). The stoichiometry of palmitoylation was determined with the liquid scintillation method according to Duncan and Gilman [24]. The specificity of the palmitoylation site was confirmed as described by Linder et al. [5].

2.2.2. Preparation of asolectin LUVs. The asolectin was further purified before use [25]. Asolectin LUVs were prepared according to Rigaud et al. [26]. Lipid compositions of the LUVs were determined by thin-layer chromatography and were phosphatidylethanolamine (35%), phosphatidylcholine (33%), phosphatidylinositol (23%) and other lipids (9%).

2.2.3. Membrane association and dissociation of $p/dG_o\alpha$.

2.2.3.1. Affinity of $pG_o\alpha$ and $dG_o\alpha$ for LUVs. 1 nM of $pG_o\alpha$ or $dG_o\alpha$ was incubated with an indicated concentration of asolectin LUVs for 90 min at 30°C. Then the $p/dG_o\alpha$-associated LUVs were pelleted by centrifugation in a Beckman TL100.3 rotor at $100\,000\times g$ for 5 min at 30°C. The supernatant was assayed for GTPγS-binding activity. To calibrate the effect of denatured protein we set a control: the same amount of $p/dG_o\alpha$ without LUVs was incubated and centrifuged exactly as described above. The supernatant GTPγS-binding activity of the control was measured. It can be assumed that the de-

*Corresponding author. Fax: (86)-10-6487 2026.
E-mail: huang@sun5.ibp.ac.cn

Abbreviations: $dG_o\alpha$, depalmitoylated $G_o\alpha$; $pG_o\alpha$, palmitoylated $G_o\alpha$; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); HB, hypocrellin B

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naturation ratio of the control was the same as that of the sample. The binding activity of LUV-bound $G_o\alpha$ was calculated by subtracting the binding activity in the sample supernatant from the binding activity in the control supernatant.

2.2.3.2. Dissociation of p/d$G_o\alpha$ from membranes. 2 μM d$G_o\alpha$ or 0.1 μM p$G_o\alpha$ was incubated with 2 mM LUVs in 40 μl buffer A at 30°C for 90 min. Then the LUV-bound $G_o\alpha$ was separated from p/d$G_o\alpha$ by centrifugation as described above. The pellet was washed twice briefly and suspended with 1.5 ml buffer A. After incubation at 30°C for the time indicated, the LUV-bound $G_o\alpha$ was pelleted down and measured for GTPγS-binding activity. To calibrate artifacts of protein denaturation, $G_o\alpha$ without LUVs was also incubated and measured for GTPγS-binding activity as described above.

2.2.4. Monolayer insertion experiments of p/d$G_o\alpha$. Construction and characterization of the monolayer trough have been described in full detail [27]. The surface pressure of the monolayer was measured by the Wilhelmy method. The increment of surface pressure ($\Delta\pi$) was recorded and plotted against the initial monolayer surface pressure.

2.2.5. Fluorescence measurement. The intrinsic fluorescence emission spectrum of $G_o\alpha$ (40 μg/ml, 500 μl) in buffer A was measured on a Hitachi 4500 spectrofluorometer at 30°C with a 5×5 mm cuvette.

2.2.6. Data analysis

2.2.6.1. Membrane association and dissociation. The data were fitted to the simple bimolecular association model:

$$R+L \rightleftharpoons RL \tag{1}$$

where R, L and RL refer to receptor ($G_o\alpha$), ligand (LUV) and receptor–ligand complex (LUV-bound $G_o\alpha$) respectively. At equilibrium, the concentration of the receptor–ligand complex is given by the following expression:

$$RL = \frac{R_t \times L}{(K_d + L)} \tag{2}$$

where R_t is the total receptor concentration and K_d is the dissociation constant. $t_{1/2}$ of the dissociation was calculated according to the following equation:

$$RL_t = RL_0 \cdot e^{-K_{2l} \cdot t} \tag{3}$$

where RL_0 is the concentration of receptor–ligand complex at time zero.

