Transducin Activation by Nanoscale Lipid Bilayers Containing One and Two Rhodopsins

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Nanodiscs are nanometer scale planar membranes of controlled size that are rendered soluble in aqueous solution via an encircling amphipathic membrane scaffold protein “belt” (Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002) Nano. Lett. 2, 853–856). Integral membrane proteins can be self-assembled into the Nanodisc bilayer with defined stoichiometry, which allows an unprecedented opportunity to investigate the nature of the oligomerization state of a G-protein-coupled receptor and its coupling to heterotrimeric G-proteins. We generated Nanodiscs having one and two rhodopsins present in the 10-nm-diameter lipid bilayer domain. Efficient transducin activation and isolation of a high affinity transducin-metarhodopsin II complex was demonstrated for a monodisperse and monomeric receptor. A population of Nanodiscs containing two rhodopsins was generated using an increased ratio of receptor to membrane scaffold protein in the self-assembly mixture. The two-rhodopsin population was isolated and purified by density gradient centrifugation. Interestingly, in this case, only one of the two receptors present in the Nanodisc was able to form a stable metarhodopsin II-G-protein complex. Thus there is clear evidence that a monomeric rhodopsin is capable of full coupling to transducin. Importantly, presumably due to steric interactions, it appears that only a single receptor in the Nanodiscs containing two rhodopsins can interact with G-protein. These results have important implications for the stoichiometry of receptor-G-protein coupling and cross talk in signaling pathways.

A growing pool of evidence suggests the facile homo- and heterodimerization of G-protein-coupled receptors (GPCRs) with potential functional implications for cellular signaling. These conclusions are based largely on coimmunoprecipitation, resonance energy transfer between labeled GPCRs in cells, functional complementation, and in vitro analysis of isolated receptors (1). For example, purified leukotriene receptor BLT1 has been shown to exist as a dimer in detergent, which forms a pentameric complex of dimer with heterotrimeric G-protein and demonstrates cooperative interaction with ligand (2, 3). Oligomeric arrays of rhodopsin, a class A GPCR of the visual transduction system, have been observed in the atomic force microscope using native disk membranes (4–6). Rhodopsin dimers have been observed in uranyl formate-stained preparations by electron microscopy (8) and have also been studied in detergent-solubilized preparations (9, 10). Recent evidence for self-association of rhodopsin in liposomes has been obtained by fluorescence techniques (11) consistent with earlier work (12). Other investigations, using detergents with varying properties, isolated and characterized rhodopsin in different oligomeric states, with the conclusion that oligomers activate transducin more efficiently (13). In contrast to these studies, rhodopsin has long been thought to function as a monomer based on extensive biophysical evidence, as reviewed in Ref. 14.

To resolve the important question of GPCR oligomerization in cellular signaling, we utilized a self-assembled system consisting of a membrane bilayer of 1-palmitoyl-2-oleoyl phosphatidylcholine and an encircling membrane scaffold protein (MSP) (15, 16) to precisely control the stoichiometry of the GPCR bovine rhodopsin. Nanodiscs were developed to solubilize membrane proteins as nanometer-sized membrane particles as an effective alternative to detergent (15). The Nanodisc is composed of phospholipid and two copies of an MSP that stabilize the discoidal bilayer (15, 16). Nanodiscs have been used to control the oligomerization state of bacteriorhodopsin (17) and have been recently shown to be useful in dissecting the exact requirements for the particular oligomerization state of bacterial chemotactic receptors needed for specific signal transduction function (18). Using Nanodisc methodology, we isolated Nanodiscs containing one and two rhodopsin molecules and compared their functional interactions with transducin.

