# Assembly of an Activated Rhodopsin–Transducin Complex in Nanoscale Lipid Bilayers

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ABSTRACT: The formation and characterization of an activated complex of the visual pigment rhodopsin and its downstream signaling partner transducin have been the subject of intense focus by several research groups. While the subunit composition of the activated complex is still the subject of some controversy, our laboratory [Xie, G., D'Antona, A. M., Edwards, P. C., Fransen, M., Standfuss, J., Schertler, G. F. X., and Oprian, D. D. (2011) *Biochemistry 50*, 10399−10407] and that of Ernst et al. [Ernst, O. P., Gramse, V., Kolbe, M., Hofmann, K. P., and Heck, M. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 10859−10864] find that the two proteins are present in a 1/1 molar ratio. Unfortunately, these data could not distinguish a ratio of 1/1 from ratios of 2/2, 3/3, etc. For this reason, we reinvestigated the issue of stoichiometry of the activated complex, exploiting the ability of Nanodisc lipid bilayers to isolate single molecules of rhodopsin. We show here that the purified complex in Nanodiscs contains an activated rhodopsin with a covalently bound *all-trans*-retinal chromophore, that transducin has an empty nucleotide-binding pocket, that the isolated complex is active and dissociates upon addition of guanine nucleotide, and that the stoichiometry corresponds to exactly one molecule of rhodopsin and one molecule of transducin.
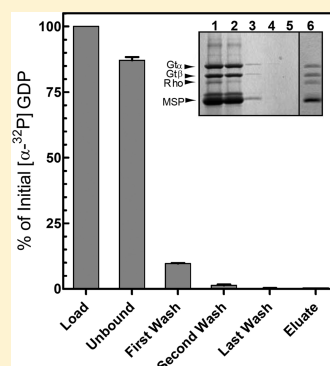
The visual pigment rhodopsin of vertebrate rod photoreceptor cells is a prototypical member of the large class of G protein-coupled receptors.[1] It is composed of an apoprotein, opsin, and an 11-*cis*-retinal chromophore covalently attached to a Lys residue (Lys296) in the seventh transmembrane (TM) segment of the protein by means of a protonated Schiff base linkage.[2] The chromophore undergoes photoisomerization to the *all-trans* form, triggering a conformational change in rhodopsin that leads to activation of the G protein transducin (Gt) and initiation of a signaling cascade that leads ultimately to closure of cGMP-gated channels in the rod outer segment, hyperpolarization of the plasma membrane, and inhibition of the release of glutamate from the synaptic terminus.[3]

The critical interaction of Gt with activated rhodopsin, or metarhodopsin II (R*),[4] has been the subject of numerous studies, the most recent of which have focused on isolation and purification of the activated complex in a detergent solution and determination of its subunit composition. While the stoichiometry of rhodopsin and Gt in the activated complex remains a subject of some controversy,[5−7] our laboratory and others report a molar ratio of 1/1 for rhodopsin and Gt.[8,9] Importantly, however, these data do not unambiguously determine the absolute subunit composition in the complex, as a 1/1 molar ratio cannot be distinguished from ratios of 2/2, 3/3, etc. For this reason, we re-examined the question of subunit stoichiometry, this time exploiting the ability of Nanodiscs to isolate single molecules of rhodopsin.[10−15]

We report here the purification and characterization of an activated complex of R* and Gt in Nanodiscs using native Gt and a constitutively active mutant of rhodopsin[16] containing also an engineered disulfide bond known to confer heightened thermal stability on the protein,[17] as was previously described for purification of the complex in a detergent solution.[9] We show that the Nanodisc complex forms only if rhodopsin is in the activated state and the nucleotide-binding site in Gt is empty. Most importantly, we show unambiguously that the complex is composed of one molecule of rhodopsin and one molecule of Gt.

■ EXPERIMENTAL PROCEDURES

Materials. Anti-rhodopsin monoclonal antibody 1D4[18,19] was from the National Cell Culture Center (Minneapolis, MN). CNBr-activated Sepharose 4B was from GE Healthcare. The 1D4-Sepharose 4B used for purification of rhodopsin and the R*−Gt complex in Nanodiscs was prepared as previously described.[20] 1D4 peptide, a synthetic octapeptide (ETSQVA-PA) corresponding to the 1D4 epitope of the eight C-terminal amino acids of opsin, was used for elution of rhodopsin from the 1D4-Sepharose 4B matrix.[20] Concanavalin A-Sepharose 4B (ConA-Sepharose) was a product of Sigma. 11-*cis*- and *all-trans*-retinal were synthesized and purified according to published procedures, as described by Xie et al.[17] [α-^{32}P]GTP (3000 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Perkin-Elmer. GTP and GTPγS

were from Amersham and Sigma, respectively. The membrane scaffolding protein, MSP1D1, was expressed and purified as described previously.[21] 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Other reagents were as reported previously.[9]

Expression and Purification of Rhodopsin. All experiments in this study were conducted with the N2C/E113Q/D282C triple mutant of rhodopsin. This mutant combines the constitutive activity of the E113Q mutant[16] with the increased thermal stability of the N2C/D282C mutant.[17] The N2C/E113Q/D282C mutant was expressed transiently in HEK293S-GnT1⁻ cells following transfection with calcium phosphate.[22] The pigment was then purified from transfected cells using 1D4 immunoaffinity chromatography essentially as described previously.[9] Assembly and purification of the Rho−Gt complex on ConA-Sepharose began with the N2C/E113Q/D282C mutant that had been previously purified from HEK293S-GnT1⁻ cells using immunoaffinity chromatography on the 1D4 column.