2.2.6.2. HB quenching of tryptophan fluorescence in the $G_o\alpha$. The classical Stern–Volmer equation relates the drop in fluorescence (F_0 and F are fluorescence in the absence or presence of the quencher respectively) to the concentration of a collisional quencher, Q, as

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \tag{4}$$

where K_{sv} is the Stern–Volmer constant for the collisional quenching process. The above equation predicts a linear plot of F_0/F vs. $[Q]$ for a homogeneously emitting system. However, the fluorescence of most proteins will be heterogeneous, and besides dynamic quenching, static quenching may also occur. In such cases the appropriate form of the Stern–Volmer equation is

$$\frac{F_0}{F} = \left(\sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q]e^{V_i[Q]})} \right)^{-1} \tag{5}$$

where K_i and V_i represent the dynamic and static quenching constants for tryptophanyl residues i of the protein respectively, and f_i is the fractional contribution of tryptophan i to the total fluorescence [28].

2.2.7. Miscellaneous. The GTPγS-binding assay was conducted according to Northup et al. [29]. [^{3}H]Palmitoyl CoA was prepared from [^{3}H]Palmitate and CoA according to Duncan and Gilman [24]. The purity of the [^{3}H]palmitoyl CoA preparation was analyzed by silica gel G plates with isobutanol:H_2O:acetic acid (50:30:20) as the mobile phase, followed by fluorography. The radioactive purity was always greater than 98%.

3. Results

3.1. The non-enzymatic palmitoylation site of $G_o\alpha$ was Cys^3 at the N-terminal

Fig. 1 shows the successful non-enzymatic palmitoylation and its specificity of the purified native bovine brain $G_o\alpha$. Fluorography (Fig. 1B) revealed that $G_o\alpha$ was successfully palmitoylated (lane C), and dithiothreitol (DTT) (lane D) or hydroxylamine (lane H) removed the labeled ^{3}H, suggesting that the [^{3}H]palmitate was thioesterified. Linder et al. [5] demonstrated previously that trypsin cleaves GTPγS-activated $G_o\alpha$ producing a stable 37 kDa protein core lacking a 2 kDa N-terminal fragment. Since the only cysteine residue in the cleaved amino-terminal fragment is Cys^3, the absence of ^{3}H in the protected trypsin core (Fig. 1, lane T) implies that palmitate is incorporated at Cys^3, the site of palmitoylation in vivo. It should also be noticed that the apparent palmitoylation ratio of $G_o\alpha$ was greatly enhanced to 85% after the endogenous palmitate was removed by DTT [30], which makes it very important to study the palmitoylation of $G_o\alpha$ in vitro.

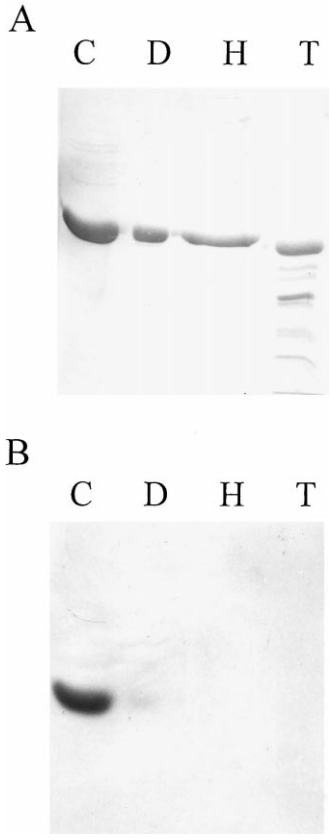

Fig. 1. Palmitoylation in vitro of bovine brain $G_o\alpha$. After palmitoylation, GTPγS–$G_o\alpha$ was incubated with 1 M Tris (lane C), 1 M hydroxylamine (lane H) or 20 mM DTT (lane D) at 30°C for 30 min, or digested with 0.1 mg/ml trypsin at 30°C for 10 min (lane T). The reactions were terminated by adding SDS–PAGE loading buffer without reducing agents. The samples were boiled immediately for 3 min and subjected to SDS–PAGE. A: Coomassie blue staining of the SDS–PAGE gel. B: Fluorography of A.