EXPERIMENTAL PROCEDURES

Materials—MSP1E3D1 was expressed and purified as described (16). POPC was obtained from Avanti Polar Lipids (Alabaster, AL), and octyl glucoside and dodecyl maltoside were obtained from Anatrace (Maumee, OH). Rod outer segments were isolated from frozen bovine retina (SPC1 Retina Inc.) and rhodopsin purified on ConA-Sepharose (GE Healthcare) using published procedures (19, 20). Transducin was puri-
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...essentially as described (21). GTPγS was from Sigma. The 1D4 monoclonal antibody was obtained from the University of British Columbia and coupled to cyanogen bromide-activated CL-Sepharose 4B (GE Healthcare) as described (22). Nonapeptide TETSQVAPA was obtained from EZBiolab, Inc. (West...}

Nanodisc Self-assembly and Purification—All rhodopsin manipulations were performed under dim red light. Nanodiscs were self-assembled at ratios of 140:1:0.1 POPC:MSP:rhodopsin for monomeric rhodopsin and 90:1:1 POPC:MSP:rhodopsin to obtain two rhodopsins per Nanodisc. Octyl glucoside (0.5 M stock in water) was added to ConA-purified rhodopsin for a final concentration of 90 mM to ensure monomerization. The rhodopsin stock (typically near 100 µM) was added to a mixture of MSP1E3D1 (typically 200 µM) and POPC/cholate (0.2 M/0.4 M sodium cholate in buffer) at the correct ratio. A protease inhibitor mixture (Roche Applied Science, Complete without EDTA) was added followed by a brief incubation on ice. An equal volume of moist Biobeads SM-2 (Bio-Rad) or Amberlite XAD-2 (Supelco), prepared by washing extensively with methanol followed by water and decantation of fines, was added to the Nanodisc assembly mixture to remove detergent. Detergent removal was allowed to proceed overnight at 4 °C with gentle agitation. The beads were removed by centrifugation. The sample was concentrated with a 10-kDa molecular mass cut-off centrifugal device and injected onto a Superdex 200 prep grade column (1.6 × 30 cm) run at 0.5 ml/min. Absorbance was continuously monitored at 630 nm, allowing determination of chromatographic peaks without bleaching. Monomer was further purified by ConA-Sepharose or 1D4 affinity chromatography essentially as described (20, 22). Nanodiscs containing one and two rhodopsins were separated on linear 10–30% (w/v) sucrose gradients centrifuged at 45,000 rpm in a type 70 Ti rotor (Beckman) for 18 h at 4 °C with slow acceleration and without braking. Gradients were fractionated from the bottom of the tube, and sucrose was removed by size exclusion chromatography.

Measurement of Nanodisc Compositions—MSP1E3D1 was quantitated by 280 nm absorbance (29,910 M⁻¹ cm⁻¹), and rhodopsin was quantitated by absorbance at 500 nm (40,600 M⁻¹ cm⁻¹). The contribution of rhodopsin to 280 nm absorbance was corrected using the ratio of absorbance A280/A500 = 1.7 (20). Lipid phosphate was determined as described (23).

Small Angle X-ray Scattering (SAXS)—ConA-purified Nanodiscs (monomer) were concentrated to 140 µM rhodopsin. SAXS data were measured on the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) beamline at the Advanced Photon Source (Argonne National Laboratory) using a photon wavelength of λ = 0.827 Å and analyzed as described (17). Atomistic models were constructed using a model POPC molecule to form a bilayer, and rhodopsin (Protein Data Bank ID 1F88; Ref. 24) was modeled into the bilayer as described (17). Scattering intensities were generated using the program Crysol, and electron density distributions were obtained using the program GNOM (25, 26).

Transducin Activation Assay—Fluorescence assays were performed at 10 °C in a thermostated, stirred cuvette in a volume of 400 µl with continuous excitation at 543.5 nm (27, 28) using buffer containing 2 mM MgCl₂. Fluorescence excitation and emission wavelengths were set to 300 and 345 nm, respectively. The transducin concentration was 300 nM, determined by titrating with known amounts of GTPγS. Reactions were started by the addition of 5 µM GTPγS from a 1 mM stock. Reaction time courses were fit to an exponential function to obtain rate constants, which were converted to the rate of G₂G₃-GTP-GS formation. Due to slow rates in 0.01% w/v dodecyl maltoside, initial rates were measured. Fluorescence was calibrated by adding known amounts of GTPγS to a reaction mixture containing G₂ and rhodopsin Nanodiscs.