Preparation of Rhodopsin-Embedded Nanodiscs. Rhodopsin-embedded Nanodiscs were prepared as described previously.[11] While the interaction of rhodopsin and arrestin is known to be highly sensitive to lipid composition,[12,13] the interaction of rhodopsin and Gt is not, and for this reason, we did not explore the effect of different lipids on the formation of the R*−Gt complex. We chose POPC for the sake of continuity with previous work.[11] POPC Nanodisc self-assembly was performed at a 70/1/0.07 POPC/MSP1D1/rhodopsin ratio to ensure that only one rhodopsin was present per Nanodisc. Rhodopsin, purified with 1D4-Sepharose, was first applied to a 40 kDa molecular mass cutoff centrifugal device (Amicon Ultra from Millipore) and washed with several volumes of 5 mM Hepes (pH 7.5) and 0.1 mM $MgCl_2$ (buffer A) containing 0.02% (w/v) dodecyl β-D-maltosde (DDM) to remove the 1D4 peptide. The protein was then mixed with MSP1D1 and POPC (Avanti Polar Lipids) and incubated for 30 min on ice. An equal volume of prewashed Biobeads SM-2 (Bio-Rad) was then added and incubated overnight at 4 °C to remove detergent. Biobeads were separated by brief centrifugation, and the supernatant fraction containing rhodopsin Nanodiscs was collected. The final concentration of rhodopsin in the Nanodisc assembly mixture was typically ∼7 μM. Stock solutions of 0.1 M POPC were prepared in 20 mM Hepes (pH 7.5), 0.1 mM $MgCl_2$, and 120 mM NaCl (buffer B) containing 0.2 M CHAPS.

Purification of Native Gt. Gt was purified from frozen bovine retinae (Schenk Packing Co.) according to previously published procedures.[23] Rod photoreceptor cell outer segment (ROS) membranes were exposed to light, washed to remove associated proteins, and then treated with 40 μM GTP in 10 mM Tris (pH 7.4), 0.1 mM EDTA, and 2 mM dithiothreitol (DTT) to elute Gt. ROS were then removed by centrifugation. The supernatant fraction was concentrated with a 50 kDa molecular mass cutoff centrifugal device, filtered through a 0.22 μm membrane filter (Steriflip from Millipore), dialyzed against 5 mM Tris (pH 7.4), 1 mM $MgCl_2$, 0.5 mM DTT, and 50% (v/v) glycerol, and then stored at −20 °C until it was used. The concentration of Gt was determined by absorption spectroscopy (ε_{280} for Gt_{GDP} of 93570 M⁻¹ cm⁻¹), the Lowry assay,[24] and active site titration with radiolabeled [³⁵S]GTPγS with a known specific activity. Concentrations determined by the Lowry assay or absorption spectroscopy were typically within 5−10% of the value determined by active site titration. The

same procedure was followed for the preparation of Gt containing [α-³²P]GDP in the nucleotide binding site except that 40 μM [α-³²P]GTP (0.31 Ci/mmol) was used instead of nonradiolabeled GTP for the release of Gt from ROS membranes.

Absorption Spectroscopy. UV−visible absorption spectra were recorded using a Hitiachi model U-3210 instrument that was specifically modified by the manufacturer for use in a dark room. Data were collected with a microcomputer and evaluated with GraphPad Prism (GraphPad Software, San Deigo, CA). All data were recorded at 25 °C with a path length of 1 cm. The molar absorption coefficient for the rhodopsin triple mutant in Nanodiscs was determined by acid trapping of the chromophore as described previously,[25] except that 50 mM sodium phosphate buffer (pH 3.5) containing 0.5% (w/v) SDS was used. Molar absorption coefficients for MSP1D1 (ε_{280} = 21430 M⁻¹ cm⁻¹) and nucleotide-free Gt (Gt_{empty}; ε_{280} = 87800 M⁻¹ cm⁻¹) were calculated with the ExPASy ProtParam tool,[26] which uses the Edelhoch method[27] with extinction coefficients for Trp and Tyr determined by Pace et al.[28] A GTP ε_{280} of 5770 M⁻¹ cm⁻¹ was measured experimentally assuming an ε_{253} of 13700 M⁻¹ cm⁻¹ and was used to calculate an ε_{280} for Gt_{GDP} of 93570 M⁻¹ cm⁻¹.

