Cooperative Binding of the Retinal Rod G-protein, Transducin, to Light-activated Rhodopsin*

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Direct measurements of the binding between light-activated rhodopsin (Rho') and transducin, the retinal rod G-protein, revealed a strongly cooperative interaction. Cooperativity was assessed by measuring the association of 125I-labeled transducin (Gt) to Rho' in urea-stripped rod outer segment membranes at equilibrium. Analysis of 125I-Gt binding curves gave a Hill coefficient of 1.8. These data were consistent with a two-site model in which binding of the first 125I-Gt to Rho' increased the binding of the second 125I-Gt by a factor of 40-fold (Kc values were 80 ± 30 and 1.9 ± 0.7 nM, respectively). The effects of GDP on the binding were also investigated. GDP decreased the affinity between Rho' and Gt by a factor of 100-fold but did not decrease the degree of cooperativity. Binding curves of 125I-Gt in the presence of 1 mM GDP showed a Hill coefficient of 1.9. The data were also consistent with a two-binding site model in which binding of the first 125I-Gt increased the binding of the second 125I-Gt by 70-fold (Kc values were 13.7 ± 5.4 and 0.20 ± 0.08 μM, respectively). The Gt subunit in the absence of Gtγ also bound Rho' in a cooperative manner. These data implicate a role for the cooperative association of Rho' and Gt in the light activation cascade of retinal rods.

Heterotrimeric G-proteins,† acting in concert with specific signal receptors, support a number of important signal transduction processes such as the action of hormones, neurotransmitters, odorants, chemoattractants, and light (for reviews see Birnbaumer et al., 1990; Simon et al., 1991). Despite the broad variety of signals and cellular responses, the basic architecture in which the activated receptor regulates its effector protein via a G-protein is conserved (Shinozawa et al., 1979). The ability of the receptor to bind to the G-protein and catalyze the exchange of GDP for GTP on its α subunit is essential in the activation cascade, and an understanding of the mechanism of this interaction has been the focus of considerable research (Gilman, 1987; Cerione, 1991).

Many important features of receptor-G-protein interactions have emerged from studies of the visual system. In retinal rod cells, the visual receptor rhodopsin (Rho) is activated by the absorption of a photon which leads to GDP/GTP exchange on the retinal G-protein (Gt) and to the dissociation of Gtα-GTP from Gtβγ and Rho'. Gtα-GTP activates cGMP phosphodiesterase by relieving the inhibitory action of the phosphodiesterase γ subunits. Rapid hydrolysis of cGMP results in closure of cGMP-gated cation channels in the plasma membrane, and the resulting hyperpolarization of the cell triggers retinal neuron activation at the cell's synaptic terminus (for review see in Stryer, 1986; Chabre and Deterre, 1989).

The mechanism of Rho'-catalyzed GTP exchange on Gt has been proposed to proceed via a Rho'-induced conformational change in which the nucleotide binding site is open and GDP binding affinity is reduced (Bennett and Dupont, 1985; Kahler et al., 1990). GDP diffuses away from this site, leaving a stable Rho'GtGTP, empty complex, which dissociates into Gtα-GTP, Gtβγ, and Rho' following GTP binding (Kuhn, 1980; Hofmann, 1985; Bornancin et al., 1989). Rebinding of GDP is thought to release Gt-GDP from Rho' without separation of the Gt subunits (Bornancin et al., 1989). Recent studies on the effect of GDP on Gt binding to Rho', detected by measuring extra-Meta II Rho formation, suggested that the Rho'-Gt-GDP complex was a transition state that could rapidly lose its GDP or dissociate into Rho' and Gt-GDP (Kahler et al., 1990; Panico et al., 1990). Three cytoplasmic loops of Rho' have been shown to be involved in the association with Gt (Takemoto et al., 1986; Konig et al., 1989; Franke et al., 1990), and bound GDP has been postulated to interfere with the binding of Gt to at least two of these loops (Kahler et al., 1990).

Sites on the Gtα subunit near the carboxyl terminus have been shown to be essential for Rho' binding (Hamm et al., 1987, 1988; Weiss et al., 1988; Watkins et al., 1985; Dhanasekaran et al., 1988). In addition, interactions of the amino terminus with Rho', possibly in combination with the Gtβ amino terminus, may also be involved in the complex (Navon and Fung, 1987, 1988; Hamm et al., 1988; Hingorani and Ho, 1987a). High affinity binding of Gt to Rho' requires the Gtβγ subunits (Shinozawa et al., 1980; Fung, 1983; Yamazaki et al., 1987; Yoshioka et al., 1989) and, although sites on Gtβγ that bind to Rho' have not as yet been identified, changes in the interactions among the three subunits upon Rho' binding are essential for nucleotide exchange (Vailancourt et al., 1990; Johnson et al., 1991). The emerging model depicts a transfer of conformational information from the Rho' binding domain of Gt, consisting of Gtα's carboxyl and amino termini and possibly the Gtβ amino terminus, to the adjacent guanine nucleotide binding domain upon Rho' binding. This results in decreased affinity for GDP and thus enhanced GDP dissociation.
There is evidence that G, forms higher oligomers in solution at concentrations much lower than those found in vivo on disc membranes (Baehr et al., 1982; Hingorani et al., 1988; Vaillancourt et al., 1990). The role of these oligomers in Rho binding, if any, has not been assessed. However, initial rates of GTP·S binding were positively cooperative with respect to G, (Wessling-Reinck and Johnson, 1987a), and the inhibition of the Rho−G, interaction by peptide fragments of Rho was cooperative with Hill coefficients of ≈2 (Konig et al., 1989). The involvement of G, oligomers in the observed cooperativity, or a potential role of Rho−oligomers in cooperative interactions, is not yet determined.

