Rhodopsin (Rho) is a G protein-coupled receptor that initiates phototransduction in rod photoreceptors. High expression levels of Rho in the disc membranes of rod outer segments and the propensity of Rho to form higher oligomeric structures are evident from atomic force microscopy, transmission electron microscopy, and chemical cross-linking experiments. To explore the structural and functional properties of Rho in n-dodecyl-β-maltoside, frequently used to purify heterogeneously expressed Rho and its mutants, we used gel filtration techniques, blue native gel electrophoresis, and functional assays. Here, we show that in micelles containing n-dodecyl-β-maltoside at concentrations greater than 3 mM, Rho is present as a single monomer per detergent micelle. In contrast, in 12 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), micelles contain mostly dimeric Rho. The cognate G protein transducin (GT) appears to have a preference for binding to the Rho dimer, and the complexes fall apart in the presence of guanosine 5'-3-(thio)triphosphate. Cross-linked Rho dimers release the chromophore at a slower rate than monomers and are much more resistant to heat denaturation. Both Rho* monomers and dimers are capable of activating GT, and both of them are phosphorylated by Rho kinase. Rho expressed in HEK293 cells is also readily cross-linked by a bifunctional reagent. These studies provide an explanation of how detergent influences the oligomer-dimer-monomer equilibrium of Rho and describe the functional characterization of Rho monomers and dimers in detergent.

Rhodopsin (Rho), the prototypical G protein-coupled receptor (GPCR), functions in the absorption of a photon in retinal rod photoreceptors (1–3). As are other GPCRs (4, 5), Rho is a seven-transmembrane-spanning helical protein (6). The high expression level of Rho, its specific localization in the internal discs of the structures termed rod outer segments (ROS), and the lack of other highly abundant membrane proteins allow Rho to be imaged in the native disc membranes by atomic force microscopy and transmission electron microscopy (7–9). These images have revealed rows of Rho dimers in native disc membranes. Subsequently, BN- and SDS-PAGE, chemical cross-linking, and proteinolysis experiments corroborated that Rho consists mainly of dimers and higher oligomers in disc membranes. Medina et al. (7) reported that Rho and photoactivated Rho (Rho*) preserved a dimeric quaternary structure in detergent.

These results are in conflict with a model of rapidly diffusing Rho in the “mosaic” fluid disc membrane (10) supported by measurements of Rho diffusion and rotation in disc membranes and by low resolution neutron diffraction studies (11–14). The concept of rapidly diffusing monomeric molecules is at variance with the dimeric forms of other GPCRs (15, 16). Moreover, the size of the G protein and arrestin surface interacting with Rho is almost twice as large as the exposed cytoplasmic surface of a single Rho molecule (17, 18).

Nonphysiological dimers of Rho can be found in its three-dimensional crystal structures (6, 19–21). The two subunits are related by a rotation of 180° about an axis in the plane of the membrane. This orientation would allow a larger than expected hydrophobic surface area to be engaged in interaction between Rho molecules residing in the native membranes (17). It would also place the cytoplasmic surfaces of the two subunits on opposite sides of the membrane. These dimers in the crystal appear to be induced by experimental crystallization conditions. Reconstitution of Rho into two-dimensional crystals also produces dimers where both Rho molecules are correctly oriented, but the dimers contact other dimers of Rho that are flipped in their orientation (e.g. see Refs. 22–24).
Progress on Rho topology and its signaling states was decisively advanced when Rho was cloned (25), expressed, and purified from heterologous systems using mild detergents (26). This procedure opened up seminal work on identification of the counter-ion of the chromophore, the role of a number of post-translational modifications, and other structural/functional properties of Rho (reviewed in Refs. 27–29). Concurrent work on other GPCRs, most notably the β-adrenergic receptor, reveals many common features among these receptors (30–32). Together, these experiments raise the question of whether Rho appears as a monomer, dimer, or higher oligomer in detergent. Another important aspect is whether the monomers in the dimer are unidirectionally oriented as observed in native disc membranes or whether they are bidirectionally oriented in a nonphysiological configuration as seen in the previously published crystal structures. Here, we describe the solubilization procedure of Rho that preserves receptor dimers in n-dodecyl-β-maltoside (DM) and oligomers in CHAPS. Different forms of Rho were also characterized in the functional assays.

MATERIALS AND METHODS

Chemicals—1,11-bis-maleimidotetraethyleneglycol (BM(PEO)₄) and dithiobis(succinimidylpropionate) (DSP) were purchased from Pierce, and CHAPS and DM were obtained from Anatrace Inc. (Maumee, OH). GTP·S was purchased from Roche Applied Science.