Gt Activation Assay. The ability of rhodopsin to catalyze the light-dependent exchange of radiolabeled [³⁵S]GTPγS for bound GDP in Gt was monitored using a filter binding assay as described previously.[29] To assay the R*−Gt complex for activity, a similar procedure was used except that the purified complex was diluted to a final rhodopsin concentration of 30−40 nM in 10 mM Tris (pH 7.5), 40 μM [³⁵S]GTPγS (1.42 Ci/mmol), 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT without additional Gt. Reaction mixtures were incubated for 5 min at 25 °C before aliquots were withdrawn and applied to nitrocellulose filters.

Stoichiometry of the Activated Complex. The relative stoichiometry of rhodopsin, transducin, and MSP1D1 in the activated complex was determined by densitometry of protein bands following electrophoretic separation on SDS−PAGE gels. The purified complex was visualized by staining with Coomasie Blue. Band densities were quantified from background-subtracted digitized gel images using Quantity One (Bio-Rad, Hercules, CA). Quantities of protein from unknown samples were determined using a standard curve with known amounts of protein as determined by UV−visible absorption spectroscopy.

■ RESULTS

Preparation of Nanodiscs Containing the Rhodopsin N2C/E113Q/D282C Triple Mutant. Nanodiscs containing the rhodopsin N2C/E113Q/D282C triple mutant (hereafter termed simply "rhodopsin") were prepared from a 70/1.0/0.07 POPC/MSP1D1/rhodopsin mixture to ensure that each Nanodisc contained only one molecule of rhodopsin, as has been described previously.[11] Empty Nanodiscs were removed by purifying the rhodopsin-embedded Nanodiscs by chromatography on a 1D4-Sepharose column.

As shown in Figure 1, absorption spectra for rhodopsin in Nanodiscs reconstituted with the 11-*cis*-retinal chromophore at pH 7.5 display an absorption maximum (λ_{max}) at 380 nm, characteristic of the unprotonated Schiff base form of the chromophore, as well as a second λ_{max} at 280 nm characteristic of protein. The A_{280}/A_{380} ratio was larger for the protein in Nanodiscs than for a detergent-solubilized preparation in DDM
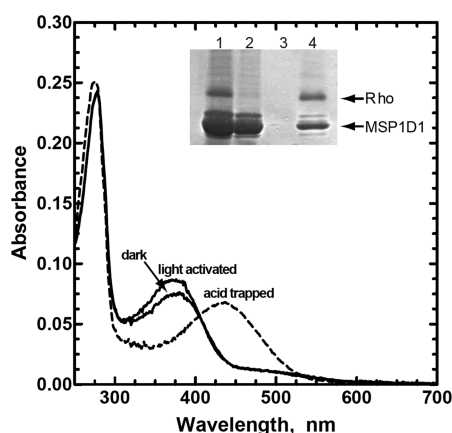
Figure 1. Preparation of rhodopsin-containing Nanodiscs. The N2C/E113Q/D282C rhodopsin triple mutant was reconstituted with 11-*cis*-retinal, inserted into the lipid bilayer of Nanodiscs, and purified by immunoaffinity chromatography on 1D4-Sepharose: (dark) absorption spectrum of the rhodopsin-containing Nanodiscs purified in the dark at pH 7.5, (light activated) spectrum following a 2 min illumination with light from a 300 W tungsten bulb, and (acid trapped) spectrum immediately following the addition of 0.5% SDS and 50 mM sodium phosphate buffer (pH 3.5) to the light-exposed sample. The acid trapped spectrum has been corrected for the effect of dilution. The inset shows the SDS−PAGE analysis of the steps used for the preparation and purification of the rhodopsin-containing Nanodiscs: lane 1, rhodopsin/MSP1D1 mixture following detergent removal before being loaded onto the 1D4-Sepharose column; lane 2, unbound material from the 1D4-Sepharose column; lane 3, last wash before elution; and lane 4, 1D4 peptide eluate containing the purified N2C/E113Q/D282C rhodopsin triple mutant in MSP1D1 Nanodiscs. Protein bands in the gel were visualized with Coomassie Blue.

micelles[9,17] because of the contribution at 280 nm from the copurified MSP1D1 (Figure 1, dark spectrum). Subtracting the contribution of rhodopsin from the absorbance at 280 nm (assuming an A_{280}/A_{380} ratio of 1.7 for purified rhodopsin) provides an initial estimation of 2 mol of MSP1D1/mol of rhodopsin, consistent with the Coomassie Blue-stained SDS−PAGE gel shown in the inset of Figure 1 and with previously published results for Nanodiscs prepared under similar conditions.[11]

Illumination of the sample increased the absorbance at 380 nm (light activated spectrum). Subsequent acid denaturation by addition of 0.5% SDS in 50 mM sodium phosphate buffer (pH 3.5) converted the λ_{max} from 380 nm to a new peak at ∼430 nm (acid trapped spectrum), characteristic of a retinylidene protonated Schiff base attached to the denatured protein.[25] The absorption coefficients at 380 nm for the dark-adapted and light-activated forms of rhodopsin in Nanodiscs were determined to be 35200 and 41500 M^{-1} cm^{-1}, respectively, using an ε_{430} of 32500 M^{-1} cm^{-1} for the chromophore under acidic and denaturing conditions.