In this report, we explore cooperative interactions in the mechanism of Rho−G, association by directly measuring the equilibrium binding of 125I-labeled G, to Rho. We find that 125I-G, exhibits cooperative binding to Rho− and that the observed positive cooperativity is best well by a two-binding site model for G,. We also report that GDP reduces the binding affinity between G, and Rho− approximately 100-fold, yet the positively cooperative binding persists. In addition, the α subunit of G, itself retains the ability to bind Rho− in a cooperative manner.

**EXPERIMENTAL PROCEDURES**

**Preparation of Bovine Rod Outer Segments**—Fresh bovine eyes were obtained from Karler Packing Co., Albuquerque, NM. Eyes (100−150) were dark-adapted after slaughter for >30 min in HEPES/Ringer buffer (25 eyes/liter; 10 mM HEPES, PH 7.5, 120 mM NaCl, 3.5 mM KCl, 0.2 mM CaCl2, 0.2 mM MgCl2, 0.1 mM EDTA, 10 mM glucose, 1 mM DTT, 0.2 mM PMSF) at room temperature and then for 2−4 h later on ice. Eyes were dissected, and the retinas were removed in the dark and placed in HEPES/Ringer buffer plus 10% sucrose (25 ml of buffer/50 retinas). All procedures done directly were visualized by infrared illumination and infrared image converters. Retinas were gently shaken for 5 min and then passed through two layers of cheesecloth. The retinal material that did not pass through the cheesecloth was shaken again and reapplicd to the cheesecloth two more times. The crude rod suspension was then layered over 37% sucrose and centrifuged for 15 min at 40,000 × g. Purified rod outer segments (ROS) were collected from the 10%/37% sucrose interface and diluted in 2 volumes of HEPES/Ringer. The ROS were pelleted by centrifuging for 10 min at 12,000 × g, and the pellet was resuspended with a 25-gauge needle in 5 ml of HEPES/Ringer.

**Purification of G, and βγ Subunits**—Crude G, was purified from ROS as described (Yamazaki et al., 1988) with modifications. The ROS were washed three times in the dark with 40 ml of isotonic buffer (10 mM HEPES, PH 7.5, 100 mM KCl, 20 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM leupeptin, 1 μM pepstatin A, centrifuging for 10 min at 12,000 × g). Pellets were resuspended with a 23-gauge needle and syringe. Washed ROS were then centrifuged for 5 min with two 100-watt tungsten lamps at a distance of 20 cm and washed once more in isotonic buffer. The rest of the procedure was done in room light. The ROS were washed three times in 40 ml of hypotonic buffer A (10 mM HEPES, PH 7.5, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM leupeptin, 1 μM pepstatin A). We observed more in 0.1 M hypotonic buffer B (hypotonic buffer A plus 250 μM GTP). Centrifugations were done for 90 min at 90,000 × g, and suspensions were done with a 25-gauge needle and syringe. Washed ROS were then centrifuged for 5 min with two 100-watt tungsten lamps at a distance of 20 cm and washed once more in isotonic buffer. The rest of the procedure was done in room light. The ROS were washed three times in 40 ml of hypotonic buffer A (10 mM HEPES, PH 7.5, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM leupeptin, 1 μM pepstatin A) and once more in 0.1 M hypotonic buffer B (hypotonic buffer A plus 250 μM GTP). Centrifugations were done for 90 min at 90,000 × g, and suspensions were done with a 25-gauge needle and syringe. Supernatants from hypotonic buffer B washes were pooled and concentrated by ultracentrifugation using Amicon YM10 filters to 1−2 ml.

The concentrated crude G, was loaded on an 80 × 0.9-cm Blue Sepharose CL-6B column (Pharmacia LKB Biotechnology Inc.) equilibrated in column buffer (10 mM HEPES, PH 7.5, 6 mM MgSO4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). The column was run in column buffer at 0.5 ml/h until the GTP peak eluted, after which a 100-ml linear gradient from 0−1.2 M KCl in column buffer was used to elute the G, subunits. The βγ subunits eluted at 0.2 M KCl, and the α subunit eluted at 0.6 M KCl. The purified subunits were pooled and concentrated by ultracentrifugation on Amicon YM10 and Centricon 10 filters to 1−3 mg/ml. Each subunit preparation was >98% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Iodination of G,**—Purified G, was desalted, and DTG was removed by passing the protein through a 25 × 0.9-cm Sephadex G-25 column (Pharmacia) equilibrated in isotonic buffer without DTT. DTT removal was necessary to avoid its reaction with the 125I-Bolton-Hunter reagent as described by the manufacturer (Du Pont-New England Nuclear). Briefly, ≈300 μl of purified G, was mixed with 250 μl of isotonic buffer without DTT for 2 h on ice. Uncoupled reagent was separated from G, by centrifuging after the reaction mixture through a Sephadex G-25 column equilibrated in isotonic buffer plus 1 mM DTT. The 125I-labeled G, was concentrated to 1−3 mg/ml by Amicon Centricon 10 filtration. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.). The specific activity was determined by counting aliquots of G, in an LKB/Wallace 1272 Clinigamma counter and was between 5,000 and 10,000 dpm/μg G, using a molecular weight of 40,000 for G, (Medynski et al., 1986). Such a specific activity corresponds to a mol ratio of 0.0005–0.001 mol of labeled reagent/mol of G, (Konig et al., 1989). The sample was stored at 4°C and was found to retain full binding capacity for ≈10 days. All experiments were performed within 7 days of purification.