Isolation of Rhodopsin-G₂ Complexes—For size exclusion chromatography of the rhodopsin-G₂ complex, a mixture of rhodopsin monomer Nanodiscs prepared as described above and G₂ (3.4 µM each) with or without 55 µM GTPγS was exposed to room light for 5 min and injected onto a Superdex 200 HR 10/30 column. Elution was monitored with a photodiode array detector. In the case of isolation by ConA-Sepharose, Nanodiscs were assembled by dialysis of a 2.1% CHAPS-solubilized mixture containing POPC, MSP1E3D1, and rhodopsin (solubilized from rod outer segments) in the ratio 140:1:0.1. To 340 µl of Nanodiscs (containing 7 µM rhodopsin) was added 14 µM all-trans-retinal and 14 µM G₂. Following a 30-min incubation on ice, the mixture was exposed to light from a 300-watt tungsten bulb filtered through a 480-nm cut-on filter and incubated for an additional 30 min on ice. 180 µl of ConA-Sepharose was added, and the rhodopsin was allowed to bind for 2 h on ice. The matrix was then washed extensively with 10 mM Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.01% (w/v) NaN₃ to remove unbound G₂, free Nanodiscs, and excess retinal. The rhodopsin/G₂ complex was eluted with 0.2 M methyl α-D-manno-pyranoside in the same buffer.

Transducin Binding Measurements—Transducin binding was measured using the method of extra-MI formation (28–30). Purified samples at a rhodopsin concentration of 1 µM and varying transducin in standard buffer were equilibrated at 10 °C. After measurement of a dark spectrum, the sample was exposed to light from a 100-watt tungsten lamp (Oriel) passed through a 480 ± 10 nm interference filter and mechanical shutter. Bleaching by the measuring beam was negligible. Data were fit to a quadratic equation for coupled equilibria between MI, MII, and extra-MI (30) using the equation

\[ M₂ \cdot G₂ = \frac{[R_I + G_{tot} + K_G(1/K_0 + 1)]}{2} = \frac{\sqrt{[R_I + G_{tot} + K_G(1/K_0 + 1)]^2 - 4[R_I + G_{tot}][K_G(1/K_2 + 1)]}}{2} \]
mated by fitting to the known spectrum of rhodopsin in Nanodiscs and a published spectrum of isorhodopsin in detergent (33). MI was determined from the light minus hydroxylamine difference spectrum (corrected for dilution) using an extinction coefficient of 45,000 M\(^{-1}\) cm\(^{-1}\) at 478 nm (31). After correction for isorhodopsin absorbance, the amount of MII was estimated from light minus dark difference spectra using published difference extinction coefficients (34). The amounts of MI and MII in the absence of transducin were used to calculate \(K_1\).

**RESULTS**

**Assembly of Rhodopsin into Nanodisc Structures**—Upon removal of detergent, Nanodiscs self-assemble from a mixture of phospholipid/cholate micelles and membrane scaffold protein (15, 16). Purified rhodopsin solubilized in octyl glucoside was added to the assembly mixture followed by removal of detergent with polystyrene adsorbent as described under “Experimental Procedures” (17, 35, 36). The assembly mixture contained POPC as phospholipid and the membrane scaffold protein MSP1E3D1 (16) in excess over rhodopsin to favor assembly of single rhodopsin molecules. After removal of detergent, the nanoparticles were isolated from small amounts of aggregated side products using size exclusion chromatography (15, 16) with typically 60–80% recovery of rhodopsin.

Removal of the majority of Nanodiscs without an incorporated receptor was accomplished by affinity chromatography on concanavalin A-Sepharose or 1D4 immunoaffinity resin. ConA and 1D4 affinity purification were found to yield samples of similar purity. Fig. 1A shows an analytical size exclusion profile of material purified by immunoaffinity eluting at an apparent Stokes diameter of 12 nm. The traces at 500 and 280 nm are superimposable, suggesting the absence of a significant fraction of Nanodiscs with no incorporated rhodopsin. Absorbance spectra of material purified sequentially through 1D4 affinity and ConA-Sepharose chromatography are illustrated in Fig. 1B. Material purified by 1D4-chromatography was able to bind quantitatively to ConA, demonstrating that both the N-terminal and the C-terminal faces of rhodopsin are available for interaction. As expected, MSP and rhodopsin copurify as indicated by SDS-PAGE shown in the inset.