Activation of Gt in the Presence of the 1D4 Antibody. Our approach for the isolation of the R*−Gt complex is to make use of the tight binding between R* and Gt in the absence of nucleotide to form the complex and then exploit the specific interaction between rhodopsin and the 1D4 antibody for purification. This strategy rests on the principle that the binding of the 1D4 antibody to the carboxyl terminus of rhodopsin does not interfere with the Gt binding site. As shown in Figure 2, the ability of R* in Nanodiscs to activate Gt is unaffected by antibody concentrations of up to 1 μM, where

>95% of the rhodopsin is expected to be bound to the antibody.[30]
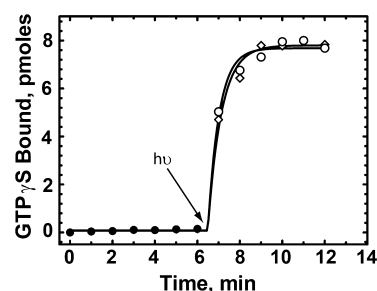
Figure 2. Effect of the 1D4 antibody on rhodopsin-catalyzed activation of Gt. Activation of Gt was monitored by following the binding of [35S]GTPγS over time in the presence (◇ and ◆) or absence (○ and ●) of 1 μM 1D4 antibody using a filter binding assay as described in Experimental Procedures. Each reaction mixture contained 5 nM rhodopsin triple mutant in Nanodiscs reconstituted with 11-*cis*-retinal, 1 μM Gt, and 3 μM GTP: (filled symbols) reaction in the dark and (empty symbols) reaction after the mixture had been exposed to light (300 W tungsten bulb for 30 s for 6.5 min). The residual 1D4 peptide was present at a concentration 20-fold lower than that of the antibody combining sites.
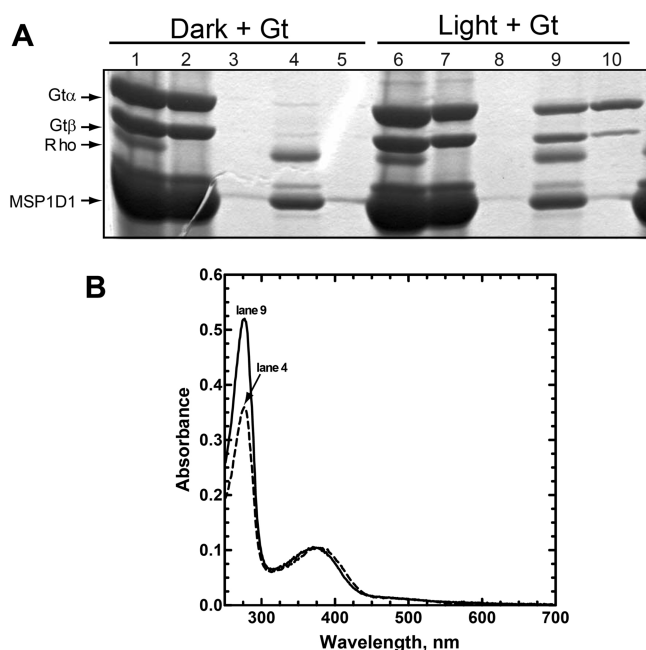
Purification and Characterization of a R*−Gt Complex in Nanodiscs. Initial attempts to prepare the R*−Gt complex in Nanodiscs were performed using mutant rhodopsin that had been reconstituted with 11-*cis*-retinal in the dark. Following the removal of the detergent, the mixture of empty and rhodopsin-containing Nanodiscs was combined with a 2-fold molar excess (relative to rhodopsin) of Gt in buffer B, incubated on ice for 15 min under dim-red light, and then illuminated with a 300 W tungsten lamp for 2 min. Samples were then incubated on ice for an additional 15 min and applied to a 1D4-Sepharose immunoaffinity column for purification (Figure 3A, lane 6). The nonbinding fraction (Figure 3A, lane 7) typically contained an amount of Gt that was visibly smaller than the amount applied to the column, as well as small amounts of rhodopsin. The column was then washed sequentially with buffer B, buffer B containing empty Nanodiscs (50 μM) to remove nonspecifically bound Gt, and then buffer B again (Figure 3, lane 8) before elution of the complex by incubation with the 1D4 peptide (Figure 3A, lane 9). Gt can be eluted alone from the column by incubation with GTPγS (Figure 3A, lane 10). In this case, it is clear that some of the $\beta\gamma$-subunit is retained on the column presumably as a result of nonspecific binding of the isoprenylated γ-subunit to the immobilized Nanodiscs (Figure 3A, lane 9 vs lane 10). Importantly, the ability to purify the complex is completely dependent on light activation of rhodopsin (Figure 3A, lanes 9 and 10 vs lanes 4 and 5).

The UV−visible absorption spectrum of the purified, light-activated R*−Gt complex is characterized by an A_{280}/A_{380} ratio of 5 (Figure 3B, lane 9 spectrum). The increased absorbance at 280 nm, compared to that of the dark sample from lane 4 of Figure 3A (Figure 3B, lane 4 spectrum; A_{280}/A_{380} ratio of 3.4), is attributed to the presence of Gt in the light-activated sample. An A_{280}/A_{380} ratio of 5 is consistent with a rhodopsin/Gt molar ratio of 1/1 [based on absorption coefficients determined for Gt_empty, MSP1D1, and rhodopsin (see Experimental Procedures)].