**Determination of the Active Fraction of G,**—The percent active fraction of the purified G, and 125I-labeled G, was determined by its ability to bind GTP·S (Kroll et al., 1989). Briefly, 100 ng G, or 400 ng G, (∼1 μCi) and 200 ng Rho in urea-stripped ROS membranes (UROSM, see below) in isotonic buffer at 23°C were illuminated in room light. After 1 min, the reaction was started by the addition of 1 μM GTP·S (25 pmol/ml GTP·S), resulting in a final volume of 1.4 ml. At time points between 1 and 60 min, 100-μl aliquots were filtered through Millipore 0.45-μm HAWP filters. Filters were washed four times with 5 ml of isotonic buffer, GTP·S was eluted (30 min) with 1 ml of 10% sodium dodecyl sulfate in scintillation vials and counted, after the addition of 10 ml of scintillation fluid, by a dual label liquid counting method. At equilibrium, 67−83% of the UROSM G, bound GTP·S, and 100% of the 125I-G, bound GTP·S. The iodination procedures resulted in losses of ≈40% of the G, α. This appears to be a preferential loss of inactive G, α, which resulted in a greater active fraction of 125I-Ga. Concentrations of G, were corrected for this percent activity.

**Preparation of Urea-stripped ROS Membranes**—ROS membranes were depleted of endogenous G, and other peripheral proteins by treatment with 5 M urea (Yamazaki et al., 1982). All steps were carried out in the dark. ROS (1 ml, ∼100 μM Rho) were washed three times with 10 mM of hypotonic buffer B. Centrifugation for all washes was for 20 min at 90,000 × g, and pellets were resuspended with a 25-gauge needle. The GTP-washed ROS were then incubated in 20 ml of 5 M urea in hypotonic buffer B at pH 6.1 for 1 h on ice. It has recently been reported that Rho is most stable at pH 6.1 (Khan et al., 1981). After centrifuging, the pellets were washed once each with 10 ml of hypotonic buffer A and isotonic buffer. Finally the pellet was resuspended in isotonic binding buffer (isotonic buffer plus 1 mg/ml bovine serum albumin) and layered over 20% sucrose in this same buffer. The sample was centrifuged for 20 min at 43,000 × g, and the pellet was resuspended in isotonic binding buffer. Thus, a subpopulation of larger membrane fragments which readily pellet through 20% sucrose was selected. Without this precaution, sedimentation resistant vesicles could complicate the 125I-G, binding assay. The rhodopsin concentration was determined by difference spectroscopy in the presence of 1% Triton X-100 and 50 mM hydroxylationine (Bowell et al., 1981).

Nucleotide diphosphokinase activity of the UROSM was measured by formation of [3H]GTP from 1 mM [3H]GDP. ROS or UROSM, at 10 μM Rho, were incubated in isotonic binding buffer with 1 mM purified [3H]GDP (specific activity, 6,800 dpm/nmol) in the dark for 10 min at 23°C. The reaction was stopped by the addition of 10 ml of cold 50 mM 1 M LiCl. The GTP spot was cut out, placed in a scintillation vial with 1.0 ml of 100 mM MgCl2 (shaking, 1 h). [3H]GTP was measured by scintillation counting after the addition of 10 ml of scintillation fluid.

**Purification of GDP**—GDP obtained from Boehringer Mannheim
was found to contain 80.1% GTP contamination as measured by ion pairing high performance liquid chromatography as described by Panico et al. (1990). This GDP was purified as described except that tetraethylammonium bicarbonate was replaced by triethylammonium bicarbonate in the column buffer. Following this procedure GTP contamination was <0.002% as reported by Panico et al. (1990). Following this procedure GTP contamination was <0.002% as reported by Panico et al. (1990). This GDP was purified as described except that volume of hundred nM Rho in UROSM was added in the dark giving a final volume of 320 μl. Samples at various 125I-Gα concentrations were incubated for 20 min at 30°C in the dark, during which time they were divided into three 90-μl aliquots. One aliquot was illuminated for 1 min by two 100-watt tungsten lamps at a distance of 20 cm, which bleached 50% of the Rho pool. This aliquot and one dark aliquot were immediately centrifuged for 20 min at 43,000 × g to pellet the UROSM. The third aliquot was reserved for total 125I-Gα determination. The free concentration of Gα was determined by removing three 20-μl aliquots from the supernatants of the centrifuged samples and measuring 125I-Gα by counting in the γ counter. The total concentration of Gα was measured by removing similar aliquots from the uncentrifuged sample and counting. The amount of Gα bound to the UROSM pellets was then determined by subtracting the average of the three 20-μl total counts aliquots from a similar concentration range that would be expected if the iodination did not alter the Rho'–Gα interaction (Fig. 1). The inhibition data are not shown). Thus, the iodination appears to have no effect on the ability of 125I-Gα to bind to Rho'. It has been reported previously that modification of lysine residues on Gα with fluorescein 5'-isothiocyanate did not inhibit its interaction with Rho' (Hingorani and Ho, 1987b). Since the N-hydroxysuccinimide ester of the Bolton-Hunter reagent also reacts with primary amino groups (Bolton and Hunter, 1973), it was expected that labeling Gα in this manner would not interfere with binding to Rho'. Accordingly, unlabeled Gα was found to inhibit the binding of 125I-Gα to Rho' over a concentration range that would be expected if the iodination did not alter the Rho'–Gα interaction (Fig. 1). The inhibition data are fit well by a two-site model of Gα binding to Rho' (see below) in which the two binding affinities are equal for both labeled and unlabeled Gα. K values are the dissociation constants shown in the equilibrium reactions, and the other parameters as described above.