Antibodies and Resins—The anti-Rho (C-terminal) monoclonal antibody 1D4 was a generous gift from Dr. P. Hargrave (University of Florida). The anti-Rho (N-terminal) monoclonal antibody B6-30N was a generous gift from Dr. Heidi E. Hamm (Vanderbilt University Medical Center). The anti-Rho (C-terminal) monoclonal antibody recognized a C-terminal epitope of peripherin that was generated by a conventional hybridoma technique. The anti-Gt α subunit (Gtα) monoclonal antibody was a generous gift from Dr. Heidi E. Hamm (Vanderbilt University Medical Center). The anti-Gt β subunit (Gtβ) polyclonal antibody was a generous gift from Dr. S. L. McIntosh (University of Minnesota, Minneapolis, MN). The anti-Rho (N-terminal) monoclonal antibody B6-30N was a generous gift from Dr. P. Hargrave (University of Florida). The C6 monoclonal antibody that recognized a C-terminal epitope of peripherin was generated by a conventional hybridoma technique. The anti-Gt α subunit (Gtα) monoclonal antibody was a generous gift from Dr. Heidi E. Hamm (Vanderbilt University Medical Center). The anti-Gt β subunit (Gtβ) polyclonal antibody was a generous gift from Dr. S. L. McIntosh (University of Minnesota, Minneapolis, MN).

Chemical Cross-linking of Expressed Rho with DSP—Bovine ROS membranes were prepared from fresh retinas under dim red light according to the procedure of Papermaster (34). Cross-linking reactions of ROS membranes with BM(PEO)₄ were performed in 100 mM Na₂HPO₄, pH 7.0, containing 150 mM NaCl. BM(PEO)₄ was solubilized in H₂O to a final concentration of 10 mg/ml (stock solution). Washed ROS membranes at Rho concentrations of 0.4 mg/ml were treated with 125-fold molar excess of DSP on ice for 30 min. Control experiments included ROS membranes and MeSO₄ only. The reaction was stopped by adding from a stock solution of 1 M Tris-HCl, pH 7.5, to a final concentration of 100 mM. To remove unreacted cross-linker, membranes were washed three times with 100 mM Na₂HPO₄ buffer, pH 8.3, containing 150 mM NaCl. ROS membrane pellets were stored at ~80 °C or used immediately.

Chemical Cross-linking of Expressed Rho with DSP—The HEK293 cell pellet was homogenized with 100 mM sodium phosphate buffer, pH 8.0. A cell suspension containing 350 µg/ml Rho was incubated with a 288-fold molar excess of DSP (50 mM stock solution) on ice for 30 min. After incubation, the reaction was quenched by the addition of 1 mM Tris-HCl buffer, pH 7.5, to a final concentration of 100 mM, and cell membranes were washed with phosphate buffer. The cell membrane pellets were stored at ~80 °C or used immediately for Rho purification by affinity chromatography using the 1D4 antibody, which targets the C-terminal region of Rho.

Rho IDA Affinity Chromatography—The cross-linked or control (non-cross-linked) cell membranes from HEK cells were lysed with a buffer consisting of 100 mM BisTris propane (BTP), 20 mM DM, pH 7.5, and protease inhibitors (complete protease inhibitor mixture tablets; Roche Applied Science) and centrifuged at 100,000 × g at 4 °C for 30 min using a Beckman ultracentrifuge. The supernatants were loaded on a 1D4-coupled CNBr-activated Sepharose 4B column (Amersham Biosciences), and the flow-through fraction was removed onto the same column three times. The beads were washed with buffer containing 10 mM BTP, 100 mM NaCl, and 2 mM DM, pH 7.5, until the absorbance at 280 nm was below 0.01. Finally, purified Rho was eluted with 100 µM peptide TETSQVAP (from the Rho C-terminal sequence) in the previous buffer at room temperature. The purified Rho concentration was determined using a Hewlett-Packard 8452A UV-visible spectrophotometer.

ROS Membrane Solubilization—ROS membranes cross-linked or cross-linked with DSP and BM(PEO)₄ were solubilized in 10 mM BTP, 7.5, containing 100 mM NaCl and 10 mM DM or 40 mM CHAPS. The membrane suspensions were incubated on ice for 30 min and then centrifuged to remove insoluble material at 100,000 × g for 15 min using a Beckman ultracentrifuge. The supernatant was used for purification of Rho-detergent complexes and cross-linked Rho-detergent complexes by size exclusion chromatography.

Purification of Gt—Gt was extracted from fresh ROS membranes (from 200 retinas) with 80 ml of ice-cold water. The membrane suspension was centrifuged at 45,000 × g for 30 min, and the supernatant was collected. The extraction was repeated twice. The combined supernatants were centrifuged at 45,000 × g for 30 min to remove ROS membrane contaminants; mixed with buffer to the final concentrations of 1 M NaCl, 2 mM MgCl₂, 1 mM DTT, and 0.5 mM benzamidine, pH 7.5; and loaded on hexyl-agarose resin (MP Biomedicals, Inc., Irvine, CA) equilibrated with the same buffer as described previously (35). Bound proteins were eluted by the stepwise addition of 75 mM NaCl and then 300 mM NaCl in the same buffer. Fractions containing Gt and phosphodiesterase (PDE) eluted with 300 mM NaCl were dialyzed against 10 mM BTP, 100 mM NaCl, 1 mM DTT, and 0.5 mM benzamidine, pH 7.5, and used in the Gt activity assay. Gt was purified from PDE by size exclusion chromatography using a Superdex 200 10/50 GL column (Amersham Biosciences) in the same buffer. The protein concentration was estimated using extinction coefficients determined according to the published method (36) or quantitated with a two-dimensional Quant kit (Amersham Biosciences).