3.2. Palmitoylation increased the affinity of $G_o\alpha$ for LUVs

Site-directed mutation and Triton X-114 partition experiments [3,6] suggest that palmitoylation increases the hydrophobicity of Gα. However, these experiments were largely qualitative, and inconsistent results make this issue more puzzling. Comparing the affinity of d$G_o\alpha$ and p$G_o\alpha$ for membranes quantitatively would help to clarify the role that pal-

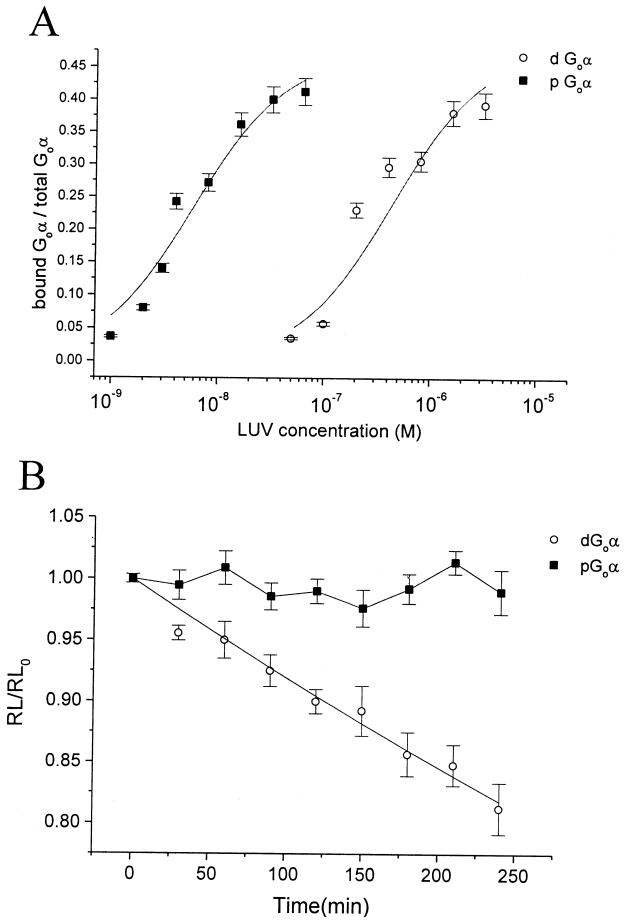

Fig. 2. A: Affinity of $pG_o\alpha$ and $dG_o\alpha$ for LUVs. After palmitoylation, the membrane association affinity of $G_o\alpha$ was increased about 50 times. B: The membrane association of $pG_o\alpha$ is more stable than that of $dG_o\alpha$. $dG_o\alpha$ dissociates slowly from LUVs with a dissociation half-life of 825 min, while no dissociation of $pG_o\alpha$ was observed.

mitoylation plays in the membrane association of Gα. Affinity studies of p/$dG_o\alpha$ for LUVs with the defined phospholipid components described above were carried out by measuring the apparent K_d constant.

From Eq. 2 above, we may predict that the $G_o\alpha$–LUVs association would reach 100%, but we found that this was not the case in our experiment. As shown in Fig. 2A, at 1 μM LUVs, the bound ratio reaches approximately 45%, and this ratio is not increased even when the LUV concentration was increased to 2 mM (data not shown). Thus, Eq. 2 is modified to:

$$\frac{RL}{R_t} = \frac{B_{max}}{(1 + K_d)} \quad (6)$$

where B_{max} is the maximum association ratio. The apparent K_d of $dG_o\alpha$ is 0.273 μM, while that of $pG_o\alpha$ is 5.77 nM. These data indicate that the membrane avidity of $G_o\alpha$ is increased significantly (about 50-fold) after palmitoylation (Fig. 2A).

3.3. Palmitoylated $G_o\alpha$ exhibited longer membrane association

As shown in Fig. 2B, we measured the dissociation of $pG_o\alpha$ and $dG_o\alpha$ directly. The K_{21} and $t_{1/2}$ of $dG_o\alpha$ are 8.4×10^{-4} and 825 min respectively, while no significant dissociation of $pG_o\alpha$ was detected within the time scale. The results also indicated that $pG_o\alpha$ exhibited a much longer membrane association than $dG_o\alpha$.