RESULTS

Inhibition of 125I-Gα Binding by Unlabeled Gα—The ability of unlabeled Gα to compete with 125I-Gα for binding to UROSM was assessed to determine if the labeling procedure had any effect on the ability of 125I-Gα to bind to Rho'. It has been reported previously that modification of lysine residues on Gα with fluorescein 5'-isothiocyanate did not inhibit its interaction with Rho' (Hingorani and Ho, 1987b). Since the N-hydroxysuccinimide ester of the Bolton-Hunter reagent also reacts with primary amino groups (Bolton and Hunter, 1973), it was expected that labeling Gα in this manner would not interfere with binding to Rho'. Accordingly, unlabeled Gα was found to inhibit the binding of 125I-Gα to Rho' over a concentration range that would be expected if the iodination did not alter the Rho'–Gα interaction (Fig. 1). The inhibition data are fit well by a two-site model of Gα binding to Rho' (see below) in which the two binding affinities are equal for both labeled and unlabeled Gα (Fig. 1, dashed line). Dark binding of 125I-Gα to UROSM was also readily inhibited by unlabeled Gα (data not shown). Thus, the iodination appears to have no effect on the binding properties of Gα. Binding of 125I-Gα to ROS Membranes—Equilibrium binding of 125I-Gα to UROSM was measured by reconstituting UROSM, which are devoid of endogenous Gα, with increasing concentrations of 125I-Gα at excess concentration of GDPγS. Bound 125I-Gα was quantitated from the loss of free 125I-Gα from the supernatant fraction after centrifugation because of association with UROSM in the pellet. Binding was found to reach equilibrium after 20 min at 30°C in the dark. Light
FIG. 1. Unlabeled G, inhibition of 125I-G, binding to Rho'. 125I-G,G (45 nM), G,βγ (400 nM), and UROSM (100 nM Rho) at the concentrations of unlabeled Gt indicated, were assayed for 125I-G, binding to UROSM as described under "Experimental Procedures." Binding caused by the interaction of 125I-G, with Rho' was determined by subtracting the dark binding from the light binding at each unlabeled Gt concentration (O). The dashed line represents the inhibition predicted from Equation 3 with Kd = 80 nM and Kd = 2 nM as determined from the curve fits of the data in Fig. 2B. The data are representative of four individual experiments.

The results of binding experiments are given in Fig. 2A. The data show a large enhancement of high affinity binding in the light because of 125I-G, association with Rho'. In addition, a nonlinear least squares fit of the data to the Hill equation (Equation 1) revealed positive cooperativity in the light binding data (napp = 1.6 ± 0.2) which was not apparent in the dark (napp = 0.64 ± 0.6). The difference between the light and dark binding curves yielded the binding caused by 125I-G, interaction with Rho' (Fig. 2B). Subtraction of dark binding from light binding to obtain the Rho'-specific binding is valid if illumination adds Rho' sites but does not significantly decrease the number of dark binding sites available. This appears to be the case, for when UROSM were heat denatured at 95 °C for 5 min, light binding was abolished, whereas no change in dark binding occurred (data not shown). These results can be justified if the dark binding of Gt to the membrane is via interactions with lipid and not rhodopsin or another membrane protein.

The light-induced binding curve was also sigmoidal in nature, and fitting the data to the Hill equation gave an napp of 1.84 ± 0.05. A Hill plot of the light-induced binding data confirmed the cooperativity, yielding an napp of 1.8 in the linear range of the data (Fig. 2C). The napp values were consistently within this range showing little variation between Gt preparations (1.85 ± 0.12, n = 4). Since the Hill coefficient provides a lower limit for the order of cooperativity, an napp of 1.8 suggests at least two sites of cooperative interaction but does not rule out a greater number of interacting sites. To address the number of allosteric sites to which Gt bound, the data were compared with what would be predicted by a simple two-interaction site model. In this model, the binding of the first molecule of Gt to either of the two sites facilitates the binding of a second molecule of Gt to the remaining free site. The fit of the data to Equation 2 was excellent, resulting in KD values of 80 ± 30 and 1.9 ± 0.7 nM for the binding of 125I-G, Gt to the first and second sites, respectively (Fig. 2B).

To assess further the positive cooperativity and the validity of the two-site model, the results were expressed in Scatchard plot format. The convex curvature of this plot of the data confirmed the positively cooperative nature of the interaction (Fig. 3). In Fig. 3, the data were normalized for Bmax (23 nM) and KD (50 nM) for comparison with the model and to determine the ratio of KD to KD more accurately (Endrenyi et al., 1971). Lines represent data calculated at KD/KD ratios ranging from 0.25 to 80 as determined from Equation 2. The experimental data have a similar shape to the lines calculated from the model, and the maximum of the curve occurs when half-maximal binding is reached. These observations are further evidence that the two-site model accurately describes the binding data. In addition, the ratio of KD to KD which best fits the data is ≈40, which is in good agreement with the curve fit values of KD ≈80 nM and KD ≈2 nM from Fig. 2B.

The maximal amount of Rho' able to bind to 125I-G, was found, from the saturation point of the fitted binding curves, to be 23% of the total Rho' in the UROSM. The reason for fractional participation of Rho' in binding to Gt is not known. However, it is possible that a fraction of the UROSM vesicles have become inverted upon disruption of the discs and that their Gt binding surfaces face the interior of the UROSM vesicles, rendering them inaccessible to 125I-G,. An alternative explanation is that Rho' forms oligomeric structures upon 125I-G, binding to give the two cooperative binding sites.