The stoichiometry of rhodopsin in immunoaffinity-purified material was found to be 0.9 rhodopsin per Nanodisc containing two MSP molecules as quantitated by optical absorbance. The measured stoichiometry of slightly less than unity may be due to systematic error in the extinction coefficients of rhodopsin and MSP or the presence of a small amount of bare Nanodiscs or opsin. Phospholipid in the affinity-purified rhodopsin Nanodiscs was determined to be 196 ± 20 POPC by phosphate analysis. Bare Nanodiscs contain 250 POPC per Nanodisc (16), suggesting that insertion of rhodopsin into the Nanodisc displaces approximately 50 phospholipids. Displacement of similar amounts of phospholipid from the Nanodisc structure has been observed with bacteriorhodopsin (17).

Small angle x-ray scattering was used to characterize the Nanodisc assemblies (16, 37). Fig. 2 shows electron density probability distributions calculated from scattering intensities for ConA-purified material in *panel A* and for bare Nanodiscs in *panel B*. The region of negative contrast for bare Nanodiscs is a characteristic of a phospholipid bilayer organization arising from the lipid acyl chains (25, 38, 39). Nanodiscs containing incorporated rhodopsin have an increased contrast in this region due to the presence of protein within the bilayer domain. The radii of gyration for bare Nanodiscs, 63 Å, and rhodopsin Nanodiscs, 52 Å, also indicate the presence of increased electron density near the center of the particle. The overall particle size is ~130 Å for the Nanodisc structures, in good agreement with the apparent size obtained by size exclusion chromatography. Similar electron density distributions were obtained using calculated scattering amplitudes from atomistic models of bare Nanodiscs as well as those for a single incorporated GPCR (Fig. 2, dashed lines) as has been described (17).

**Transducin Binding to Monomeric Rhodopsin Nanodiscs**—Rhodopsin-transducin complexes were isolated by ConA affinity chromatography, as shown in Fig. 3A, *inset*. The starting material contained an excess of bare Nanodisc, which was removed along with unbound transducin with a wash step. Rhodopsin-transducin complexes were eluted specifically with α-methyl mannoside. The observed spectrum indicates an MII · G\(_i\) complex absorbing at 380 nm along with minor fraction of photoproducts absorbing at higher wavelengths. Formation of an MII · G\(_i\) complex can also be observed as an increase in particle size, as shown in Fig. 3B. The apparent size of non-photolyzed rhodopsin Nanodiscs absorbing at 500 nm
shifts from 12.2 to 13.2 nm for the complex monitored at the MII absorbance peak at 380 nm. The presence of GTPγS prohibits an observable complex formation.

**Two Rhodopsin Molecules in Nanodiscs**—Nanodiscs containing two rhodopsin molecules were assembled and isolated to address the issue of functionality of dimer versus monomer. Nanodiscs were assembled as for monomer but by using a stoichiometry of two rhodopsins per Nanodisc component rather than an excess of lipid and membrane scaffold protein. The assembled Nanodisc mixture was isolated by size exclusion followed by sucrose density gradient ultracentrifugation. The results of ultracentrifugation are shown in Fig. 4 for Nanodiscs assembled at a ratio of two rhodopsins per Nanodisc and at a ratio of 0.2 rhodopsin per Nanodisc (monomer). The monomeric rhodopsin in Nanodiscs (found to be 0.8 rhodopsin/Nanodisc) fractionates as a lower density peak when compared with Nanodiscs containing two rhodopsins (found to be 1.9 rhodopsin/Nanodisc). The apparent size based on size exclusion chromatography of the Nanodiscs containing two rhodopsins appears slightly larger than rhodopsin monomer Nanodiscs and with 143 ± 7 phospholipids per Nanodisc when compared with 196 ± 20 for monomer, as expected for incorporation of an additional rhodopsin molecule.

**G-Protein Coupling to Rhodopsin in Nanodiscs**—Rate constants were measured using the fluorescence increase of Gα tryptophans upon GTPγS binding in the presence of catalytic amounts of rhodopsin, excess GTPγS, and a fixed amount of transducin (Fig. 5). The initial rates are linear with rhodopsin concentration. No measurable rate was observed in the absence of rhodopsin. The measured rates for monomer correspond to an apparent second order rate constant of \(2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) at 10°C, indicating that rhodopsin monomer in Nanodiscs efficiently catalyzes GDP/GTP exchange. Assays performed at 20°C exhibit ~2-fold increases in rates (provided as supplemental data). Rates using dodecyl-maltoside solubilized rhodopsin at dodecyl maltoside concentration near the critical micelle concentration are presented for comparison. Excess detergent micelles are known to decrease the rate of transducin activation due to phase partitioning effects (28). Similarly, bare Nanodiscs decrease the rate of activation of G, with half-inhibition at 280 nm bare Nanodisc (provided as supplemental data).