The R*−Gt complex could also be prepared from Nanodiscs containing rhodopsin that had been reconstituted and activated

Figure 3. Preparation and purification of a R*−Gt complex. Rhodopsin reconstituted with 11-*cis*-retinal in Nanodiscs was mixed with a 2-fold excess of Gt and then either incubated in the dark or illuminated for 2 min with light from a 300 W tungsten bulb, as indicated in the figure. The mixture was then applied to a 1D4-Sepharose immunoaffinity column for purification as described in Experimental Procedures. (A) Coomassie Blue-stained SDS−PAGE gel containing samples from each step in the purification procedure: lanes 1−5, sample in the dark; lanes 6−10, sample exposed to light; lanes 1 and 6, material loaded onto the 1D4-Sepharose column; lanes 2 and 7, unbound material; lanes 3 and 8, last wash; lanes 4 and 9, 1D4 eluate; and lanes 5 and 10, GTPγS eluate. Only the α- and β-subunits of Gt are shown; in this and subsequent figures, the γ-subunit is not shown because it runs off the end of the gel. Molecular masses are as follows: Gtα, 39966 Da; Gtβ, 37377 Da; Rho (N2C/E113Q/D282C mutant), 38984 Da; and MSP1D1, 24662 Da. The band migrating just above MSP1D1 on the gel is an impurity from the MSP1D1 preparation and has not been identified. As noted in the text, the smaller yield of Gtβ from the GTPγS elution, here (lane 10) and in Figure 7, presumably reflects nonspecific binding of the more hydrophobic, isoprenylated γ-subunit to immobilized Nanodiscs on the column. (B) UV−visible absorption spectra for the purified samples from lanes 4 and 9 from panel A, as indicated in the figure.

with *all-trans*-retinal (Figure 4). The subunit composition and spectral properties of the *all-trans*-retinal-activated complex were very similar to those of the light-activated complex (Figure 4, panel A vs panel B), including the ability to acid trap a protonated form of the retinylidene chromophore (Figure 4, acid trapped spectra) demonstrating that the retinal is covalently bound in both complexes.

To eliminate concern that the 1D4 antibody used in purification, which like Gt binds to the cytoplasmic surface of rhodopsin, might influence the subunit stoichiometry of the complex, an alternate purification procedure was employed using ConA-Sepharose. ConA targets carbohydrate moieties on the extracellular surface of rhodopsin, opposite the binding site for Gt. Other than the affinity support and associated eluant, all other procedures for purification of the activated complex were the same as those described above using 1D4-Sepharose. As shown in Figure 5, the subunit composition for the activated complex purified using ConA is very similar to that of the
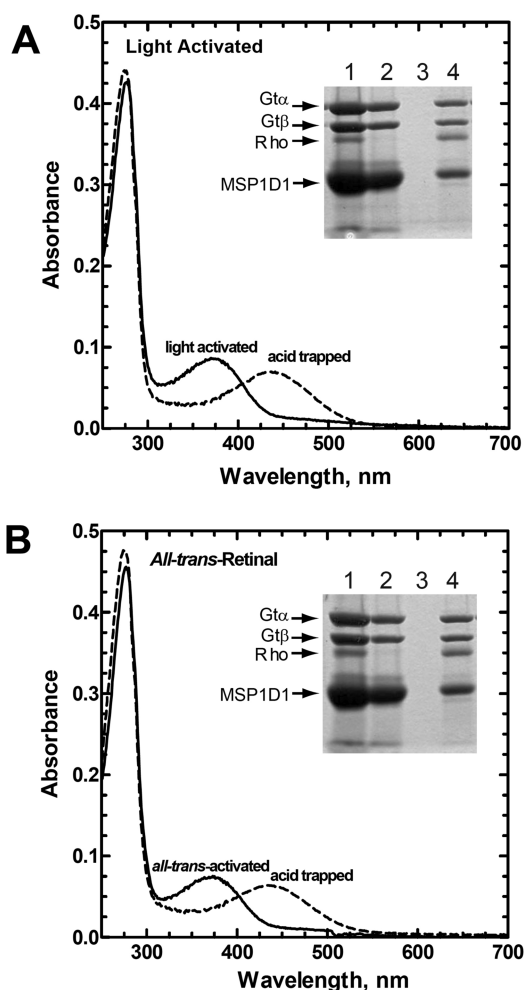

Figure 4. Formation of a R*−Gt complex following activation of rhodopsin with *all-trans*-retinal. The figure shows absorption spectra for the purified complex before and after acidification with 50 mM sodium phosphate buffer (pH 3.5) containing 0.5% (w/v) SDS. Spectra have been corrected for dilution. Insets show SDS−PAGE analysis of the steps used for the preparation and purification of the R*−Gt complexes in Nanodiscs (procedures in the legend of Figure 3 and Experimental Procedures): lane 1, material before being loaded onto the 1D4-Sepharose column; lane 2, unbound material; lane 3, last wash; and lane 4, 1D4 peptide eluate. Protein bands in the gel were visualized with Coomassie Blue. Only the α- and β-subunits of Gt are shown. (A) Formation of the R*−Gt complex with light-activated rhodopsin (rhodopsin sample reconstituted with 11-*cis*-retinal). (B) Formation of the complex using rhodopsin activated with *all-trans*-retinal.

sample purified on the 1D4 column (Figure 4B, inset). Thus, the use of the 1D4 antibody does not appear to significantly influence subunit stoichiometry in the activated complex.