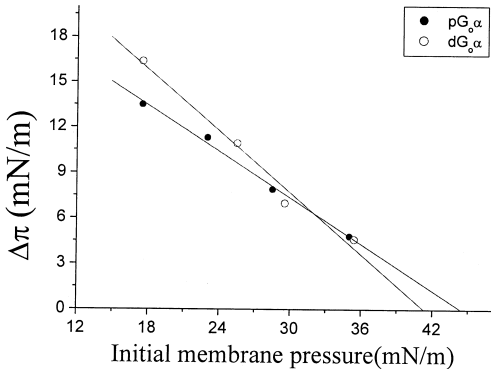

Fig. 3. Measurement of the limiting membrane pressure of $pG_o\alpha$ and $dG_o\alpha$. At the initial surface pressure indicated, 4 μg of $pG_o\alpha$ or $dG_o\alpha$ was injected into the subphase and the increment of surface pressure was recorded at equilibrium.

3.4. Ability of monolayer insertion of p/$dG_o\alpha$

The data above demonstrated that the association of $G_o\alpha$ with membranes could be promoted with palmitoylation. To gain further insight into the $G_o\alpha$–membrane interaction, insertion of $pG_o\alpha$ or $dG_o\alpha$ into the asolectin monolayer was further compared by the membrane-balance technique.

After injection of $pG_o\alpha$ or $dG_o\alpha$ into the subphase, the surface pressure of the asolectin monolayer increased slowly and stabilized at about 90 min, while no surface pressure increase was observed when the same volume of buffer or bovine serum albumin was injected (data not shown). More insight into the monolayer insertion can be gained by measuring the limiting insertion pressure ($\Delta\pi$), which is defined as the maximal surface pressure allowing protein insertion. As shown in Fig. 3, the limiting surface pressures for $pG_o\alpha$ and

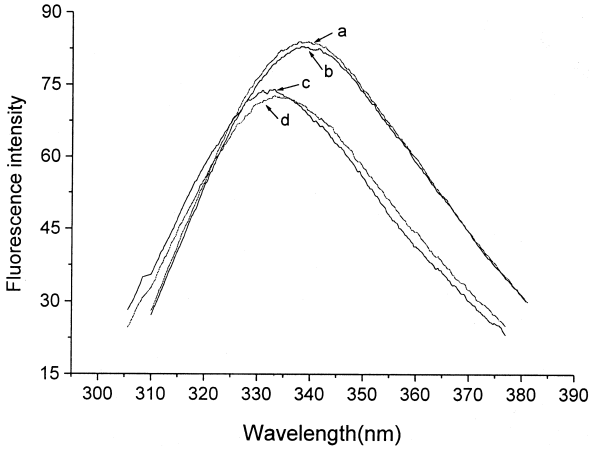

Fig. 4. Tryptophan emission spectrum of $pG_o\alpha$ and $dG_o\alpha$ before and after membrane association. 20 μg of $pG_o\alpha$ (a), $dG_o\alpha$ (b), LUV-bound $pG_o\alpha$(c) or LUV-bound $dG_o\alpha$ (d) was added to 500 μl buffer A in a 5×5 mm cuvette, and the tryptophan emission spectrum was recorded with a Hitachi 4500 spectrofluorometer at 30°C. The excitation wavelength was 295 nm. The emission spectra were monitored from 310 nm to 380 nm.

