Biochemistry (Moscow), Vol. 68, No. 1, 2003, pp. 121-128. Translated from Biokhimiya, Vol. 68, No. 1, 2003, pp. 145-153.

Evidence for Disaggregation of Oligomeric $G_o\alpha$ Induced by Guanosine-5′-3-O-(thio)triphosphate Activation

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Received March 16, 2002
Revision received April 25, 2002

Abstract—Myristoylated $G_o\alpha$ was expressed in and highly purified from *Escherichia coli* strain JM109 cotransformed with pQE60 ($G_o\alpha$) and pBB131 (N-myristoyltransferase, NMT). Non-denaturing gel electrophoresis and gel filtration analysis revealed that the $G_o\alpha$, in its GDP-bound form, could form oligomers involving dimer, trimer, tetramer, pentamer, or hexamer and guanosine 5′-3-O-(thio)triphosphate (GTPγS) activation induced disaggregation of the $G_o\alpha$ oligomers to monomers. The $G_o\alpha$ was crosslinked by a cross-linker, N,N′-1,4-phenylenedimaleimide (*p*-PDM), yielding multiple crosslinked products. In contrast, no obvious cross-linking occurred when $G_o\alpha$ was pretreated with GTPγS. Immunoblot analysis also demonstrated oligomerization of the purified $G_o\alpha$ proteins and its disaggregation triggered by GTPγS. These results provided direct evidence for the "disaggregation–coupling" theory and the disaggregation action of GTPγS may further elucidate the regulatory role of GDP/GTP exchange in G protein-coupled signal transduction pathways.

Key words: $G_o\alpha$, GTPγS, chemical cross-linking, oligomerization and disaggregation, GDP/GTP exchange

G protein-coupled signal transduction pathways are composed of receptors, heterotrimeric guanine-nucleotide-binding proteins (G proteins) and effectors [1-3]. Stimulation of G proteins with activated receptors results in regulation of a variety of enzymes and ion channels. G proteins are inactive in the GDP-bound state and are activated by receptor-catalyzed guanine nucleotide exchange resulting in binding of GTP to the α subunit. The binding of GTP leads to dissociation of Gα·GTP from Gβγ subunits and activation of downstream effectors by both Gα·GTP and free Gβγ subunits [3]. Since G proteins cannot diffuse freely on the membrane, the model of "subunit dissociation" does not explain satisfactorily how activation of a receptor can theoretically cause many Gα proteins to be activated and the signals are produced in rapidly (fractions of seconds) reversible fashion. G-protein-coupled receptors (GPCRs) have recently joined the list of cell surface receptors that are known to dimerize. Dimerization has been shown to alter the ligand-binding, signaling, and trafficking properties of these receptors [4, 5]. In the latest crystallographic studies, it was found that the N-terminal tail of phospholipase C-β, the effector of G_q, could also mediate dimerization [6]. However, the exact structure that G proteins behave as in GDP-bound form and GTP-bound form remains unclear and has not been reported in publications. Rodbell and his colleagues made a series of studies using *n*-octyl glucoside to extract G_s, G_o, and G_i or using digitonin to extract G_s, G_o, G_i, and G_q from rat brain synaptoneurosomes [7, 8], or using *n*-octyl glucoside to extract $G_s\alpha$ from rat liver membrane [9]. They found that the G proteins in detergent extracts of cell membrane displayed multimeric structures and disaggregated to monomers when activated by GTP. These results proposed the hypothesis that G proteins are multimeric in GDP-bound form and disaggregate to monomers when activated by GTP. But most of their experiments were carried out on cell membrane level and using detergent extracts, which cannot provide powerful and direct evidence that G proteins in themselves exist as oligomeric structures rather than heterotrimeric monomer structures and that GTP activation leads to disaggregation of its oligomers. It is well known that GDP/GTP exchange in G protein-coupled signal transmembrane transduction is a key event in modulating the

Abbreviations: BCIP) 5-bromo-4-chloro-3-indolyl-phosphate; BS) bis(sulfosuccinimidyl)suberate; DTT) dithiothreitol; G protein) guanine nucleotide binding protein; GPCRs) G-protein-coupled receptors; GTPγS) guanosine-5′-3-O-(thio)triphosphate; IPTG) isopropyl-β-D-thiogalactoside; NBT) nitro blue tetrazolium; NMT) N-myristoyltransferase; *p*-PDM) N,N′-1,4-phenylenedimaleimide.

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0006-2979/03/6801-0121$25.00 ©2003 MAIK "Nauka/Interperiodica"

inactivation and activation of G proteins. Since Gα subunit is an important catalytic subunit of heterotrimeric G proteins, in this work we investigated oligomerization of the $G_o\alpha$ in GDP-bound form and disaggregation of the $G_o\alpha$ in GTP-bound form with highly purified $G_o\alpha$ from *E. coli*. The results obtained here may provide insight for the understanding of the structures of G proteins in signal transmembrane transduction and further elucidation of how GDP/GTP exchange plays an important role in G protein-coupled signal transduction.