ROS Membrane Fractionation—ROS membranes (prepared as described above) were harvested onto a nitrocellulose membrane, purified Rho monomer, and the BM(PEO)₄-treated Rho dimer and analyzed by size exclusion chromatography. Specifically, ROS membranes containing 100 µg of Rho were mixed with 150 µg of Gt in 10 mM BTP buffer, pH 7.5, containing 100 mM NaCl, 1 mM MgCl₂, and 1 mM DTT; bleached three times by a photographic flashlight from a 5 cm distance; and incubated on ice for 15 min to form the complex. The excess Gt was washed out. ROS membranes with bound Gt were solubilized in 10 mM BTP buffer, pH 7.5, containing 100 mM NaCl, 1 mM MgCl₂, and 1 mM DTT supplemented with 10 mM DM. The proteins were loaded onto a gel filtration column and eluted with 10 mM BTP, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, and 3 mM DM, pH 7.5. Rho BM(PEO)₄ dimers or Rho monomers (50 µg each) were mixed with 75 µg of the GT mixture. The complexed proteins were immediately loaded onto a gel filtration column.

To measure Gt dissociation from the complex with Rho, 100 µM GTP·S (Sigma) was added after complex formation and removal of unbound Gt. After 30 min of incubation on ice, 10 mM DM was added to solubilize ROS membranes. Then the proteins were loaded onto a gel filtration column equilibrated with 10 mM BTP, 100 mM NaCl, 1 mM MgCl₂, 3 mM DTT, 3 mM DM, and 10 mM GTP·S, pH 7.5. The elution profile of GT was monitored by absorbance at 280 nm and the absorbance at 420 nm. The elution profile of GT was measured with the PerkinElmer Life Sciences 50B luminescence spectrophotometer, employing excitation and emis-
sion wavelengths at 300 and 345 nm, respectively (37–39). No signals from Rho without transducin were detected in the control experiment.

Size Exclusion Chromatography—Gel filtration chromatography was performed using a Superdex 200 10/300 GL column (Amersham Biosciences), equilibrated with 10 mM BTP, 100 mM NaCl, and either 12 mM CHAPS detergent or DM at concentrations of 0.1, 1, or 3 mM. The column was run at room temperature with a flow rate of 0.4 ml/min. Size exclusion chromatography was used to separate the Rho dimers obtained in cross-linking experiments from the monomers and for the Rho-Gt binding experiments. The absorbance at 280 and 500 nm of the eluate was monitored by a Cary 50 Bio UV-visible spectrophotometer (Variant Inc., Walnut Creek, CA). Fractions containing proteins were analyzed by SDS-PAGE and immunoblotting with monoclonal anti-Gt/H9251 antibody (gift from Dr. H. E. Hamm, Vanderbilt University Medical Center) and polyclonal anti-Gt/H9252 antibody (gift from Dr. O. Kisselev, St. Louis University School of Medicine). The void volume of the column (blue dextran 2000; Amersham Biosciences) was in fraction 23, and the column volume (NaN₃) was eluted in fraction 62. The column was calibrated using ferritin (450 kDa), IgG (158 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and cytochrome c (12.5 kDa). The standard proteins were run through the column with or without 3 mM DM. The detergent did not change the migration of the standard soluble proteins. The linear calibration curve representing the logarithm of molecular mass as a function of the fraction number was used to calculate the molecular mass of the Rho monomer-detergent complexes and Rho dimer-detergent complexes.

SDS-PAGE, BN-PAGE, and Immunoblotting—Protein separation was performed on 10% SDS-PAGE. Coomassie Blue R250, silver staining, and immunoblotting (Immobilon-P polyvinylide difluoride; Millipore Corp.) were carried out according to standard protocols. Alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Promega) was used as secondary antibody. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color development substrate (Promega). Linear gradients of acrylamide (5–13%) BN-PAGE were prepared as described by Schagger and von Jagow (40) but with the addition of 0.1% DM or 0.1% CHAPS. Gels were run in the dark.

Protein Concentration Measurements and Rho Stability—Rho concentration was determined using a PerkinElmer Life Sciences Lambda 800 UV-visible spectrophotometer in the range of 250–650 nm. Freshly purified Rho samples were incubated at 50 °C while monitored by UV-visible absorption spectra, with or without 20 mM neutral NH₂OH. The recorded absorbance was read at 504 nm for 2 h. An S.D. value was calculated from three sets of data from parallel experiments.

Meta II Decay Rate—All measurements were performed in 0.1 mM Rho dissolved in buffer consisting of 10 mM BTP, 100 mM NaCl, and 2 mM DM, pH 6.0, which favors the formation of Meta II, the activated, signaling form of Rho. A PerkinElmer Life Sciences 50B luminescence spectrophotometer was used to measure the intrinsic fluorescence increase due to Trp residues, which is correlated with the decrease in the protonated Schiff base concentration (data not shown, consistent with Refs. 41–44). Rho monomers or dimers purified by size exclusion chromatography were bleached by a Fiber-Lite illuminator for 15 s immediately before the fluorescence measurements. Bleaching was carried out from a distance of 15 cm to prevent heat accumulation, and a thermostat was applied to stabilize the temperature of the cuvette at
Fluorometer slit settings were 2.5 nm at 295 nm for excitation, and 8.0 nm at 330 nm for emission.