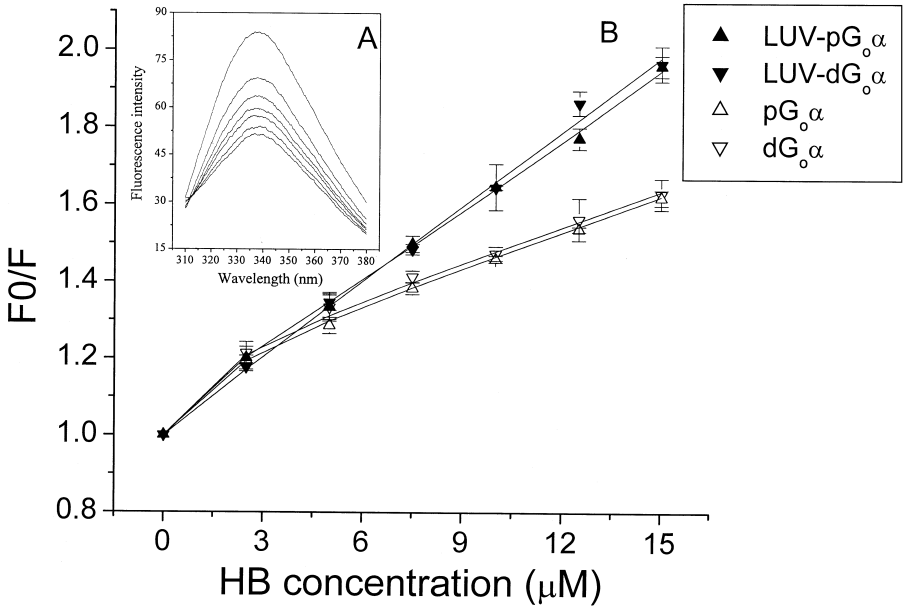

Fig. 5. HB quenching of the tryptophan fluorescence of $G_o\alpha$. A: Fluorescence emission spectrum of $dG_o\alpha$ at various concentrations of HB. From top to bottom the HB concentrations are 0, 2.5, 5.0, 7.5, 10, 12.5 and 15 μM respectively. The profiles of the fluorescence quench for $pG_o\alpha$, LUV–$dG_o\alpha$ and LUV–$pG_o\alpha$ are similar to that of $dG_o\alpha$. B: To quench the intrinsic fluorescence of $G_o\alpha$, HB was added to the concentrations as indicated and mixed thoroughly, and then the emission spectrum was recorded at 295 nm excitation. Peak values of the emission spectrum were fitted to Eq. 5.

$dG_o\alpha$ were 44.43 mN/m and 41.3 mN/m respectively, which indicates the stronger insertion ability of $pG_o\alpha$ in comparison with $dG_o\alpha$.

3.5. *Conformation of $pG_o\alpha$ and $dG_o\alpha$ associated with membranes*

From the results above, it is interesting to further explore whether the conformations of $pG_o\alpha$ and $dG_o\alpha$ change upon membrane association; this can be done by fluorescence measurement. Fig. 4 clearly shows that the intrinsic fluorescence intensities of tryptophans in $pG_o\alpha$ and $dG_o\alpha$ decreased significantly when they were bound to the membrane. Furthermore, the emission maxima of $pG_o\alpha$ and $dG_o\alpha$ were significantly blue-shifted to 333 nm and 336 nm, respectively, from 340 nm, which indicated that both $pG_o\alpha$ and $dG_o\alpha$ adopted a conformation with more exposure of the tryptophan to a relatively hydrophobic environment. An interesting phenomenon is that there is no difference between the emission spectra of $pG_o\alpha$ and $dG_o\alpha$ in solution, but there was a small but repeatable difference (3 nm shifted) between the emission maxima of membrane-bound $pG_o\alpha$ and $dG_o\alpha$. This would imply that after binding to the membrane, the conformations of $pG_o\alpha$ and $dG_o\alpha$ were different, while there was no conformational difference when they were in solution.

To further explore the conformational change of $p/dG_o\alpha$ bound to the membrane, the intrinsic fluorescence of $G_o\alpha$ was quenched with HB. HB is a photosensitive pigment with an excitation wavelength at 490 nm and a maximum emission wavelength at 630 nm. The application of HB to quench the fluorescence of Trp residues embedded in the hydrophobic domain of membrane proteins has also been reported recently [31]. As shown in Fig. 5, the quenching data of HB were fitted to Eq. 5 with the assumption that V_i is equal to $0.1K_i$ [28] and the results are shown in Table 1. It can be seen from the results in Table 1 that after membrane association, the K_1 of $pG_o\alpha$ decreased from 7.5339×10^5 M to 20 M, while the K_1 of $dG_o\alpha$ increased from 6.1669×10^5 M to 22.9358×10^5 M; the K_2 of $pG_o\alpha$ increased from 2.281×10^4 M to 7.943×10^4 M, while the K_2 of $dG_o\alpha$ increased from 2.266×10^4 M to 5.022×10^4 M. In agreement with the emission spectrum results, the quenching data also revealed that $pG_o\alpha$ and $dG_o\alpha$ underwent a conformational alteration after binding to the membrane.