MATERIALS AND METHODS

Materials. Tris, dithiothreitol (DTT), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), and goat anti-rabbit IgG conjugated with alkaline phosphatase were purchased from Promega (USA). Guanosine 5′-3-O-(thio)triphosphate (GTPγS), Hepes, and BSA were from Boehringer Mannheim (Germany). N,N′-1,4-phenylenedimaleimide (*p*-PDM) and urea were from Aldrich (USA). Urease from jack bean and albumin from chicken egg were from Sigma (USA). β-Mercaptoethanol was from Merck (Germany). Nitrocellulose membrane was obtained from Gelman (USA). Rabbit anti-mouse polyclonal antibody specific to $G_o\alpha$ was from Santa Cruz Biotechnology (USA). Plasmid pQE60 ($G_o\alpha$) was a generous gift of Professor Susanne Mumby (University of Texas, Southwestern Medical Center) and plasmid pBB131 (N-myristoyltransferase, NMT) was a generous gift of Professor Gordon (University of Washington). Superdex^{TM} 200 HR 10/30 gel filtration column was purchased from Amersham Pharmacia Biotech Inc. (England). All other chemicals were of reagent grade.

Expression and purification of myristoylated $G_o\alpha$. Myristoylated $G_o\alpha$ was prepared by the method of Mumby [10] with a few modifications. *Escherichia coli* strain JM109 was cotransformed with pQE60 ($G_o\alpha$) and pBB131 (NMT) and grown in T_7 enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, and 50 mM KH_2PO_4, pH 7.2) supplemented with 50 μg/ml ampicillin and 50 μg/ml kanamycin. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 60 μM to induce synthesis of NMT and $G_o\alpha$. The cells were harvested and lysed by lysozyme. The lysate was centrifuged at 30,000*g* for 1 h. The supernatant was applied to a DEAE-Sephacel column and eluted with 300 mM NaCl. The DEAE eluate was brought to 1.2 M in $(NH_4)_2SO_4$ and 25 μM in GDP and applied to a phenyl-Sepharose column and eluted with a gradient of $(NH_4)_2SO_4$ (1.2 to 0 M). In this step, myristoylated $G_o\alpha$ and non-myristoylated $G_o\alpha$ were separated. The pooled myristoylated $G_o\alpha$ was dialyzed against buffer Q (50 mM Tris-HCl, pH 8.0, 0.02 mM EDTA, 1 mM DTT) overnight to remove $(NH_4)_2SO_4$. The protein sample was subsequently applied to a Q-Sepharose column and eluted with a gradient of NaCl (0-300 mM) in buffer Q. The protein fractions were assayed by SDS-PAGE and the fractions of highly purified $G_o\alpha$ were pooled and stored in liquid nitrogen.

GTPγS binding activity and nonenzymatic palmitoylation of myristoylated $G_o\alpha$. [^{35}S]GTPγS binding assays were generally conducted according to the method of Northup [11].

Nonenzymatic palmitoylation of the myristoylated $G_o\alpha$ was generally conducted according to the method of Duncan [12].

Continuous non-denaturing gel electrophoresis. The procedures used were almost the same as described in [13] with a few modifications. The pH value of all the buffers was adjusted to 8.0. $G_o\alpha$·GDP (20 μl of 10 μM) was mixed with 5 μl of 5× sample buffer (300 mM Tris-HCl, pH 8.0, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and subjected to 3-20% non-denaturing gel electrophoresis with constant voltage of 200 V for 5 h. $G_o\alpha$·GTPγS was prepared as following. $G_o\alpha$·GDP (10 μM) in buffer (20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 7.5 mM $MgCl_2$, 500 μM GTPγS) was incubated at 30°C for 20 min. $G_o\alpha$·GTPγS (20 μl of 10 μM) mixed with 5 μl of 5× sample buffer was subsequently subjected to electrophoresis under the same conditions as above.

Western blotting. Blots were processed as described in [13]. $G_o\alpha$·GDP or $G_o\alpha$·GTPγS (20 μl of 2 μM) was resolved on 3-20% polyacrylamide gel. The proteins on the transferred membranes were incubated with the rabbit anti-mouse polyclonal antibody specific to $G_o\alpha$ (1 : 100) at 37°C for 2 h and goat anti-rabbit IgG conjugated with alkaline phosphatase (1 : 7000) at 37°C for 2 h, respectively.

Cross-linking of $G_o\alpha$ by *p*-PDM. Cross-linking of the $G_o\alpha$ was performed as described in [14]. In brief, the purified $G_o\alpha$ was dialyzed for 4 h against buffer A (20 mM Hepes, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$) to remove DTT. $G_o\alpha$·GDP or $G_o\alpha$·GTPγS (5 μM) was subjected to cross-linking by adding 75 μM *p*-PDM in dimethylformamide at 20°C for 30 min. The reaction was quenched by adding 8 mM β-mercaptoethanol.

Gel filtration. $G_o\alpha$ (100 μl of 5 μM) was injected onto Superdex^{TM} 200 HR 10/30 column using an ÄKTApurifier HPLC system (Amersham Pharmacia Biotech Inc.). Elution was conducted with buffer A at 0.5 ml/min. Eluted proteins were monitored at 280 nm. The molecular weight markers including urease from jack bean and albumin from chicken egg were injected onto the same column in parallel experiments to characterize the elution position.