The rhodopsin present in Nanodiscs at the stoichiometry of a dimer is approximately half as active (49%) as monomer in catalyzing GTPγS exchange. A partially diffusion-limited reaction could explain this result since the particle concentration is half that of monomer at equal rhodopsin concentration, and the apparent second order rate constant approaches the range of a diffusion-limited reaction in solution for particles the size of Nanodiscs and transducin heterotrimer. However, the results detailed below indicate that diffusion-limited reaction conditions are not the most likely explanation.

Transducin interaction with rhodopsin was quantitated using the method of extra-MII formation. In this experiment,
rhodopsin in Nanodiscs was photolyzed by different timed exposures to light in the presence of varying amounts of transducin. The absorbance change at 380 nm with respect to a sample containing no transducin is a measure of extra-MII. A, rhodopsin monomer Nanodisc. B, two rhodopsins per Nanodisc. C, light minus dark difference spectra obtained with monomer (circles) and two rhodopsins per Nanodisc (squares) in the absence (open symbols) and presence (filled symbols) of excess Gt at 10 °C, with 60 s of light exposure. Rhodopsin concentration was 1.4 μM, and Gt concentration was 2 μM. D, plot of maximum values of ΔA380 by extrapolating to saturating transducin concentration for samples containing two rhodopsins per Nanodisc versus one rhodopsin per Nanodisc exposed to the same amount of light. Each data point is a different light exposure time. The least squares fit to a line has a slope of 0.45 ± 0.03. mOD, milliunit optical density.

TABLE 1

Photoproducts obtained in the presence or absence of excess Gt after a 60-s light exposure at 10 °C

| Product* | Monomer | Two rhodopsins | Monomer + Gt | Two rhodopsins + Gt |
|----------|---------|----------------|--------------|---------------------|
| Rhodopsin | 23 (4)  | 22 (3)         | 9 (2)        | 18 (5)              |
| Iso rhodopsin | 21 (4) | 22 (3) | 3 (1) | 10 (3) |
| MI        | 39 (3)  | 51 (3)        | 10 (1)       | 39 (1)              |
| MII       | 17 (3)  | 4 (2)         | 77 (2)       | 34 (2)              |
| Fraction MII* | 0.35 (0.06) | 0.09 (0.04) | 0.91 (0.01) | 0.49 (0.02) |
| K₁*       | 0.45 (0.1) | 0.09 (0.04) |

* Product is expressed as percentage of all species with error from separate determinations in parentheses.

* Fraction MII = MII/(MI + MII + Gt).

* K₁ = MII/MI.

determined in a separate experiment. The data of Fig. 6 were fit as described under “Experimental Procedures” to obtain a dissociation constant (K₁) for transducin-MII interaction of 40 ± 19 nm for rhodopsin monomer (three separate experiments) and 22 ± 15 nm for Nanodiscs containing two rhodopsins (two separate experiments). Thus the estimated K₁ values for one and two rhodopsins in a Nanodisc are similar. An unanticipated result is that the amount of MII · Gt formed at near saturating transducin is less for the Nanodiscs containing two rhodopsins when compared with monomer, as shown in Fig. 6, A and B. Fig. 6C shows difference spectra obtained in the presence and absence of near saturating transducin. Fig. 6D is a plot of extra-MII formed with Nanodiscs containing two rhodopsins versus rhodopsin monomer at saturation with transducin. The light exposure time is different for each data point. The plot fits to a slope of 0.45, suggesting that only about half of the rhodopsin present in Nanodiscs containing two rhodopsins is available to interact with transducin. Analysis of photoproducts obtained after a 60-s exposure to light is presented in Table 1. The total fraction of MII forms, φ = (MII · Gt + MII)/(MI + MII + Gt), approaches unity for monomer at near saturating Gt (φ = 0.91), whereas for Nanodiscs with two rhodopsins, the value is half (φ = 0.49). Assuming a one-to-one complex of transducin with rhodopsin, the saturation value should be the same for both samples. It is therefore likely that the activities presented in Fig. 5 reflect the ability of only half of the rhodopsin in Nanodiscs having two rhodopsins to participate in catalysis of GTPγS exchange.