4. Discussion

Unlike myristoylation and prenylation, the physiological role of palmitoylation in signal transduction of the G protein is elusive. It is well agreed that palmitoylation of Gα subunits is regulated by their activation states [11]. However, the functional consequences of this regulation are largely unknown. Until now, most of the experiments on the relationship be-

Table 1
HB quenching of the fluorescence of two $G_o\alpha$ tryptophan residues

	f_1	K_1 ($\times10^5$ M)	f_2	K_2 ($\times10^4$ M)
$pG_o\alpha$	0.19164	7.5339	0.80845	2.281
$dG_o\alpha$	0.19025	6.1669	0.80979	2.266
LUV–$pG_o\alpha$	0.13615	0.0002	0.86581	7.943
LUV–$dG_o\alpha$	0.07244	22.9358	0.92755	5.022

f: fractional contribution of the fluorescence of tryptophan; K: Stern–Volmer constant of the HB quenching of tryptophan.

tween palmitoylation of $G_o\alpha$ and its association with the membrane have been carried out at a cytological level and the data of these experiments are largely controversial and to some extent difficult to explain [4,6,13,16,17]. Therefore, in vitro study of the palmitoylation and membrane association of $G_o\alpha$ would be helpful to clarify the role of palmitoylation of $G_o\alpha$ in its membrane association.

In this paper we provided direct evidence that the palmitoylation of $G_o\alpha$ may facilitate its membrane association. Consistent with the additional hydrophobicity introduced by palmitoylation, we found that palmitoylation increased the avidity of $G_o\alpha$ for membranes. Moreover, palmitoylated $G_o\alpha$ exhibited a much longer membrane association than depalmitoylated $G_o\alpha$. However, it should be pointed out that although the membrane avidity of $G_o\alpha$ decreased significantly after depalmitoylation (with K_d approximately 10^{-6} M), it is still much higher than the accessible lipid concentration in a typical cell (approximately 10^{-3} M)[32]. Thus, after depalmitoylation, most $G_o\alpha$ should remain membrane-bound, rather than being released from the membrane to the cytosol. Furthermore, the membrane association of $dG_o\alpha$ is still much more stable than that of monoacylated lipopeptides (with half-life <1 s) [33,34] and should be stable enough for most biological processes. Moreover, considering that both the GTPase cycle and palmitoylation turnover are within the time scale of several minutes [11], most of the $dG_o\alpha$ will be re-palmitoylated before release. Therefore, our data suggest that activation and depalmitoylation may not actually result in the release of $G\alpha$ from the membrane to the cytosol, which is consistent with the observation of Mumby's group [19].

It is established that agents known to interact only with the phospholipid headgroup do not induce a surface pressure increase in the monolayer [35]. We found that the surface pressure of asolectin monolayers actually increased after the injection of $G_o\alpha$ into the subphase while no surface pressure increment was observed after the injection of buffer or bovine serum albumin. This indicates that at least part of $G_o\alpha$ was inserted into the monolayer, and the most likely candidates are the acyl modifications. Further measurement of the limiting insertion pressure also indicated that the limiting insertion pressure of $pG_o\alpha$ is higher than that of $dG_o\alpha$ (Fig. 3), which would mean that the membrane insertion ability of $pG_o\alpha$ is stronger than that of $dG_o\alpha$.

The data mentioned above also show that the affinity of $dG_o\alpha$ is much higher than previously estimated [36], and the dissociation of $G_o\alpha$ is much slower than that of the lipopeptide, which may indicate that acylation promotes the membrane association of $G\alpha$ by other mechanisms besides hydrophobicity change after palmitoylation. Therefore, studies and comparison of conformation changes of $pG_o\alpha$ and $dG_o\alpha$ upon membrane association would further provide new clues to reveal how palmitoylation influences the membrane association of $G\alpha$.