Protein concentration determination. The protein concentration was determined by the Bradford method using BSA as a standard [15].

RESULTS

Purification of myristoylated recombinant $G_o\alpha$. *E. coli* cells expressing $G_o\alpha$ were harvested, lysed, and centrifuged. The supernatant was applied to DEAE-Sephacel, phenyl-Sepharose, and Q-Sepharose in series to get highly purified $G_o\alpha$. SDS-PAGE showed only one band with purity greater than 95% (Fig. 1a, lane G) and the band could be further recognized by anti-$G_o\alpha$ antibody (Fig. 1b). The [^{35}S]GTPγS binding activity of the purified $G_o\alpha$ was 20 ± 1.3 nmol/mg protein, so the GTPγS binding in moles per mole of protein was 0.8. The recombinant $G_o\alpha$ was successfully palmitoylated and the palmitoylation ratio was greater than 99% as conducted according to reference [16]. Since only myristoylated $G_o\alpha$ can be palmitoylated and non-myristoylated $G_o\alpha$ fails to incorporate palmitate [12], our results demonstrated the recombinant $G_o\alpha$ was myristoylated. The highly purified $G_o\alpha$ facilitated further study on oligomerization and disaggregation of the $G_o\alpha$.

Continuous non-denaturing gel electrophoresis of oligomeric $G_o\alpha$. Non-denaturing gel electrophoresis is one of the available approaches to detect oligomerization or disaggregation of proteins or protein subunits. Figure 2 shows oligomerization of the $G_o\alpha$ examined by 3-20% non-denaturing gel electrophoresis. It can be clearly seen that the $G_o\alpha$·GDP exhibited several bands with molecular weight above 66 kD (Fig. 2a, L2). As the molecular weight of monomeric $G_o\alpha$ is about 39 kD, it was readily determined that $G_o\alpha$·GDP may exist as dimer, trimer, tetramer, pentamer, and hexamer mainly according to molecular weight standards. Moreover, the intensity of the bands became weaker while oligomeric grade increased. When the $G_o\alpha$ was pre-activated with GTPγS, the $G_o\alpha$ formed mainly monomers with few oligomers existing (Fig. 2a, L1). The similar results of the oligomerization of the $G_o\alpha$·GDP (Fig. 2b, L3) and its disaggregation (Fig. 2b, L4) induced by GTPγS could be further confirmed with immunoblot analysis.

Oligomerization of protein is different from its aggregation. Proteins in oligomerization should be still in their active state. When protein is denatured, it tends to aggregate without discrete bands. In order to distinguish the oligomerization and aggregation in the samples as indicated in Fig. 3a, [^{35}S]GTPγS binding activity of those

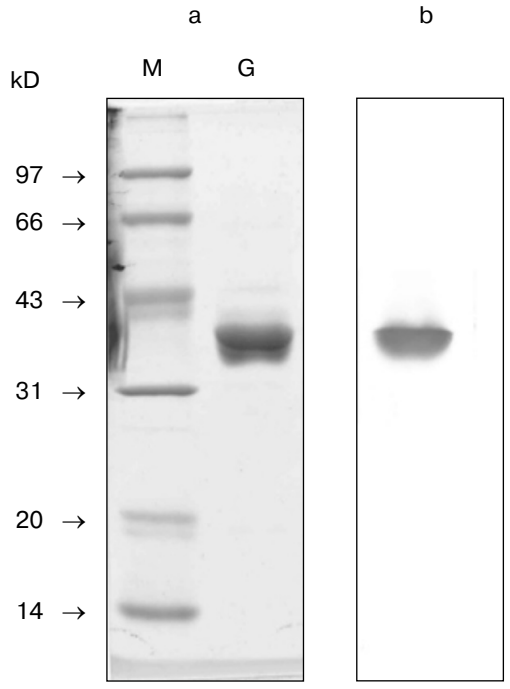

Fig. 1. a) SDS-PAGE of purified recombinant $G_o\alpha$ in 12% polyacrylamide gel. Lanes: M) molecular weight standards; G) recombinant myr-$G_o\alpha$. b) Western blotting of the purified $G_o\alpha$ with the specific antibody as described in "Materials and Methods". The proteins were electrophoretically transferred to nitrocellulose membrane in a Bio-Rad Mini Trans-Blot Cells apparatus with constant voltage of 100 V for 2 h. NBT and BCIP were added to detect protein bands.