DISCUSSION

GPCR oligomerization is a topic of great interest due to the potential importance of the functional consequences of such interactions in signal transduction pathways. We chose to use Nanodiscs, which can provide distinct advantages over detergents and liposomes, to observe and compare the function of rhodopsin in Nanodiscs containing one and two rhodopsin molecules. Nanodiscs are stable entities in which rhodopsin can be isolated at a stoichiometry of one and two per nanobilayer. Importantly, excess amphiphile, either detergent or lipid, is detrimental to G-coupling assays, and thus care was taken to remove these and bare Nanodiscs from the samples. The largest well characterized Nanodisc formed with the membrane scaffold protein MSP1E3D1 was

FIGURE 6. Transducin binding monitored by extra-MII formation. A and B, rhodopsin (1 μM) was equilibrated with the indicated concentrations of transducin at 10 °C. The samples were photolyzed for 1 (crosses), 3 (squares), 6 (diamonds), 20 (triangles), and 60 s (circles) of total exposure time with 480 nm light. The change in absorbance at 380 nm with respect to a sample containing no transducin is a measure of extra-MII. A, rhodopsin monomer Nanodisc. B, two rhodopsins per Nanodisc. Data were fit as described under “Experimental Procedures” to obtain K₀ = 30 nm (monomer) and 18 nm (two rhodopsins per Nanodisc). C, light minus dark difference spectra obtained with monomer (circles) and two rhodopsins per Nanodisc (squares) in the absence (open symbols) and presence (filled symbols) of excess Gt at 10 °C, with 60 s of light exposure. Rhodopsin concentration was 1.4 μM, and Gt concentration was 2 μM. D, plot of maximum values of ΔA380 by extrapolating to saturating transducin concentration for samples containing two rhodopsins per Nanodisc versus one rhodopsin per Nanodisc exposed to the same amount of light. Each data point is a different light exposure time. The least squares fit to a line has a slope of 0.45 ± 0.03. mOD, milliunit optical density.
used to provide sufficient bilayer area to incorporate putative dimers (16, 17, 37). A chemically defined and pure phospholipid and affinity-purified rhodopsin were used to form the Nanodiscs, from which we have isolated separate populations of Nanodiscs containing one and two rhodopsin molecules.

The expected size, composition, and organization of the Nanodiscs containing rhodopsin monomer were confirmed by size exclusion chromatography, SAXS, and chemical analysis. The size of the particle is slightly larger than the well-characterized Nanodisc without incorporated rhodopsin due to the extramembrane regions of the photoreceptor. The particles contain fewer phospholipids than bare Nanodiscs, as expected, due to displacement by one rhodopsin molecule. SAXS indicates a bilayer particle with increased electron density in the bilayer core region. Calculated scattering and electron density distribution using atomistic models are qualitatively similar with differences due to imperfections of the model bilayer parameters. These structural studies point to Nanodisc self-assembly resulting in the incorporation of rhodopsin into a native-like bilayer environment as has been extensively discussed previously for other integral membrane proteins (17, 18, 36, 41–43). The Nanodiscs containing two rhodopsins are clearly separated from monomer on a sucrose density gradient and have the expected size and stoichiometry of MSP, lipid, and rhodopsin. We therefore conclude that the two preparations contain one rhodopsin as a monomer and two rhodopsins as a potential dimer in the phospholipid bilayer Nanodisc, providing a well-defined system for analysis and functional comparisons.

A stable rhodopsin-transducin complex was isolated as presented in Fig. 3, consistent with a high affinity one-to-one complex with rhodopsin monomer. The complex exhibited an MII spectral signature and was sensitive to GTPγS, indicating a specific association with light-activated monomeric rhodopsin. The 40 ± 19 nm dissociation constant estimated from measurements of extra-MII formation is comparable with values for rod disk membranes measured or estimated from the literature (10–100 nm) (29, 32, 44, 45). The estimated KD probably reflects an upper limit because the amount of metarhodopsin I actually increases in the presence of Gt due to photoregeneration of rhodopsin and isorhodopsin from MI when Gb is absent (Table 1).