Nucleotide-Binding State of Gt in the Complex. It is well established that rhodopsin catalyzes the release of GDP from Gtα upon formation of the R*−Gt complex. To determine if nucleotide was released in the Nanodisc preparations, Gt loaded with $[\alpha$-^{32}P]GDP with a known specific activity in the nucleotide-binding pocket was used to form the activated complex. As shown in Figure 6, most of the GDP (87 ± 1.2%; n = 3) was found in the unbound fraction from the 1D4-Sepharose column, 9.7 ± 0.3% (n = 3) in the first wash, and none in the eluate, which contained the expected content of Gt in the complex (Figure 6, inset, lane 6).
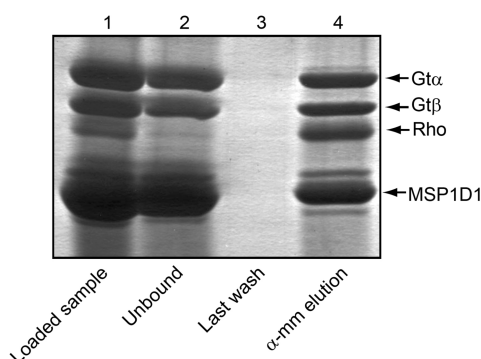
Figure 5. Purification of the R*−Gt complex using ConA-Sepharose. Rhodopsin in Nanodiscs was reconstituted with *all-trans*-retinal in the presence of excess Gt as described in Experimental Procedures and then purified by chromatography on ConA-Sepharose. Steps in the purification procedure were analyzed via Coomassie Blue-stained SDS−PAGE gels: lane 1, material applied to the ConA-Sepharose column; lane 2, unbound material; lane 3, last wash; and lane 4, methyl α-D-mannopyranoside eluate. Only the α- and β-subunits of Gt are shown.
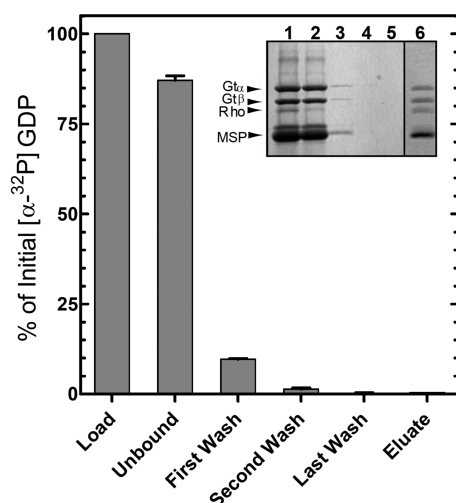
Figure 6. Nucleotide release during formation of the R*−Gt complex. The activated complex was purified on 1D4-Sepharose as described in the legend of Figure 3 and Experimental Procedures using a Gt sample containing $[\alpha\text{-}^{32}\text{P}]$GDP in the nucleotide-binding pocket. Radioactivity in the fractions was monitored by liquid scintillation counting. Values are expressed as a percent of the total $[\alpha\text{-}^{32}\text{P}]$GDP in the loaded sample. Error bars represent the standard deviation ($n = 2$). The inset shows the SDS−PAGE analysis of the steps in the purification of the complex as visualized by Coomassie Blue staining: lane 1, material applied to the 1D4-Sepharose column; lane 2, unbound material; lane 3, first wash; lane 4, second wash; lane 5, last wash; and lane 6, 1D4 peptide eluate.

Therefore, the nucleotide-binding pocket of Gt in the activated complex is empty.

Stoichiometry of Subunits in the R*−Gt Complex. Initial experiments, described above, used the ratio of the absorbance at 280 nm to that at 380 nm in the UV−visible absorption spectrum of the purified preparations to determine the subunit composition from the known absorption coefficients for rhodopsin, transducin, and MSP1D1. The amount of Gtα in the complex was also determined by simply diluting a sample with a known amount of rhodopsin (from the ε_{380}) into a reaction mixture containing 40 μM $[^{35}\text{S}]$GTPγS