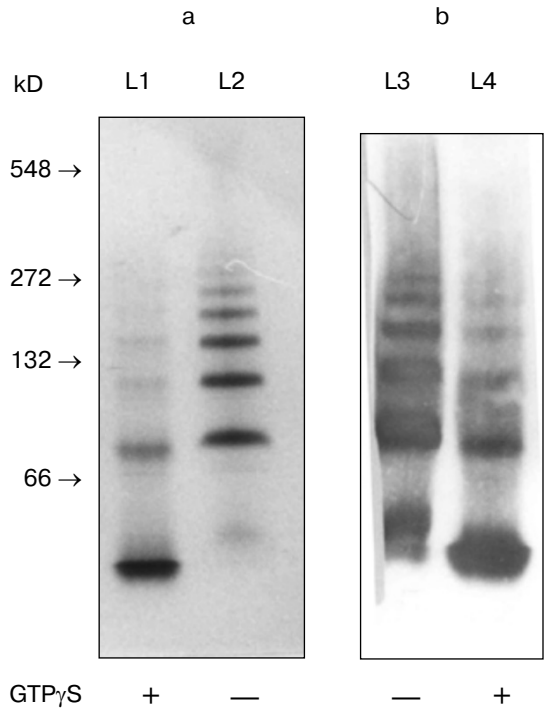

Fig. 2. Oligomerization of $G_o\alpha$ analyzed by non-denaturing gel electrophoresis: a) 3-20% non-denaturing polyacrylamide gel; b) Western blotting performed as above following 3-20% non-denaturing gel electrophoresis. GTPγS (+), $G_o\alpha$ was activated by 500 μM GTPγS before electrophoresis (lanes L1 and L4); GTPγS (–), $G_o\alpha$ was not activated by GTPγS before electrophoresis (lanes L2 and L3).

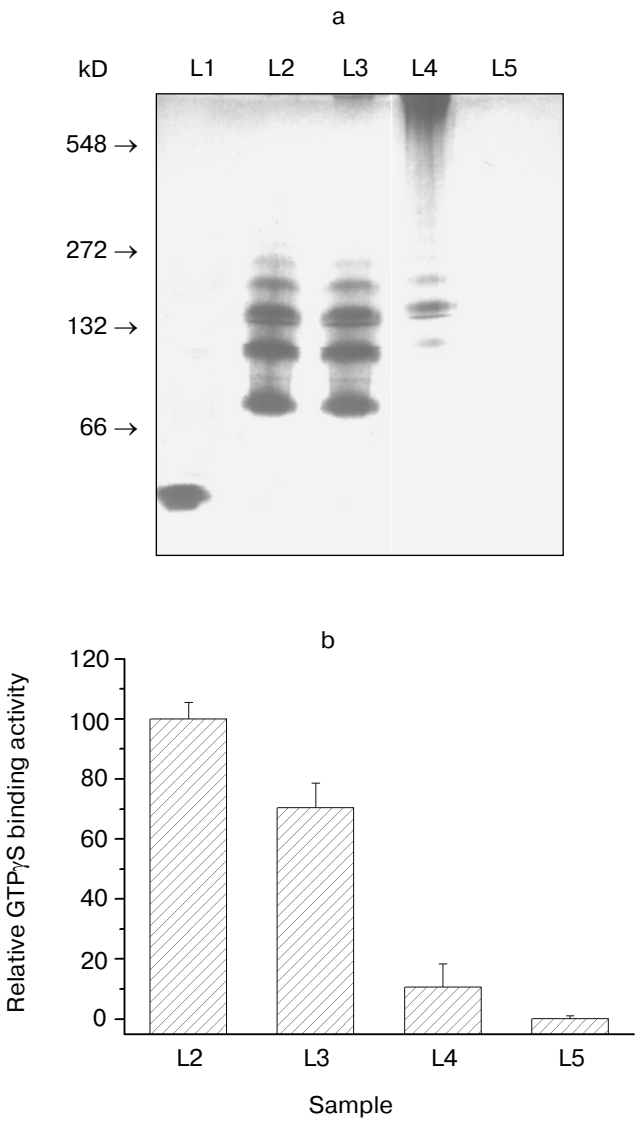

Fig. 3. Behavior of $G_o\alpha$ in oligomerization or aggregation analyzed by non-denaturing gel electrophoresis (a) and its GTPγS binding activity (b). a) 3-20% non-denaturing polyacrylamide gel. Lanes: L1) $G_o\alpha$ activated by 500 μM GTPγS; L2) $G_o\alpha$·GDP; L3) $G_o\alpha$·GDP frozen-thawed rapidly in liquid nitrogen for 4 times; L4) $G_o\alpha$·GDP denatured by 5 M urea and then dialyzed to remove urea; L5) $G_o\alpha$·GDP incubated at 75°C for 20 min. b) Assay of [^{35}S]GTPγS binding activity of the corresponding samples in Fig. 3a. The activity assay was conducted as indicated in "Materials and Methods" and the [^{35}S]GTPγS binding activity of the $G_o\alpha$ without any treatment was taken as 100%.

samples was analyzed. It can be seen (Fig. 3b) that the proteins with higher activity showed significant oligomers (L2, L3), the proteins with less activity showed aggregation (L4), even the denatured proteins could not enter the gel (L5).