The concentration of GDP which is free in this reconstituted system can be estimated by assuming that GDP is released from G,G only after interacting with Rho'. The maximal concentration of Gt bound to Rho' is 23 nM at saturation; therefore the free GDP concentration varies between zero and 23 nM depending on the Gt bound to Rho'. This small amount of free GDP did not dissociate the Rho' -G, complex (see Fig. 4).

Effect of GDP on the Rho' 125I-G, Complex—The addition of GDP to reconstituted 125I-G, in UROSM inhibited the light-induced binding (Fig. 4). Half-maximal inhibition occurred at 1.4 ± 0.3 μM GDP, and the inhibition saturated at 100 μM GDP. These results are similar to results obtained from GDP inhibition of extra MII formation (Kahlert et al., 1990; Panico et al., 1990). The inhibition data were described in terms of the two-site model and were found to be in good agreement with the model. The dashed line in Fig. 4 represents the fit of the data to Equation 4 using the KD values for Gt binding to Rho' in the absence of GDP from Fig. 2 and KD values for binding in the presence of GDP from Fig. 5. The resulting KD for GDP binding to Gt was 0.36 ± 0.05 μM. This value...
Fig. 2. Binding of $^{125}$I-G, to ROS membranes. Panel A, $^{125}$I-G,$\alpha$ at the concentrations indicated, G,$\beta$ (400 nM, a saturating concentration, see Fig. 7) and UROSM (200 nM Rho) were assayed for $^{125}$I-G, binding to UROSM as described under "Experimental Procedures"; O, light; A, dark. Dashed lines represent nonlinear least squares fits of the data to the Hill equation ($n_{app}$ dark = 0.64 ± 0.6, $n_{app}$ light = 1.6 ± 0.2). Data are representative of six individual experiments. Panel B, binding caused by the interaction of G, with Rho' was assessed by subtracting the curve fit of the light data from the curve fit of the dark data in panel A) at the concentrations indicated (O). The dashed line represents the nonlinear least squares fit of the data to Equation 2. Values obtained from the curve fit were $B_{max}$ = 23 ± 0.1 nM, $K_{app}$ = 80 ± 30 nM, and $K_{app}$ = 1.9 ± 0.7 nM. Fitting the data to the Hill equation gave a similar curve with an $n_{app}$ = 1.84 ± 0.05. Panel C, Hill plot of the $^{125}$I-G, Rho' binding data from panel B (O). Data points in the linear range of the Hill plot (between 10 and 90% saturation) are shown. $Y$ represents the amount of $^{125}$I-G, bound at a particular concentration of $^{125}$I-G, and $Y_{max}$ represents the $^{125}$I-G, bound at saturation as determined by the curve fit in panel B. The dashed line represents the linear least squares fit of the data ($n_{app}$ = 1.8).
FIG. 3. Scatchard plot of $^{125}$I-Gt binding to Rho'. Data from Fig. 2B are presented in Scatchard plot format (○). Bound/free data are normalized for Kd and Bmax (80 and 23 nM, respectively, from the fit of the data in Fig. 2B), and bound data are normalized for to compare with Equation 2. Lines represent calculated bound/free versus bound at different $K_d$/$K_d'$. The graphs, in ascending order, correspond to the following ratios of $K_d$ to $K_d'$: 0.25, 10, 20, 30, 40, 50, 60, and 80.

The graphs compare favorably with the 0.2 μM $K_d$ for GDP binding reported previously (Bennett and Dupont, 1985). In the derivation of Equation 4, it was assumed that the $K_d$ values for the binding of Gt to Gt to the second site were independent of the GDP ligation state of Gt to the first site (see “Experimental Procedures”). The validity of this assumption is supported by the data since the requirement that $K_d$/$K_d'$ is satisfied within experimental error by the $K_d$ values measured in Figs. 2B and 5B. The resulting $K_d$ value for GDP binding to Gt is in agreement with direct measurements of this $K_d$ (Bennett and Dupont, 1985).

Inhibition of $^{125}$I-Gt binding to ROS Membranes in the Presence of 1 mM GDP—The capacity of GDP to inhibit Rho'-Gt interactions has led to the hypothesis that Rho'-Gt, GDP exists as a transition state complex (Kahlert et al. 1990) in the following equilibrium:

$$Rho' + Gt.GDP \rightarrow [Rho' . Gt . GDP] \; \rightarrow \; Rho' . Gt + GDP$$

MODEL 3

It was reasoned that by adding saturating amounts of GDP to the reconstituted system, the equilibrium would be shifted to the left and that, at high concentrations of Gt.GDP, the binding affinity of the Rho'-Gt.GDP complex might be measurable. The results shown in Fig. 4 suggested that at 1 mM GDP, the binding equilibrium would be saturated with respect to GDP. Therefore, 1 mM GDP was added to $^{125}$I-Gt-reconstituted UROSM, and the binding was measured as before (Fig. 5A). Much higher concentrations of $^{125}$I-Gt were required to
The data, and 1.9 from the slope of the linear portion of the Hill plot (Fig. 5, B and C). Fitting the Rho'·G·GDP binding data to Equation 2 gave $K_{d1}$ and $K_{d2}$ values of 13.7 ± 5.4 and 0.20 ± 0.68 μM, respectively. These dissociation constants are ≈100-fold greater than those measured in the absence of GDP, but despite the large reduction in affinity of the Rho'·G·GDP complex, the positive cooperativity is not diminished.