Rhodopsin monomer in a Nanodisc is functional in coupling to G-protein. A comparison with the catalytic rate of GTPγS exchange in detergent-solubilized systems is informative. Published rate constants using detergent-solubilized material in different aggregation states (13) may be compared with the present results. Faster GTPγS exchange rates for oligomer in 50 μM hexadecyl maltoside (11 nm Gt/nM rho/min) and 0.5 mM tetradecyl maltoside (1.8 nm Gt/nM rho/min) were attributed to higher order oligomers when compared with monomers and dimers in 3 mM dodecylmaltoside (0.8 nm Gt/nM rho/min). However, an alternative interpretation is that inhibition by excess detergent occurs, with detergent concentrations differing by up to 60-fold. In comparison, the rate constant is 15-fold greater for monomer in Nanodiscs (37 nm Gt/nM rho/min) with respect to 0.3 mM dodecylmaltoside (2.6 nm Gt/nM rho/min, this work). Rhodopsin has been observed to exist as a mixture of monomers, dimers, and higher oligomeric forms at concentrations of dodecylmaltoside lower than 3 mM (9, 13). Thus a much faster rate is seen with pure monomeric rhodopsin in Nanodiscs when compared with a mixture of monomer, dimer, and oligomer in 0.3 mM detergent. The observations of a high affinity complex and rapid GTPγS exchange show that rhodopsin monomer is fully capable of highly efficient coupling to transducin.

Nanodiscs containing two molecules of rhodopsin were isolated using density gradient centrifugation. Activity in the GTPγS exchange assay was lower in comparison with monomeric rhodopsin in Nanodiscs, by half, in contrast to the published reports (9, 13) utilizing detergent as discussed above. The dissociation constant for MII · Gt is similar to that of monomer (Fig. 6, A and B). The MII-to-MI equilibrium is shifted somewhat toward MI when two rhodopsins are present in a Nanodisc. What is particularly interesting is that near saturating transducin concentrations, the MII/MI equilibrium is not completely shifted to MII in the case two rhodopsins in a Nanodisc. In fact, about half (45%, Fig. 6D) of the rhodopsin in the preparation appears to form MII · Gt in comparison with the monomeric form, which is almost fully shifted toward the MII · Gt state with increasing transducin concentration.

The results for two rhodopsins per Nanodisc are consistent with a dimeric form interacting with one transducin. Such an interaction has been postulated based on models of rhodopsin oligomers (7, 46) derived from atomic force microscopy measurements of paracrystalline arrays of rhodopsin in native disc membranes (4, 6). There is also precedence for a heteropentameric complex of G-protein heterotrimer and GPCR dimer based on biochemical and biophysical studies with purified leukotriene B4 receptor (2). However, the orientation of the two rhodopsins, parallel or anti-parallel, in the Nanodiscs is not yet known. It is possible that dimers do not form and two monomers are present but that steric constraints prohibit interaction with more than one transducin at a time. Another interpretation is that a random orientation would result in two equal populations, parallel and anti-parallel, one of which cannot interact with transducin. It is noteworthy that the phospholipid-to-rhodopsin ratio used to generate two rhodopsins per Nanodisc lies within the range in which self-association of rhodopsin in liposomes has been detected (11, 12). Whether the aggregated state in liposomes reflects a specific and physiological dimeric or oligomeric interaction is also unknown.

It is clear from early studies of rhodopsin-transducin interaction that the stoichiometry of transducin to light-activated rhodopsin in rod outer segments is one-to-one (44). We have shown that dimerization is not necessary for efficient coupling of rhodopsin to G-protein. We have also presented evidence that is consistent with the idea of formation of a pentameric complex of rhodopsin dimer and transducin heterotrimer in which only one rhodopsin is in the active MII state. Whether surface-associated arrays of rhodopsin dimers in the native membrane promote a more rapid response to photoactivation
or a regulatory role in visual signal transduction is a question that remains to be addressed.

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