Cross-linking of $G_o\alpha$ by *p*-PDM. *p*-PDM (12 Å) is a homobifunctional cross-linking reagent reacting with sulfhydryl group. Previous studies suggested that *p*-PDM was effective in cross-linking G proteins with sulfhydryl reactive groups [14]. In the chemical cross-linking experiments, we found that Tris and DTT might interfere with *p*-PDM to gain crosslinked products. Thus it was necessary to dialyze the purified $G_o\alpha$ proteins in Tris buffer against Hepes buffer to remove DTT and Tris. The $G_o\alpha$ with or without dialysis was crosslinked by *p*-PDM and subjected to SDS-PAGE. The dialyzed $G_o\alpha$ displayed obvious crosslinked products, while the $G_o\alpha$ without the dialysis displayed no crosslinked products (data not shown). This suggested that it was essential to establish an optimum reaction condition of the cross-linker used in order to realize cross-linking of defined proteins.

We further explored the effect of GTPγS activation on cross-linking of the $G_o\alpha$ either in its latent form, $G_o\alpha$·GDP, or in its activated form, $G_o\alpha$·GTPγS, with the cross-linker *p*-PDM. The $G_o\alpha$ was subjected to cross-linking and then analyzed by 3-20% SDS polyacrylamide gel electrophoresis as shown in Fig. 4. It revealed that $G_o\alpha$·GDP displayed large crosslinked products with molecular weight much greater than that of $G_o\alpha$ monomers (Fig. 4a, L3), while $G_o\alpha$·GTPγS displayed no large crosslinked products with only one band approximately above the monomer (Fig. 4a, L2). This result seemed to be similar to the observation of Park in which the GTPase Rab3A was crosslinked by bis(sulfosuccinimidyl)suberate (BS) [17]. Figure 4b shows the western blotting analysis of 3-20% SDS polyacrylamide gel performed as above. It was clear that $G_o\alpha$·GDP could

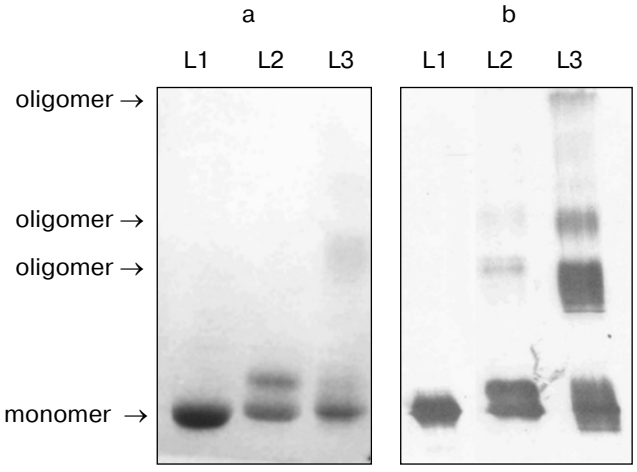

Fig. 4. Cross-linking of $G_o\alpha$ with *p*-PDM analyzed by SDS-PAGE (a) and Western blotting (b) on 3-20% denaturing polyacrylamide gel. Each protein sample (in 20 μl) was mixed with 5 μl of 5× sample buffer (300 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue), heated at 100°C for 4 min and subjected to 3-20% SDS-polyacrylamide gel electrophoresis for 5 h at 200 V. Lanes: L1) $G_o\alpha$·GDP as a control; L2) $G_o\alpha$ activated by 500 μM GTPγS preceding to cross-linking; L3) $G_o\alpha$ not activated by GTPγS preceding to cross-linking.