Phosphorylation of Rho Dimer and Monomer—Freshly separated Rho monomers or dimers (2 μg each) were mixed in 20 mM BTP, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, 0.1 mM [γ-32P]ATP (100 to ~150 cpm/pmol) and purified Rho kinase (0.1 μg of protein), and the assay was carried out as described previously (45). The reacted mixture was analyzed by SDS-PAGE using 12.5% gel. The gels were stained with Coomassie Blue and dried, followed by performance of an autoradiogram.

RESULTS
Two cross-linking reagents were used to explore Rho oligomerization in native membranes and in mild detergents. DSP is a selective cross-linker for primary amines, such as Lys residues (Fig. 1A). There are many more exposed Lys residues than exposed Cys (Fig. 1C) in the model. Any link between Lys₁₄₁, Lys₂₃₁, Lys₂₄₅, and Lys₂₄₈ from monomer a and Lys⁶₆, Lys⁶₇, and Lys₃₃₉ from monomer c is possible (Fig. 1C, yellow...
Lys<sup>325</sup> and Lys<sup>311</sup> are too far from the other monomers to allow cross-linking. The second cross-linker is BM(PEO)<sub>4</sub>, which is a cross-linking reagent for cysteinyl sulfhydryl groups (Fig. 1B) used to specifically link Cys<sup>140</sup> and/or Cys<sup>316</sup>, two reactive Cys residues. The model of the arrangement of Rho dimers in the native membranes (17) suggests that only one possibility for bridging Cys<sup>140</sup>–Cys<sup>140</sup> from Rho monomers exists (Fig. 1C, green circle).

Cross-linking of ROS Membranes—ROS membranes isolated from bovine retinas were cross-linked with DSP. As shown in Fig. 2A, lane 1, Rho dimers and higher oligomers were formed concurrently with a decrease in the amount of Rho monomers compared with the untreated control (Fig. 2A, lane 5). To discriminate between cross-link products and nonspecific oligomerization, we took advantage of the fact that DSP is a thiol-cleavable cross-linker. Rho dimers obtained with DSP were reduced by DTT, and the majority of them returned to the monomeric form (Fig. 2A, lanes 3 and 4), showing that the cross-linking was a result of chemical reaction and not nonspecific aggregation. In control experiments, we tested whether Rho cross-linked to peripherin, another protein of disc membranes. Although segregation of both proteins in disc membranes cannot be excluded, highly mobile monomeric Rho would collide with peripherin as frequently as with itself. Peripherin was cross-linked to form higher order structures in conditions where Rho was mostly dimeric; however, no heterodimer was observed (Supplemental Fig. 1).

Bleaching of ROS membranes has a slight influence on the DSP cross-linking reaction (Fig. 2A, lane 2, lane 6). When the sample of Rho was exposed to light in the membranes (Supplemental Fig. 2A) or when it was purified in a monomeric form (Supplemental Fig. 2B) and applied on the gel filtration column, low levels of the oligomeric forms were observed only when samples were exposed to light without the reducing agent. Thus, bleaching of Rho preparations without the reducing agent leads to light-induced oxidation and formation of the oligomer, whereas in the presence of the reducing agent, bleaching has no effect on oligomerization (Supplemental Fig. 2). These data are consistent with Medina et al. (7) for the nonreducing conditions but differ from their interpretation that light causes oligomerization of Rho.

Despite the fact that the modification conditions are difficult to optimize, since modification of two potential cross-linking side chains individually would prevent formation of the cross-
link product between them, remarkable stoichiometry (>80%) was observed with the fresh preparations of ROS. The effect of DM on Rho cross-linking showed that at low detergent concentration (<1 mM), Rho could be cross-linked, whereas at higher DM (≥3 mM), no Rho dimer was formed. The cross-linking reaction in DM concentrations close to its critical micelle concentration proceeded as in the control (Fig. 2B, lane 2; without DM, lane 1). In 1 mM DM, the Rho dimer could still be formed, but higher oligomers were weakly visible (lane 3), whereas in the higher DM concentrations, the dimer was not formed (lanes 4 and 5). The corresponding non-cross-linked controls are shown in lanes 6–10 (Fig. 2B). Size exclusion chromatography was used to separate DSP dimers and monomers because solubilized Rho exists in solution as so-called “mixed micelles” consisting of detergent micelles, lipids, and Rho (47). Rho DSP dimers and Rho monomers were separated according to their molecular mass (Fig. 2C). For noncross-linked ROS membranes, almost all of the Rho migrated through the column as monomers (Fig. 2C). The dimer-DM micelle complexes were eluted at a molecular mass of ~140–160 kDa, and the monomer-DM micelles were eluted at ~90–100 kDa, as determined by calibration of the column with proteins of known molecular mass (Fig. 2E). The molecular mass of the Rho monomer and dimer estimated by SDS-PAGE is ~36 and 72 kDa, respectively.³ The aggregation number of DM is 132 (48), and the molecular mass of DM micelles is 67,399 Da (48). The sizes of the Rho monomer and Rho dimer complexes with DM seen in the size exclusion chromatography experiments (~90–100 and ~140–160 kDa, respectively) agree with the sums of the protein and micellar molecular masses (103,500 Da (monomer) and 149,500 Da (dimer)). It should be noted that mixed lipid-detergent-protein micelles may have aggregation numbers for the detergent in the range of n = 111–215 (49–51).