Scatchard analysis of the binding of $^{125}$I-G to Rho' in the presence of 1 mM GDP confirmed the positive cooperativity as seen by the convex curvature of the data (Fig. 6). The data also have the same general shape as the lines generated from Equation 2 and are best fit by a $K_{d1}/K_{d2}$ ratio of 70. This ratio is in good agreement with the values of 15.7 and 0.2 μM for $K_{d1}$ and $K_{d2}$, respectively, and is similar to the $K_{d1}/K_{d2}$ ratio of 40 found in the absence of GDP. Thus, notwithstanding the large decrease in affinity, the binding of Rho' to G·GDP exhibits a high degree of positive cooperativity that fits well the two-binding site model. Therefore, even the reduced binding domains between Rho' and G·GDP must still be sufficient to elicit cooperative interactions.

Examination of the saturation points of the curves shows that, as was the case without added GDP, only partial participation of Rho' was observed. Here, 27% of the total Rho' was bound at saturation (Fig. 5B). The dark binding curve saturated at 11.9 ± 1.6 pmol of $^{125}$I-G, bound (Fig. 5A), which is ≈60% of the 20 pmol of total Rho in the sample. This suggests that there is ≈1 dark G, binding site for every two Rho molecules in UROSM. By comparison, in the native disc membrane, there is found ≈1 G, molecule for every 10 Rho molecules (Hamm and Bownds, 1986). Thus, in native disc membranes, only a fraction of the G, binding sites are occupied, which agrees with previous measurements of dark binding. By comparison, in the presence of 1 mM GDP, only a fraction of the G, binding sites are occupied, which agrees with previous measurements of dark binding. By comparison, in the presence of 1 mM GDP, only a fraction of the G, binding sites are occupied, which agrees with previous measurements of dark binding.

**Binding of $^{125}$I-Gα to Urea-stripped ROS Membranes without Gβγ**—$^{125}$I-Gα required Gβγ for high affinity binding to Rho'. As shown in Fig. 7, no binding was observed at 50 nM $^{125}$I-Gα and 100 nM Rho' in the absence of Gβγ. The addition of Gβγ dramatically increased binding. Half-maximal binding occurred at 45 nM Gβγ, and the binding saturated at ≈300 nM Gβγ. Fitting the data to the Hill equation again revealed the cooperative nature of the interaction ($n_{app} = 1.65 ± 0.22$), which simply reflects the increasing $G_{βγ}/G_{βγ}$ concentration as $G_{βγ}$ is increased. Thus, questions arose as to the ability of $^{125}$I-Gα to bind to Rho' in the absence of Gβγ, for which indirect evidence has been obtained by others (Wesling-Resnick and Johnson, 1987a; Cerione, 1991), and as to the necessity of Gβγ to form cooperative interactions with Rho'.
These questions were addressed by measuring the binding of \({}^{125}\text{I}-\text{Gt}\) to UROSM in the absence of \(\text{Gt}\beta\gamma\) (Fig. 8). \({}^{125}\text{I}-\text{Gt}\) showed very low ability to bind UROSM both in the dark and in the light. In fact, light binding was approximately 1,000-fold weaker than in the presence of \(\text{Gt}\beta\gamma\). Binding did not saturate even at 12 \(\mu\text{M} \, {}^{125}\text{I}-\text{Gt}\), and because of the prohibitive amounts of \({}^{125}\text{I}-\text{Gt}\) required to attain higher concentrations, saturation of the binding was not obtained. Curve fits of the data to the Hill plot again revealed no cooperativity in the dark (\(n_{\text{app}} = 1.02 \pm 0.16\)). The Hill coefficient was not significantly greater in the light (\(n_{\text{app}} = 1.15 \pm 0.24\)) because of the large dark component in the light binding. However, binding to Rho' did show some degree of cooperativity (\(n_{\text{app}} = 1.5 \pm 0.07\)), which was also seen in a Hill plot of the data (\(n_{\text{app}} = 1.5\)). It was not clear whether the decreased degree of cooperativity was inherent in the weak \(\text{Rho'}-\text{Gt}\) interaction or whether it was a result of the inability to saturate the binding. Whatever the reason, it appears that \({}^{125}\text{I}-\text{Gt}\) does bind Rho' in a cooperative manner.

It has been recently reported that \(\text{Gt}\alpha\) can interact with Rho' and undergo nucleotide exchange to some extent (Ceronne, 1991). If this is the case, then it would be expected that GDP would decrease the binding of \(\text{Gt}\alpha\) to Rho'. Measurements of light-induced binding in the presence of 1 mM GDP did show decreased binding to Rho' (Fig. 9). The binding curve in the presence of 1 mM GDP was shifted by a factor of 4 to higher \({}^{125}\text{I}-\text{Gt}\) concentrations. This is consistent with the ability of Rho' to catalyze GDP exchange on \(\text{Gt}\alpha\). The degree to which GDP decreases \(\text{Gt}\alpha\) binding is, however, some 25-fold less than in the presence of \(\text{Gt}\beta\gamma\).