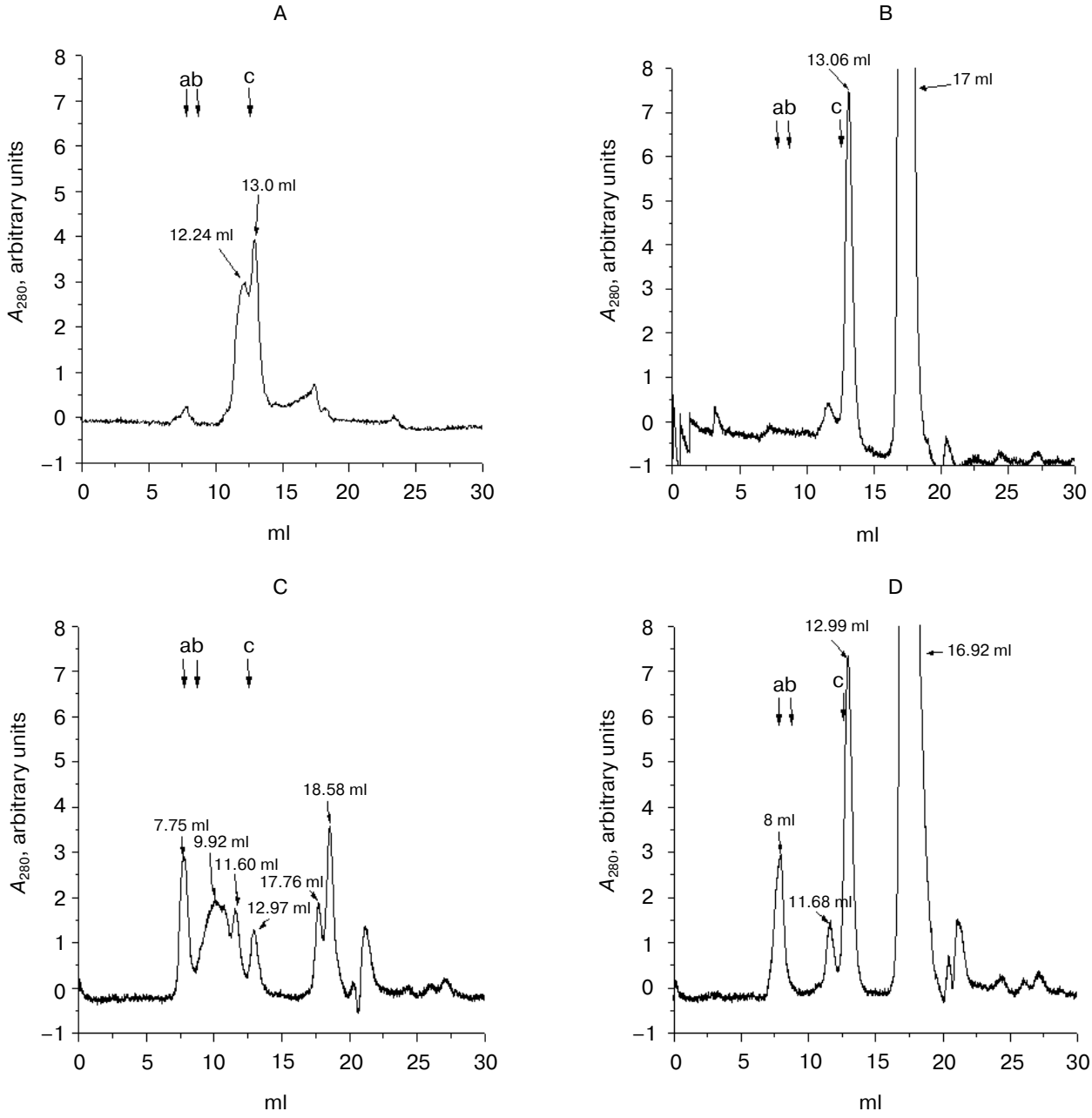

Fig. 5. Gel filtration of $G_o\alpha$ in the absence or presence of a cross-linker *p*-PDM on Superdex^{TM} 200 HR 10/30 column. A) Uncrosslinked $G_o\alpha$·GDP; B) uncrosslinked $G_o\alpha$·GTPγS ($G_o\alpha$ was activated by 500 μM GTPγS); C) crosslinked $G_o\alpha$·GDP; D) crosslinked $G_o\alpha$·GTPγS. The arrows indicate the elution position: a) urease from jack bean (hexamer, 545 kD, 7.81 ml); b) urease from jack bean (trimer, 272 kD, 8.69 ml); c) albumin from chicken egg (monomer, 45 kD, 12.64 ml).

yield significant intermolecular crosslinked products (Fig. 4b, L3), which reinforced the results of L3 in Fig. 4a, but the $G_o\alpha$·GTPγS exhibited very few and faint crosslinked products (Fig. 4b, L2) as showed in the case of L2 in Fig. 4a.

Gel filtration chromatography. From the above results of the native gel electrophoresis combined with western blotting assay, we suppose that $G_o\alpha$ proteins themselves can form oligomers that are sensitive to disaggregation by GTPγS. These results can be further confirmed by gel filtration chromatography of $G_o\alpha$ in the presence or absence of the cross-linker *p*-PDM. Figure 5 shows the gel filtration elution profiles of the uncrosslinked or crosslinked $G_o\alpha$·GDP or $G_o\alpha$·GTPγS. It can be seen that uncrosslinked $G_o\alpha$·GDP displayed two coterminous peaks, the elution volumes of which were 12.24 and 13.0 ml, respectively (Fig. 5A), and

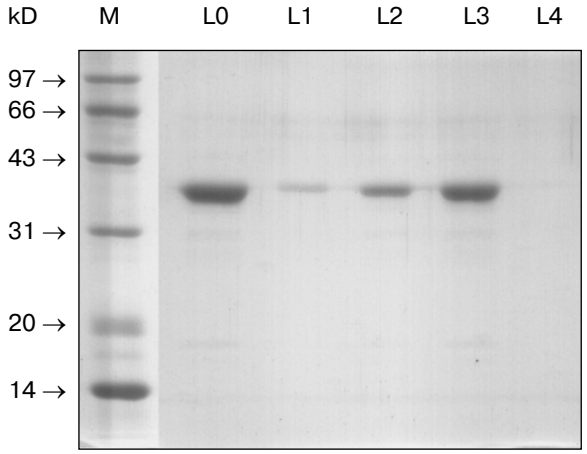

Fig. 6. SDS-PAGE of the fractions eluted from the gel filtration column. Lanes: L1 and L2) fractions corresponding to 12.24 and 13.0 ml elution peak of $G_o\alpha$·GDP, respectively (Fig. 5A); L3 and L4) fractions corresponding to 13.06 and 17.0 ml elution peak of $G_o\alpha$·GTPγS, respectively (Fig. 5B); L0) $G_o\alpha$·GDP without gel filtration as a control. The electrophoresis of the samples was performed the same as that in Fig. 1.

uncrosslinked $G_o\alpha$·GTPγS displayed two significant separate peaks, the elution volumes of which were 13.06 and 17.0 ml, respectively (Fig. 5B). The eluate was concentrated and subjected to SDS-PAGE analysis. As shown in Fig. 6 that L1, L2, and L3 corresponding to the elution peaks of 12.24 and 13.0 ml (Fig. 5A) and 13.06 ml (Fig. 5B) all exhibited one protein band with the same electrophoretic mobility as the control $G_o\alpha$ (Fig. 6, L0). However, no protein bands were observed on lane 4 (L4) corresponding to the elution peak of 17.0 ml (Fig. 5B) which might be an absorption peak of GTPγS.