Thus, at high concentrations of DM (≥3 mM), Rho probably exists as monomers present in different vesicles that are unable to cross-link because of the distance between the micelles.

³ Note that bovine opsin is composed of 348 amino acids (39,007 Da) with a calculated molecular mass of 42,002 Da, including double palmitoylation, acetylation, and glycosylation. Increased binding of SDS to opsin increases protein mobility during SDS-PAGE.
To explore this possibility, DSP Rho dimers (Fig. 2F, lane 1) and monomers (Fig. 2F, lane 2) purified by gel filtration chromatography in 3 mM DM were subjected to a cross-linking reaction with DSP. No dimers or higher oligomers were formed.

The Effect of DM Concentration on the Separation of Rho Monomers and Dimers—ROS membranes cross-linked with DSP were solubilized, and the proteins were fractionated using gel filtration chromatography equilibrated with different concentrations of DM. In low DM concentration (0.1 mM), below its critical micelle concentration, Rho appeared in earlier fractions, almost in front of the column (Fig. 3A, left panel), suggesting the presence of higher oligomers. The concentration of 1 mM DM was intermediate (Fig. 3B, left panel), but at ≥3 mM DM, Rho was present in the individual detergent micelles, thus appearing as DSP-cross-linked dimers and monomers (Fig. 3C, left panel). The best separation was obtained for a micelle/Rho ratio of 1:1 in the presence of 3 mM DM (Fig. 3C, left panel). In control experiments, the DSP Rho dimers collapsed into monomers when reduced with DTT (Fig. 3, right panel).

In addition, BN-PAGE was used for analysis of the distribution between monomers and dimers after gel filtration chromatography in 3 mM DM (Fig. 3D). BN-PAGE reliably represents the oligomeric states of the membrane proteins (52). Dimers and monomers were observed that corresponded to the gel filtration separation. Upon the addition of DTT, the majority of DSP-cross-linked dimers dropped back to the monomer form (Fig. 3D, right panel).

Finally, ROS membranes cross-linked with noncleavable BM(PEO)₄ were solubilized and purified by gel filtration chromatography in 3 mM DM (Fig. 3E, left panel). The protein distribution was almost the same as in the case of DSP-cross-linked Rho. DTT did not reduce the BM(PEO)₄ Rho dimer (Fig. 3E, right panel).

Oligomeric Structure of Rho in CHAPS—We also investigated the effect of n-octyl-β-D-glucoside, n-dodecyl-N,N-dimethylamine-N-oxide, and tetraethylene glycol monooyctyl ether on size exclusion separation of cross-linked Rho (data not shown). Rho in most detergents exhibited similar properties to Rho in DM. However, in 12 mM CHAPS, Rho was again eluted almost in front of the column (Fig. 4A, left panel). DSP-linked Rho dimers were reduced to the monomer after DTT addition as monitored by SDS-PAGE (Fig. 4A, right panel). Even when noncross-linked ROS membranes were solubilized in CHAPS, Rho did not dissociate into monomers (Fig. 4B), in contrast to similar experiments in the presence of DM (Fig. 2D).

Two mechanisms could account for this observation; either CHAPS preserves the oligomeric structure, or this detergent extracts lipids and induces Rho to form Rho aggregates. To address this issue, we performed the gel filtration experiments using increasing concentrations of CHAPS (Supplemental Fig. 4). The change from 12 to 40 mM CHAPS led to separation of trimers and higher order complexes from the monomer and dimer, but these last two were not separated. Only the addition of DM allowed the differences to be readily observed. Taking into account that CHAPS micelles are smaller than those formed by DM (53), the results suggest that this detergent does not dissociate Rho into monomeric units. It was expected that if nonspecific aggregates were formed as those induced by SDS, Rho would not dissociate into monomeric units. This conclusion is further supported by findings that CHAPS and DM do not induce time-dependent aggregation, as would be expected if nonspecific aggregates were formed (Supplemental Fig. 5).

BN-PAGE confirmed that Rho in 12 mM CHAPS exists as higher aggregates, indicated by the arrows (Fig. 4C). There are no visible Rho monomers on the gel expected at ~50–60 kDa. The same effect of CHAPS on membrane protein solubilization was observed for bovine cytochrome c oxidase (54). To confirm that Rho did not dissociate to the monomer in 12 mM CHAPS, proteins in fraction 32 (Fig. 4C) were subjected to BN-PAGE (Fig. 5, lane 4) together with control Rho DSP dimer (lane 2) and monomer (lane 3) purified by gel filtration chromatography in 3 mM DM. The smallest unit of Rho solubilized in CHAPS appeared to be the oligomer (the aggregation number for a CHAPS micelle is 10; thus, molecular mass is 6.15 kDa) (53), and there may have been an exchange of detergent during electrophoresis. The gel calibration demonstrated that Rho DSP dimer (lane 2) and monomer (lane 3) purified by gel filtration in 3 mM DM were consistent with the micelles containing Rho monomers having approximate molecular masses of about 80 kDa, whereas micelles containing Rho dimers have molecular masses of about 140 kDa.