**DISCUSSION**

Equilibrium measurements of the binding of \({}^{125}\text{I}-\text{Gt}\) to Rho' have revealed a highly cooperative interaction. The resulting Hill coefficient was 1.84 \(\pm 0.12\). This value is similar to the \(n_{\text{app}}\) of 1.97 \(\pm 0.22\) reported from initial rates of GTPyS binding with respect to \(\text{G}\) concentration (Wessling-Resnick and Johnson, 1987a). These values of \(n_{\text{app}}\) suggest at least two cooperatively interacting sites but do not rule out the possibility of a greater number of sites. However, the binding data are well described by a two-site interaction model in which the binding of the first \({}^{125}\text{I}-\text{Gt}\) increases the binding of the
FIG. 8. $^{125}$I-G$_{\alpha}$ binding to ROS membranes in the absence of G$_{\alpha}$. Panel A, $^{125}$I-G$_{\alpha}$ at the concentrations indicated, and UROSM (2 $\mu$m Rho) were assayed for $^{125}$I-G$_{\alpha}$ binding to UROSM as described under "Experimental Procedures". O, light; A, dark. The dashed lines represent nonlinear least squares fits of the data to the Hill equation ($n_{\text{app}}$, dark = 1.02 ± 0.16, $n_{\text{app}}$, light = 1.15 ± 0.24). Data are representative of two individual experiments. Panel B, binding caused by the interaction of $^{125}$I-G$_{\alpha}$ with Rho was assessed by subtracting the curve fit of the light binding data from the curve fit of the dark binding data in panel A at the concentrations indicated (O). The dashed line represents the nonlinear least squares fit of the data to the Hill equation ($n_{\text{app}}$, 1.50 ± 0.02). Panel C, Hill plot of the $^{125}$I-G$_{\alpha}$-Rho binding data from panel B (O). Data points in the linear range of the Hill plot (between 10 and 90% saturation) are shown. $Y$ represents the amount of $^{125}$I-G$_{\alpha}$ bound at a given concentration of $^{125}$I-G$_{\alpha}$, and $Y_{\text{max}}$ represents the $^{125}$I-G$_{\alpha}$ bound at saturation as determined by the curve fit in panel B. The dashed line represents the linear least squares fit of the data ($n_{\text{app}}$ = 1.5).
second \(^{125}\text{I}-\text{G} \approx 40\)-fold. At saturating concentrations of GDP, measurements of Rho\(^{-}\)-G\(_{\alpha}\)-GDP trimeric complexes yielded binding affinities \(\approx 100\)-fold weaker than in the absence of GDP, but the cooperativity of the interaction was not reduced. In fact, binding of G\(_{\alpha}\) to the second site was 70-fold greater than binding to the first site. The large difference in binding energy between Rho\(^{-}\)-G\(_{\alpha}\)-empty and Rho\(^{-}\)-G\(_{\alpha}\)-GDP could serve as the driving force for Rho\(^{-}\)-catalyzed nucleotide exchange on G\(_{\alpha}\) (Bennett and Dupont, 1985). The dissociation constants for the second site in the absence and presence of GDP (2 and 200 nM, respectively) are in excellent agreement with dissociation constants reported by Bennett and Dupont (1 and 200 nM, respectively), determined by using the binding signal from light-scattering measurements.

It has been postulated that in the presence of GDP, G\(_{\alpha}\) can only associate with one of three cytoplasmic loops of Rho\(^{-}\) that are available to bind G\(_{\alpha}\) in the absence of GDP (Kahlert et al., 1990). Whatever the nature of the weaker interaction in the presence of GDP, the reduced contacts are still sufficient to induce cooperativity, as evidenced by the 70-fold increase in affinity for the second site in the presence of 1 mM GDP. This is consistent with the observation that single peptides, corresponding to each of the three cytoplasmic loops of Rho\(^{-}\), inhibited Rho\(^{-}\)-G\(_{\alpha}\) binding in a cooperative way (Konig et al., 1989).

A question arising from this study concerns the nature of the two binding sites. How are the sites formed, and what is the oligomeric structure of the Rho\(^{-}\)-G\(_{\alpha}\) complex? Three plausible mechanisms for two-site binding are depicted in Fig. 10. In panel A, each Rho\(^{-}\) molecule has two binding sites for G\(_{\alpha}\). At first glance, this possibility seems weak since mapping of the interaction domains of Rho\(^{-}\) and G\(_{\alpha}\) has revealed a specific, circumscribed binding site that appears to leave little room for a second G\(_{\alpha}\) molecule. However, without further structural analysis, this possibility cannot be ruled out, especially when portions of G\(_{\alpha}\) bound to the first site are allowed to make up part of the second site.

In panel B, the binding of the first G\(_{\alpha}\) molecule to Rho\(^{-}\) opens up a site on G\(_{\alpha}\) for a second G\(_{\alpha}\) molecule to nest, thus forming a G\(_{\alpha}\) dimer.\(^{2}\) Oligomers of G\(_{\alpha}\) have been reported in solution (Baehr et al., 1982; Hingorani et al., 1988; Vaillancourt et al., 1990), but the effects of Rho\(^{-}\) on oligomerization have not been addressed. It is interesting to note that in cross-linking studies, although all three subunits can be cross-linked to each other using conventional cross-linking reagents (Hingorani et al., 1988), oligomeric forms of G\(_{\alpha}\) were the predominant cross-linked species when highly specific photoactivatable cross-linking reagents were used (Vaillancourt et al., 1990). Thus, the G\(_{\alpha}\) subunit could contain a site for G\(_{\alpha}\) dimer association. This would be consistent with the data in Fig. 8 showing that G\(_{\alpha}\), without G\(_{\beta}\gamma\), can bind Rho\(^{-}\) cooperatively and with steady-state kinetics of GTP\(_{\gamma}\)S binding which showed cooperativity with respect to G\(_{\alpha}\) alone (Wessling-Resnick and Johnson, 1987a).