with a known specific activity and quantifying the amount of bound GTPγS by separation of the protein onto nitrocellulose filters and scintillation counting. The Gt/rhodopsin molar ratio determined by this method was 1.01 $\pm$ 0.06 [standard error of the mean ($n = 3$)], in good agreement with the spectrophotometric determination described above.
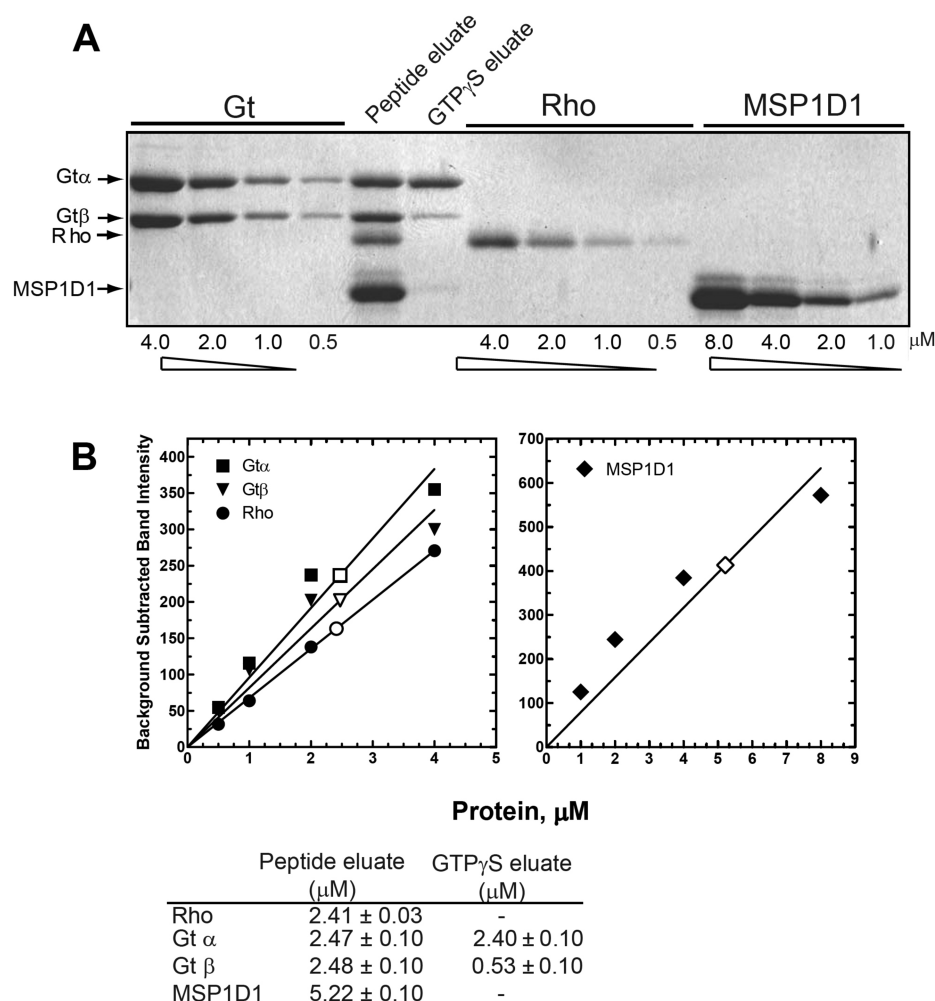
A final determination of subunit stoichiometry in the purified complex was made by densitometry of band intensities following separation by SDS−PAGE (Figure 7). Gels containing samples of the purified complex from the 1D4 support and known standards for Gt, rhodopsin, and MSP1D1 were scanned, and bands in the digitized images were integrated. As shown in Figure 7, the data support a 1/1/2 complex of Gt, R*, and MSP1D1. Analysis of the GTPγS eluate confirmed a 78% loss of Gβ as compared to Gα of the same sample (see above).

■ DISCUSSION

While the biochemical literature on the interaction of rhodopsin and Gt is extensive and dates back to the early 1980s,[4,31−33] there remains some controversy about the stoichiometry of the activated complex of these two proteins. Rhodopsin is known to form higher-order aggregates in rod outer segment disc membranes,[34] and there are reports suggesting that the functional unit is comprised of two rhodopsin molecules and one Gt.[6,7] On the other hand, the fact that rod photoreceptor cells can respond to a single photon[35] suggests that one molecule of rhodopsin is sufficient to activate Gt. In addition, there appears to be general agreement in the field that a single molecule of rhodopsin is sufficient to activate Gt in *in vitro* assays.[8,10,11,15]

We previously reported the purification of a R*−Gt complex in a detergent solution using either ConA- or 1D4-Sepharose as the supporting affinity matrix.[9] The complex, which was formed from the N2C/E113Q/D282C rhodopsin triple mutant and native Gt, contained a covalently bound *all-trans*-retinal chromophore in rhodopsin and an empty nucleotide-binding pocket in Gt. It was active, dissociated upon addition of guanine nucleotide, and was characterized by a 1/1 molar ratio of rhodopsin to Gt. A similar complex, with a 1/1 molar ratio of subunits, was also purified and characterized using native rhodopsin.[9] The 1/1 stoichiometry agreed well with previous studies by Ernst et al.[8] using size-exclusion chromatography to prepare the complex from native rhodopsin and Gt. Importantly, however, none of these studies unequivocally identified the number of protein subunits in the activated complex, as a molar ratio of 1/1 cannot be distinguished from 2/2, 3/3, and higher-order aggregates. For this reason, we used Nanodiscs to isolate single molecules of rhodopsin. We previously reported the preparation of a R*−Gt complex in Nanodiscs using native rhodopsin but did not rigorously determine subunit stoichiometry in that study.[11]