Based on the results of Figs. 2 and 4, we demonstrated that $G_o\alpha$·GDP in itself can form oligomers and disaggregated to monomers when activated by GTPγS. Therefore, it could be deduced from the results of Fig. 6 that both the eluate of the 13.0 ml peak in Fig. 5A and the 13.06 ml peak in Fig. 5B were monomeric $G_o\alpha$; the eluate of the 12.24 ml peak in Fig. 5A was oligomeric $G_o\alpha$ with high molecular weight and less elution volume.

Figures 5C and 5D show the elution profiles of the crosslinked $G_o\alpha$·GDP and $G_o\alpha$·GTPγS. Crosslinked $G_o\alpha$·GDP displayed a distinct elution profile (Fig. 5C) compared to that of $G_o\alpha$·GDP (Fig. 5A), while the elution profile of crosslinked $G_o\alpha$·GTPγS (Fig. 5D) was similar to that of the uncrosslinked $G_o\alpha$·GTPγS (Fig. 5B). These results demonstrated that $G_o\alpha$·GDP could be crosslinked strongly by *p*-PDM, while $G_o\alpha$·GTPγS very weakly. Since the elution position of the same protein changes very little under the same experimental conditions, we get the following conclusions, according to the comparison of elution profiles between crosslinked and uncrosslinked $G_o\alpha$, that the eluate of 12.97 ml peak in Fig. 5C and the eluate of 12.99 ml peak in Fig. 5D were corresponding to monomeric $G_o\alpha$, because the elution volumes were almost equal to 13.0 ml in Fig. 5A and 13.06 ml in Fig. 5B, respectively. Thus the 13.0 ml peak must be the elution position of monomers under this condition. The eluate of the 16.92 ml peak in Fig. 5D might be derived from GTPγS. In terms of the characteristics of the gel filtration chromatography technique, the protein eluted below 13 ml volume has higher molecular weight than that of monomeric $G_o\alpha$; the protein eluted above 13 ml volume has lower molecular weight than that of monomeric $G_o\alpha$. Therefore, the eluates of 17.76 and 18.58 ml peak in Fig. 5C were derived from small molecules. Those elution peaks less than 12.97 ml (Fig. 5C) were derived from intermolecular crosslinked products with molecular weight all above 39 kD. It should be pointed out that the eluate of the 7.75 ml peak (Fig. 5C) appeared prior to the hexamer of urease from jack bean, which indicated the formation of very large crosslinked structures with molecular weight greater than 545 kD that failed to enter the stacking gel and were not detected on the gel.

DISCUSSION

It is well known that heterotrimeric GTP-binding proteins, commonly termed G proteins, serve as transducers between activated receptors and effectors [1-3]. G proteins consist of α, β, and γ subunits. During the past decade more attention has been paid to the studies of structural change of G proteins in signal transduction. A widely held theory termed "subunit dissociation" suggests that an activated receptor turns a G protein on, promoting exchange of GTP for GDP bound to the Gα subunit, followed by dissociation of Gα·GTP from Gβγ [3]. Much experimental evidence consistent with this opinion have implicated heterotrimer as being the natural structure of G proteins located in cell membrane responsible for receptor-mediated actions of external signals. Although these propositions have been widely accepted, some of the underlying assumptions have been challenged in part because of findings that G proteins can form large structures much greater than heterotrimer. It should be mentioned here that some of the experiments from Rodbell's laboratory about this issue were carried out with G-protein in detergent extracts of cell membranes [7, 8], which would make the system more complicate. Until recently, GPCRs were thought to function as monomers. However, a growing number of biochemical, biophysical, and functional studies suggest that GPCRs form functional dimers [4, 5]. In crystal structure, the dimerization of G protein effector has also been examined [6]. This proposes the possibility that oligomerization may be a common property of proteins related to G protein-coupled signal transduction.

Whether Gα subunits in themselves can form oligomers still remains to be elucidated. Gα is a catalytic subunit of G proteins. The GDP/GTP exchange of Gα is a key step in G-protein-coupled signal transduction. It is necessary to explore the organization of G proteins, especially of Gα subunits, in different guanine nucleotide bound forms. Thus, studies with highly purified Gα proteins would be very important and helpful to clarify these key events. In this paper, we presented several lines of direct evidence for the oligomerization of the purified $G_o\alpha$ in itself by combining different approaches of non-denaturing gel electrophoresis, gel filtration chromatography, chemical cross-linking by *p*-PDM, and immunoblot analysis. The evidences obtained were the following.