Cross-linking of Rho Expressed in HEK293—Opsin expressed in HEK293 cells was regenerated with 9-cis-retinal and then cross-linked with DSP in the cell membranes. Next, Rho was purified by 1D4 affinity chromatography and subjected to gel filtration chromatography as shown in Fig. 6A. The dimer formed after DSP cross-linking migrated faster (fractions 30–33) than the monomer (fractions 34–37). The DSP Rho dimer was reduced by DTT (Fig. 6A, right panel). Non-cross-linked Rho was eluted from the column as a monomer (Fig. 6B). The level of Rho was changed as a function of time after induction of Rho expression. Between 1–48 h of induction, significant cross-linked protein was observed in all conditions.
These results demonstrate that Rho expressed in HEK cells forms dimers as it does in ROS membranes.

**Meta II Decay**—Trp residues exhibit only weak fluorescence in dark state Rho, with the maximum fluorescence at 330 nm. Following bleaching and formation of Meta II, Trp fluorescence increases (41–43). The decay rates of Meta II Rho monomers and BM(PEO)₄, cross-linked Meta II Rho or DSP-cross-linked Meta II Rho dimers (Fig. 7A) purified by gel filtration chromatography were examined by measuring changes in Trp emission at 330 nm. When the data were fitted to a first-order reaction, relaxation times of \( \tau \) were calculated from three independent experiments.

(data not shown). These results demonstrate that Rho expressed in HEK cells forms dimers as it does in ROS membranes.

**Fig. 6.** SDS-PAGE analysis of fractions from gel filtration of Rho expressed in HEK293 cells after cross-linking with DSP. Fractions containing proteins (30 \( \mu \)l) were analyzed by SDS-PAGE under nonreducing or reducing conditions (left and right panels, respectively). Fractions containing the highest concentration of dimer and monomer are indicated with asterisks. A, immunoblotting with 1D4 anti-Rho antibodies of proteins obtained in fractions from gel filtration chromatography of noncross-linked Rho. B, immunoblotting with 1D4 anti-Rho antibodies of proteins obtained in fractions from gel filtration chromatography of noncross-linked Rho (control). Similar results were obtained in two independent experiments.

**Fig. 7.** Meta II decay of purified Rho monomers, DSP cross-linked Rho dimers, and BM(PEO)₄-cross-linked Rho dimers. A, Meta II monomers decay and B and C, Meta II dimers decay without NH₂OH (upper panels) or with NH₂OH (lower panels). The fluorescence excitation was at 295 nm, and emission was measured at 330 nm. Insets, SDS-PAGE analysis of Rho used in the experiments. The relaxation times (\( \tau \)) were calculated from three independent experiments.
τ = 22.0 min for the BM(PEO)_4 Rho dimer, and τ = 24.8 min for the DSP Rho dimer were measured. Therefore, Meta II decayed slightly faster for the Rho monomer than for both cross-linked dimers.

Because NH₂OH promotes removal of the chromophore from the binding site(s) following illumination, a rapid increase in fluorescence was seen for the Rho monomer and both cross-linked dimers in the presence of NH₂OH. No significant differences between the dimers were observed (Fig. 7B), suggesting that although cross-linking may affect normal channeling (42), the difference in Rho decay is alleviated in the presence of the strong nucleophile.

Rho Stability—Time-dependent denaturation of purified Rho monomer, DSP dimer, BM(PEO)_4 dimer, and Rho oligomers was measured. Rho dimers (Fig. 8, B and C, circles) were more stable than the monomer (Fig. 8A, circles). After a 2-h incubation at 50 °C, 60% of the Rho monomers lost their chromophores, whereas only 20% of both dimers lost theirs. The highest stability was observed for Rho oligomers (Fig. 8D, circles), indicating that the tightly packed structure of the pigment protects the protein from denaturation. This difference in stability between the monomer and synthetic dimers was diminished when a strong nucleophile, NH₂OH, was added (Fig. 8, A–C, triangles). But Rho oligomers, even in the presence of NH₂OH, were more stable than the Rho monomer and Rho dimers (Fig. 8D, triangles).