The R*−Gt complex reported here using Nanodiscs had properties that were very similar to those reported for the complex in a detergent solution.[9] The complex contains a covalently bound *all-trans*-retinal chromophore in rhodopsin and an empty nucleotide-binding pocket in Gt. It is active, dissociates upon addition of guanine nucleotide, and, most importantly, is characterized by a 1/1 molar ratio of rhodopsin to Gt. In this case, however, we may conclude that the complex is composed of one molecule of rhodopsin and one molecule of Gt because the Nanodiscs were prepared under conditions in which each contained only a single molecule of rhodopsin. This

Figure 7. Densitometric determination of the subunit stoichiometry in the R*–Gt complex. (A) SDS–PAGE analysis of the R*–Gt complex. Known amounts of Gt, the N2C/E113Q/D282C mutant (Rho), and MSP1D1 were loaded onto the gel to generate standard curves for the determination of the amount of each subunit contained in the 1D4 peptide and GTPγS eluates. (B) Quantification of subunits in the complex as determined by densitometry. Individual bands from the SDS gel in panel A were quantified as described in Experimental Procedures using ImageJ, and standard curves derived from the known amounts of the α- and β-subunits of Gt, rhodopsin, and MSP1D1 were generated (filled symbols) and used to determine concentrations of the subunits in the 1D4 peptide (empty symbols) and GTPγS eluates (not shown), as presented in the table at the bottom of the panel. Errors were determined from a least-squares fit of data to the standard curves.

result agrees well with the subunit composition determined by X-ray crystallography for an activated complex of the β_2-adrenergic receptor and Gs determined by Kobilka and co-workers; the β_2-adrenergic receptor–Gs complex is composed of a single molecule of receptor bound to a single molecule of the G protein.[36] It also agrees well with several studies showing that a single molecule of rhodopsin is sufficient for phosphorylation by rhodopsin kinase[12,14] and that rhodopsin binds arrestin in a complex composed of a single molecule of rhodopsin and a single molecule of arrestin.[12–14]

A comment on the use of a rhodopsin mutant in these studies is warranted. The N2C/E113Q/D282C triple mutant combines the properties of two previously described rhodopsin mutants: a double mutant, N2C/D282C, that contains an engineered disulfide bond between two introduced Cys residues[17] and a single mutant, E113Q, that neutralizes the Schiff base counterion.[37,38] The engineered disulfide confers enhanced thermal stability on the protein but otherwise has no effect on activity.[17] A crystal structure of this mutant in the dark state is identical to that of the native protein, with the exception of a missing carbohydrate chain normally found attached to

Asn2 and the presence a disulfide bond between the two introduced Cys residues at positions 2 and 282.[39] The E113Q mutation leads to a large change in the spectral properties of the dark state protein, shifting the absorption maximum from 500 nm for the retinylidene chromophore in the native protein to 380 nm in the mutant,[37,38] and leads to constitutive activation of the apoprotein opsin.[16] Importantly, for this study, the mutation also prolongs the lifetime of the active metarhodopsin II state.[37] The phenotypes of both mutations, N2C/D282C and E113Q, combine in the triple mutant;[9] otherwise, the protein displays wild-type properties. For example, a crystal structure of the N2C/E113Q/D282C mutant in the active state superimposes with that of the WT protein,[40] as does another mutant that combines a constitutively active mutation (M257Y) with the engineered disulfide bond between residues 2 and 282.[41] We previously used the N2C/E113Q/D282C triple mutant to determine the stoichiometry of the Rho*–Gt complex in a detergent solution to be 1/1.[9] In that study, we also showed that, under identical conditions, native rhodopsin from bovine retina and the N2C/D282C double mutant formed similar complexes with a 1/1 stoichiometry.

Thus, in conclusion, it is unlikely that use of the N2C/E113Q/D282C triple mutant affects the stoichiometry of the activated rhodopsin–Gt complex. As a final note, we had previously prepared an activated complex of native rhodopsin and Gt in Nanodiscs containing only a single rhodopsin.[11] While we did not rigorously determine the stoichiometry of the complex, Coomassie staining of bands from SDS–PAGE gels in that study[11] is consistent with the patterns reported here for the 1/1 complex with the mutant, bolstering the conclusion that use of the triple mutant does not affect the results.

While the impetus for initiating these studies was to determine the subunit stoichiometry for the activated complex, our current focus is on adapting the Nanodisc system for use with total internal reflection fluorescence microscopy to follow the interaction of R* and Gt at the single-molecule level. Nanodiscs would appear to be an ideal system for such studies.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-(γ-thio)triphosphate; ROS, rod outer segment; R*, activated rhodopsin; Gt, transducin; Gtα, α-subunit of transducin; Gtβ, β-subunit of transducin; Gt$_{GDP}$, transducin containing GDP; Gt$_{empty}$, transducin with no nucleotide bound; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; ConA-Sepharose, concanavalin A-Sepharose 4B; DDM, dodecyl β-D-maltoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEK, human embryonic kidney; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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