1. The non-denaturing gel electrophoresis (Fig. 2) and gel filtration analysis (Fig. 5) indicated that $G_o\alpha$ proteins in themselves can form oligomers. A number of regulators of signal transduction have been known to interact with G proteins [18, 19]. The multimeric structures of G proteins may favor direct protein–protein interactions and contribute to specificity and fidelity of signaling. Furthermore, oligomeric G proteins can be disaggregated to monomers when activated by GTPγS. This demonstrated that the GDP/GTP exchange plays a key role in G protein cycle. It should be mentioned that the native gel showed clear distinct bands like ladders (Fig. 2a, L2), while immunoblot analysis did not show such clear bands but color of the whole developed lanes was too dark with the same quantity of $G_o\alpha$ proteins (data not shown). Therefore, we decreased the amount of $G_o\alpha$ proteins loaded on the gel, which showed better resolution as indicated in Fig. 2b, but we still can see that the area between different bands of the lanes also could be developed by anti-$G_o\alpha$ antibody, which made those bands not as clear as that in Fig. 2a. This may imply that the oligomerization and disaggregation of the $G_o\alpha$ was a dynamic and rapid process. $G_o\alpha$ proteins cannot form very stable oligomers in the case of non-denaturing electrophoresis experimental conditions. The results of the gel filtration may also confirm this situation as showed in Fig. 5A in which we did not observe several peaks of different oligomers with $G_o\alpha$·GDP. Another point is that oligomerization of the $G_o\alpha$ is quite different from aggregation of denatured proteins. The results here indicated that aggregated proteins cannot exhibit distinct bands when examined by continuous non-denaturing gel electrophoresis (Fig. 3a, L4). Under rigid denaturation condition, $G_o\alpha$ formed very large aggregates that even failed to enter the stacking gel and were not detected on the gel (Fig. 3a, L5).

2. Through chemical cross-linking of $G_o\alpha$ by *p*-PDM as a cross-linker, we found that $G_o\alpha$·GDP yielded intermolecular crosslinked products of large sizes, but pre-activation of $G_o\alpha$ by GTPγS, $G_o\alpha$·GTPγS did not show remarkable intermolecular cross-linking reactions (Fig. 4, L2 and Fig. 5D). We also observed that the quantities of monomers of $G_o\alpha$·GDP decreased with extended cross-linking time, but it should be mentioned that quantities and intensity of the crosslinked bands were not obviously increased when analyzed by SDS-PAGE (data not shown). This may imply that very large crosslinked structures appeared and failed to enter the stacking gel. This assumption was further confirmed by the gel filtration experiments of the crosslinked $G_o\alpha$·GDP. As shown in Fig. 5C, the large structures eluted in 7.75 ml appeared prior to the hexamer of urease from jack bean, which would mean formation of large crosslinked products with molecular weight greater than 545 kD. They were so large as to be unable to enter the gel and could not be detected on the gel. This may implicate that $G_o\alpha$ proteins exist as large multimeric structures in the native membrane environment. Although we did not add excess GDP or GTPγS in gel filtration buffer, $G_o\alpha$ would not release its guanine nucleotide in the presence of Mg^{2+} during the gel filtration, which would be helpful to keep the $G_o\alpha$ from denaturation [3].

3. The results obtained from native gel electrophoresis and chemical cross-linking were further reinforced by immunoblot analysis (Figs. 2b and 4b). It should be pointed out that the protein concentration in Western blotting experiments (Fig. 4b) was about 40% of that loaded on SDS polyacrylamide gel (Fig. 4a), but decrease of the protein concentration of the samples did not weaken the cross-linking of $G_o\alpha$·GDP. In contrast, immunodetection displayed more obvious crosslinked products (Fig. 4b, L3) than SDS-PAGE (Fig. 4a, L3) due to its high resolution. It could be deduced that the cross-linking was not owing to intermolecular collision [20] but owing to oligomerization of the $G_o\alpha$ proteins.

4. The $G_o\alpha$ used in this study was expressed and purified from *E. coli* without $\beta\gamma$ complex. It would be most unquestionable that formation of oligomers of the $G_o\alpha$ may not need $\beta\gamma$ complex. This also provided stronger direct evidence for the experiments of Rodbell [9] in which G proteins in *n*-octyl glucoside extracts were analyzed.

In conclusion, we provide several lines of more direct evidence that $G_o\alpha$ proteins themselves can form oligomers in inactive state and disaggregate to monomers when activated by GTPγS. The oligomerization of G proteins gives a conclusive explanation of how ligand-activated receptors catalytically activate G proteins in a very rapid manner both at the onset and the offset and with amplification of the signals generated in response to the activated process. The disaggregation of oligomeric Gα induced by GTPγS would be helpful to further understand the significance of GDP/GTP exchange mode in the G protein cycle of signal transduction in cells.

This work was generously supported by Project 39730130 from the National Natural Science Foundation of China. We thank Professor Susanne Mumby (University of Texas, Southwestern Medical Center) for a

gift of plasmid pQE60 ($G_o\alpha$) and Professor Gordon (University of Washington) for a gift of plasmid pBB131 (NMT).

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