Rho*-Gt Binding—The binding of Gt to ROS membranes (Fig. 9A), purified BM(PEO)_4 Rho* dimer (Fig. 9B), and purified Rho* monomer (Fig. 9C) was examined by gel filtration chromatography. Photoactivation of Rho changes its conformation, and Gt then binds to the cytoplasmic surface of Rho* (55). The molecular mass of Gt is 83 kDa (α subunit 39 kDa, β subunit 36 kDa, γ subunit 8 kDa) (55), and it elutes from the gel filtration Superdex 200 column in fractions 37–43 (data not shown). The Rho monomer is present mainly in fractions 34–36, and the Rho dimer is in fractions 31 and 32 (Fig. 2C). Gt bound to ROS membranes appears maximally in fraction 29–33 after gel filtration chromatography (Fig. 9A, left panel), in earlier fractions than the majority of Rho monomer. The addition of GTPγS (a nonhydrolyzable analog of GTP) leads to the separate release of Gtα and Gtβγ subunits from Rho. The dissociation of Gt is visible as a shift to the later 39–41 fractions. One logical explanation of these gel filtration experiments would be that the complex, having higher molecular mass, migrates as a Rho monomer with Gt. However, another explanation supported by experiments is also possible. At least in water, DM micelles are highly elliptical, with a shorter radius, 0.59 of the length of the longer radius, and contain n = 132 detergent molecules (48). Rho probably localizes to portions of the ellipsoid with less curvature (i.e., the longer sides of the micelle). The Rho-detergent micelle mobility would be only minimally affected when a soluble protein became bound to the surface. This prediction was observed experimentally when Fab and IgG of the B6–30 anti-Rho antibody were tested in the
gel filtration condition (Supplemental Fig. 6). It is clear from these experiments that the mobility of Fab is affected when in complex with Rho compared with the free antibody fragment. The mobility of the larger IgG fragment is only minimally affected by the presence or absence of Rho. Consistently, the mobility of Rho remains minimally affected by Fab or IgG. Thus, by analogy, the elution of the Gt-Rho* complex corresponds most likely to Gt-(Rho*)₂. In other words, the shift in the migration could be due to Gt binding and stabilizing the formation of Rho* dimers, forming a complex with a higher molecular mass. To test whether Gt is in complex with the monomeric Rho* and dimeric Rho* and whether the monomer or dimer is favored, the binding of Gt to purified BM(PEO)₄ Rho dimer and analyzed by gel filtration chromatography in 3 mM DM. C, Gt was bound to the purified Rho* monomer and analyzed by gel filtration chromatography in 3 mM DM. The upper panels show SDS-PAGE of fractions obtained from gel filtration, and lower panels show immunoblotting with anti-Gtα and anti-Gtβ antibodies. The internal standard PDE is marked with an asterisk. Similar results were obtained in three independent experiments for the same conditions.

Gt Activation by Rho*—When GTP or GTPγS is added to the complex of Rho* and Gt, the intrinsic Trp fluorescence emission of the Gt α-subunit increases. Conditions for the assay were chosen so that the transducin activation rate was the same as that determined by GTPγS-induced complex dissociation (37).
It has been shown that the dissociation of Gtβγ from Gα happens on a time scale comparable with G protein activation within a lipid milieu (56). Since this process involves not only subunit-subunit interactions but also receptor-subunit interactions, it cannot be explained adequately by a simple model where either of the interactions is rate-limiting for the activation of Gα. Rho monomer purified by gel filtration in 3 mM DM (A), ROS membranes (B), and Rho oligomer purified by gel filtration in 0.2 mM DM (C). The reaction was carried out at 20 °C in a continuously stirred cuvette with 30 nM Rho and 250 nM Gt in 10 mM BTP, 100 mM NaCl, 6 mM MgCl₂, and 3 mM DM (pH 7.5) in A or 0.2 mM DM in C. After 600 s of recordings, 5 μM GTPγS was added. The initial activation rates (kₐ) were calculated from three independent experiments.

DISCUSSION

The elementary unit of GPCRs in the membranes is assumed to be a homo- and/or heterodimeric complex (16). Further, these dimers may be organized into higher order structures (e.g. see Refs. 8, 17, 60, and 61). The oligomeric structures appear to be preserved among other GPCRs, because they are essential for biosynthesis, cellular transport, diversification, and degradation (reviewed in Ref. 16). Detergent extraction of the receptor may lead to disruption of these oligomeric and dimeric structures and could be affected by many experimental conditions, including salt, pH, or temperature. These studies are critical for further characterization of G protein receptor complexes for biochemical and structural studies. In this work, we have explored cross-linking of Rho in native membranes and shown how detergent influences the oligomer-dimer-monomer equilibrium of Rho. In addition, we describe the effect of detergents on the properties of Rho, employing functional assays. This aspect of the work is particularly interesting, since the vast majority of mutagenesis studies on Rho are performed employing detergents. Because the cross-linking is performed in the native membranes, there is no possibility of misorientation of protein as observed in the Rho crystals (6).

Rho Dimer and Monomers in Detergents—Mild detergents like DM cause the oligomeric structure of Rho to be disrupted. At sufficient concentrations of DM, Rho occurs mostly as a monomer (Fig. 2). However, further exploration is needed to resolve whether detergents at low concentration might cause aggregation during the solubilization process.