In considering G\(_{\alpha}\) dimers as a potential source of cooperative binding, it is important to address the possibility that the cooperativity is caused by the formation of G\(_{\alpha}\) dimers in solution. This dimerization would occur independently of Rho\(^{-}\) simply as a result of increasing the G\(_{\alpha}\) concentration. Such a mechanism would require G\(_{\alpha}\) dimers to bind Rho\(^{-}\) with higher affinity than G\(_{\alpha}\) monomers and would require a shift in the monomer to dimer equilibrium over the concentration range used in the binding experiments. Since we observed cooperative interactions over two very different concentration ranges in the absence or presence of GDP (1–200 nM and 0.1–10 \(\mu\)M, respectively), a preformed dimer mechanism for the cooperative binding appears unlikely. Moreover, in concentrated solutions (20–50 \(\mu\)M) G\(_{\alpha}\) behaved as a monomer when assayed by size exclusion chromatography, agreeing with results of others using size exclusion and sucrose density gradient ultracentrifugation techniques (Wessling-Resnick and Johnson, 1987b). Thus, models involving G\(_{\alpha}\) dimers in the observed cooperativity should derive from the formation of the dimer upon interaction with Rho\(^{-}\).

Both of the mechanisms depicted in panels A and B are consistent with the observation, made from light-scattering measurements (Bennett and Dupont, 1985), that the rate of formation of Rho\(^{-}\)-G\(_{\alpha}\) saturates at Rho\(^{-}\)/G\(_{\alpha}\) ratios of 1:2–4. This observation can be interpreted by the binding of one Rho\(^{-}\) molecule to two or four G\(_{\alpha}\) molecules. In addition, the mechanisms depicted in panels A and B may not be mutually exclusive, but the second G\(_{\alpha}\) binding site may be made up of interactions with both Rho\(^{-}\) and the G\(_{\alpha}\) bound to the first site.

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\(^{2}\)For the model in panel B to satisfy the requirements of Equation 4 and the data presented in Fig. 4, the binding of the second G\(_{\alpha}\) must be insensitive to the GDP ligation state of the first G\(_{\alpha}\).
Cooperative Binding of Transducin to Rhodopsin

Fig. 10. Possible mechanisms for the cooperative binding of G, to Rho'. Panel A illustrates a mechanism in which two G, binding sites exist on one Rho'. When G, binds to the first site, binding at the second site by a second G, molecule is facilitated. Panel B depicts a mechanism in which the association of the first G, molecule to Rho' opens up a site on G, for a second G, molecule to bind with high affinity, thereby forming a G, dimer. Panel C shows a mechanism in which the interaction of G, and Rho' induces the formation of a Rho' dimer. This promotes the interaction of a second G, with the free Rho' of the dimer at higher affinity.

Lastly, panel C depicts a model in which the interaction of Rho' with G, may induce association with a second Rho' to form a Rho' dimer and enhance binding of G, to the second Rho'. This scheme is consistent with classical mechanisms of cooperativity in which the cooperatively binding ligand induces changes in the interaction between subunits of the protein containing the ligand binding site (Ricard and Cornish-Bowden, 1987). Some evidence for oligomerization of Rho upon illumination comes from chemical cross-linking studies (Shaw et al., 1980). However, equatorial x-ray diffraction measurements are consistent with Rho monomers instead of Rho dimers (Blaurock, 1977; Dratz et al., 1979). Furthermore, they show only small changes at the disc surface upon total illumination of ROS (Chabre and Cavaggioni, 1975), suggesting that light does not induce dimer formation. Such diffraction measurements give a time-averaged result over the whole Rho' population and therefore cannot rule out the possibility that transient dimerization of the Rho' population that is interacting with G, occurs at any given moment. Another concern with regard to this model is that it predicts cooperative binding with respect to Rho' as well as G, Reports monitoring Rho'-G, interactions via light-scattering changes or light-induced GTPase activity did not find cooperativity with respect to Rho' (Bennett and Dupont, 1985; Guy et al., 1990). Taken together, the bulk of the evidence favors the models in panel A or B but not C as potential mechanisms for cooperative interactions between Rho' and G,.

What then is the physiological role of the cooperativity? Retinal rods respond rapidly to light stimuli even as small as a single photon. For responses to light to be fast and sensitive, Rho' must catalyze the exchange of GDP for GTP on thousands of G, molecules/s. Cooperative Rho'-G, interactions could play a role in rapid responses to light. For example, interactions between the two G, binding sites may be necessary for essential steps in G, activation to occur, e.g. release of GDP, GTP binding, or release of G,α-GTP from Rho' and G,βγ. Another possibility is that activation steps can occur without the allosteric interaction between the two G, sites, but the cooperative binding enhances the rate of activation by producing two G,α-GTP molecules/interaction with Rho'. This would increase the gain of light activation resulting in greater sensitivity in the system. To address these possibilities, it will be necessary to find a way to prevent cooperativity and to compare the rates of G, activation in systems that exhibit cooperative and noncooperative states.

Aside from potential effects on reaction rates, the cooperative binding may allow for increased regulation of the Rho'-G, interaction. For example, phosphorylation of Rho' is known to decrease G, binding and thus rates of PDE activation (Miller et al., 1986; Bennett and Sitaramayya, 1988). Does phosphorylation of Rho' produce its effects by disrupting the cooperative binding of G,? Arrestin is another known regulator of the Rho'-G, interaction (Wilden et al., 1986). Can the binding of arrestin to phosphorylated Rho' block the binding of G, to both sites? These questions must be addressed before the physiological role of positive cooperativity can be better understood. Finally, given the conserved nature of the association between different receptors and their G-proteins,
it is possible that cooperative interactions may also function in other receptor-G-protein systems.

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