It is known that bovine brain $G_o\alpha$ contains two Trp residues, that is, Trp 132 (corresponding to Trp 129 of $G_t\alpha$) and Trp 212 (corresponding to Trp 207 of $G_t\alpha$). According to the published three-dimensional structure of $G_t\alpha$ and $G_i\alpha$[37–39], Trp 132 is at the α-helical domain of $G\alpha$, and Trp 212 is at the Switch II domain. Trp 132 is more buried, while Trp 212 is relatively exposed. In Table 1 we postulated that f_1 and K_1 correspond to the fluorescence fractional contribution and Stern–Volmer constant, respectively, of Trp 212 while f_2 and K_2 correspond to Trp 132, which was based on the following two observations, as shown in Table 1: (1) in aqueous solution K_1 was greater than K_2, which meant that the Trp corresponded to K_1 was more exposed; and (2) alteration amplitude of K_1 was greater than K_2 upon membrane association, which meant that the conformational change was greater. As mentioned above, Trp 212 locates at the Switch II domain and Trp 132 at the helix domain. The Switch II domain is the interface of $G\alpha$–$\beta\gamma$ interaction; in the absence of $G_{\beta\gamma}$, the Switch II domain of GDP-bound $G\alpha$ will be randomly coiled [37]. Since no $\beta\gamma$ in our experiments was presented and $G_o\alpha$ was GDP-bound, Trp 212 should be more exposed than Trp 132 at the helix domain. Moreover, the Switch II domain is the most flexible domain in $G\alpha$, so it is more likely that K_1 corresponds to Trp 212 rather than Trp 132.

It can be seen from the results in Table 1 that after membrane association, the K_2 of $pG_o\alpha$ increased from 2.281×10^4 M to 7.943×10^4 M, while the K_2 of $dG_o\alpha$ increased from 2.266×10^4 M to 5.022×10^4 M. This may show that Trp 132 becomes more exposed to HB after membrane association. The data in Table 1 also indicate that the membrane association influences the quenching efficiency of the other tryptophan. The K_1 of $pG_o\alpha$ decreased from 7.5339×10^5 M to 20 M, while the K_1 of $dG_o\alpha$ increased from 6.1669×10^5 M to 22.9358×10^5M, which implies that after membrane association, the Switch II domain of $G_o\alpha$ underwent a dramatic conformational change. These data further confirmed the conformational change of $d/pG_o\alpha$ upon membrane association.

The results in Table 1 also show that both the fluorescence fractional contributions and Stern–Volmer constants of the two Trps of $pG_o\alpha$ and $dG_o\alpha$ are not significantly different before the membrane association. This signifies that the conformational difference between $pG_o\alpha$ and $dG_o\alpha$ in aqueous circumstances is negligible. However, the dramatic difference between K_1 of LUV–$pG_o\alpha$ (20 M) and K_1 of LUV–$dG_o\alpha$ (22.9358×10^5 M) clearly indicates the remarkable conformational difference between the membrane-bound $pG_o\alpha$ and $dG_o\alpha$. The significant conformational difference between the membrane-bound and membrane-unbound $pG_o\alpha$ and $dG_o\alpha$, and the absence of difference between the conformation of $pG_o\alpha$ and $dG_o\alpha$ in an aqueous environment would suggest that membrane association may be involved in the regulation of $G\alpha$ signaling by its palmitoylation.

Palmitoylation may indeed modulate signal transduction by regulating the membrane association or dissociation of some peripheral proteins; however, this should not be the only function of reversible palmitoylation, because many integral membrane proteins are also modified by palmitate at cysteine residues. There are accumulating data suggesting the diversity of the functions of palmitoylation [40–42]. In addition, palmitate modification may also regulate the partitioning of signaling proteins between microdomains [19]. Many signaling proteins that are both myristoylated and palmitoylated appear to be enriched at specialized plasma microdomains called detergent-insoluble glycosphingolipid-enriched membranes (DIGs) [43]. Regulated interactions between $G\alpha$ and caveolin, a protein located specifically in a kind of DIG, caveolae, have also been reported [44]. Also, the N-termini of dually acylated $G\alpha$ are found to be sufficient to target green fluorescent protein to caveolae [45]. We have also observed the interaction between $p/dG_o\alpha$ and caveolin (data not shown). Studies on how palmitoylation regulates the functions of the G protein by mod-

ulating microdomain partitioning and/or protein–protein interaction are in progress.

In summary, palmitoylation of native bovine brain $G_o\alpha$ may facilitate the membrane association of $G_o\alpha$. Conformational differences of $pG_o\alpha$ and $dG_o\alpha$ after the membrane association may also be involved in the regulation of $G\alpha$ signaling by palmitoylation.

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