Based on neutron diffraction data, pure DM micelles in water (molecular mass of a DM monomer is 510.6 Da) are elliptical, with a shorter radius 59% of the longer radius. They also have an aggregation number n = 132 (48). Thus, the molecular mass of these asymmetric DM micelles would be 510.6 × 132 = 67,399 Da. The elliptical nature of the micelles will affect the migration of the complex less if Rho is predominantly located on the longer surface of the micelles rather than bound to the edges, which would extend the micelle axial ratio further. From our gel filtration chromatography results, we found that the dimer-MC micelle complexes were eluted at a molecular mass of ~140–160 kDa and that the monomer-MC micelle complexes were eluted at ~90–100 kDa. The BN-PAGE supports our interpretation. As noted by Heuberger et al. (52), the Coomassie Brilliant Blue dye replaces DM molecules. Using the correction factor of Heuberger et al. of 1.8, our calculated molecular mass was 77 kDa for the Rho dimer and 44 kDa for the monomer, values close to those expected. Moreover, specific cross-linking of Rho into dimers very reliably confirmed where the dimers and monomers migrated in gel filtration experiments, since the dimers could be reduced to monomeric units upon reduction as shown by SDS-PAGE. Our results are consistent with the observations of Suda et al. However, molecular weight calculation could be complicated by the fact that mixed micelles of protein, lipids, and detergent and different experimental conditions yield n values for DM ranging from...
111 to 140 (see citation in Moller and le Maire (50)). Butler et al. (51) studied the *Escherichia coli* multidrug transporter ERrR in detergent and reported that the protein dimer was associated with 2–3 lipids and 206–210 DM molecules. Taking into account hydration, these numbers could be somewhat lower but higher than \( n/H_11005 \) for pure DM micelles. For cytochrome oxidase, the number of DM in the micelle was 149–215 (49, 50). For bacteriorhodopsin, \( n/H_11005 \) 207. Values of 152 and 148 DM per molecule have been reported for Ca\(^{2+}\)/ATP and reaction center, respectively (50). It should be noted that, using a different method of Rho purification, Medina et al. (7) reported that Rho is mostly a dimer in the same detergent as reported here.

In CHAPS, either the structure of the oligomer is preserved more effectively or the detergent is better able to extract phospholipids. As reported earlier, a higher detergent critical micelle concentration corresponds with a larger amount of lipid associated with the solubilized Rho, and a larger amount of lipid is reassociated with Rho upon surfactant dilution (62). The ability of CHAPS to extract higher amounts of phospholipids may better preserve the higher organization of Rho observed in the native membranes. Whether caused by extraction of lipids or some other effect, the stabilized native structure of Rho observed in CHAPS could be very useful in structural studies of the Gt complex with the dimer of Rho.

**Gt and Rho Kinase Interact with Rho Dimers and Monomers in Detergent**—It is clear from our experiments that monomeric Rho can activate Gt (Fig. 10A). However, in conditions where Rho is mostly in dimeric and oligomeric states (Fig. 10C), it appears that the oligomorized Rho is more active. This is also supported by gel filtration experiments that show a migration of Gt with the dimer of Rho rather than with the monomeric form (Fig. 9). Rho kinase efficiently utilizes both monomeric and dimeric Rho (Fig. 11). The biggest difference observed between Rho monomers and dimers is their stability (Fig. 8). This is not surprising, considering that Rho could be stabilized in the dimer because of additional interactions between molecules. As expected, chromophore release was slightly affected by whether Rho was cross-linked, because this modification could make the helices more rigid and slow the removal of the chromophore (Fig. 7).

**Dimerization of Rho in HEK 293 Cells**—The oligomerization of GPCRs is probably a biosynthetic process (reviewed in Ref. 16). Interestingly, Bouvier and co-workers (63) found that \( \beta_2 \)-adrenergic receptor homodimerization is an event occurring in the ER during biosynthesis, revealing that \( \beta_2 \)-adrenergic receptor homodimerization plays an important role in ER export and cell surface targeting. We believe that a similar mechanism could play a similar role in Rho expressed in the heterologous system and, by conjecture, in vivo. If Rho undergoes dimerization during biosynthesis, the dimer should be observed in HEK293 cells expressing Rho, where the density of Rho is low compared with the total amounts of other membrane proteins. Indeed, we observed efficient cross-linking of Rho in the heterologous expression system as compared with Rho in ROS membranes (Fig. 6). This suggests, although it does not prove, that dimerization could be a biosynthetic process and indicates a relatively high affinity of individual Rho for forming a dimer.

**Support for the Dimeric or Oligomeric Forms of Rho in Native Membranes**—Our current results further support earlier studies using atomic force microscopy, transmission electron microscopy, BN-PAGE, modeling, and cross-linking experiments that suggest that Rho forms dimeric and higher order structures in native membranes (8, 9, 17, 46, 64) (see also Ref. 7 and earlier work cited there).

In the fluid mosaic model, collisions between Rho molecules...
have been estimated to occur at a rate of up to 10^6 collisions/s (12). We do not favor this explanation for our cross-linking experiments for several reasons, although collision interaction could not be totally ruled out. First, in a control experiment, we tested whether peripherin, a 36-kDa protein, is cross-linked to Rho. Peripherin is a membrane protein that shares the same disc membranes of ROS with Rho (65, 66), but no heterodimer peripherin-Rho could be detected. Second, the cross-linking reagents ensure that two molecules of Rho are in intimate contact (Fig. 1), suggesting that at some point in time, Rho molecules form a tight complex. Because Rho is present at a 3 mM concentration in the ROS, the K_D for dimer formation must be >~10 mM to ensure that Rho molecules are virtually free and mobile. If the collision rate is as high as mentioned above with such a low K_D, the dissociation rate would appear equal enough rapidly; thus, there would be insufficient time for the relatively slow reaction of Lys and Cys at neutral pH to occur. EPR data with Rhodopsin in Mild Detergents

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REFERENCES