Rhodopsin–G-Protein Interactions Monitored by Resonance Energy Transfer†

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ABSTRACT: Resonance energy transfer measurements were implemented to monitor the specific interactions between G-protein and rhodopsin in phospholipid vesicles reconstituted with the purified proteins. Fluorescently labeled G-protein was extracted from bleached rod outer segments (ROS) reacted with several sulfhydryl reagents: *N*-(1-pyrenyl)maleimide (P), monobromobimane (B), 7-(diethylamino)-3-(4-maleimidylphenyl)-4-methylcoumarin (C), and *N*-(4-anilino-1-naphthyl)maleimide (A). Limited labeling of ROS, resulting in the modification of less than a single –SH residue per G-protein molecule and less than 0.2 residue per rhodopsin, did not impair the specific in situ interactions between rhodopsin and G-protein. This was demonstrated by preservation of their light-activated tight association and Gpp(NH)p binding and their fast dissociation with excess GTP. The distribution of fluorescent label among the three subunits of G-protein revealed a highly reactive –SH group in the γ subunit accessible to labeling when G-protein was bound specifically to bleached rhodopsin. Recombination of purified fluorescent derivatives of G-protein with purified rhodopsin reconstituted in lipid vesicles restored the light-activated Gpp(NH)p binding to a level comparable to that measured with unlabeled G-protein. Similar observations were obtained with ROS depleted of peripheral proteins. Likewise, modification of up to two –SH groups per rhodopsin molecule with the fluorescent reagents did not affect the functional recombination of G-protein with rhodopsin in reconstituted lipid vesicles or in depleted ROS. Interactions between rhodopsin and G-protein were monitored by resonance energy transfer measurements, with the following fluorescent conjugates as donor/acceptor couples: P–rhodopsin/C–G-protein, P–rhodopsin/B–G-protein, and P–G-protein/C–rhodopsin. Energy transfer was detected in bleached recombinants of these pairs, reflecting the binding of G-protein to membranes. Specific binding and nonspecific binding of G-protein were discerned by the reduction of energy transfer induced by an excess of (a) nonhydrolyzable analogues of GTP, which promote the dissociation of the rhodopsin–G-protein complex, and (b) unlabeled G-protein, which competes for binding sites in rhodopsin. To a first approximation, these measurements placed the labeled –SH groups of G-protein and rhodopsin 38–46 Å apart.

The fast and highly amplified activation of the cyclic-GMP phosphodiesterase in retinal rod cells by excited rhodopsin is a central process in visual transduction. The subsequent hydrolysis of cyclic GMP leads to the closure of the Na^+ channels in the photoreceptor plasma membrane [Fesenko et al., 1985; Yau and Nakatani, 1985; Haynes et al., 1986; Zimmerman & Baylor, 1986; reviewed by Stryer (1986)].

The photoactivation of the phosphodiesterase requires the presence of a GTP binding protein (G-protein).[1] The coupling role of the G-protein is achieved through a sequence of changes in the conformations and interactions of rhodopsin and G-protein. G-Protein binds with high affinity to photolyzed rhodopsin (Godchaux & Zimmerman, 1979; Kuhn, 1980), undergoes a conformational change (Fung & Nash, 1983; Halliday et al., 1984; Navon & Fung, 1984), and subsequently exchanges bound GDP for GTP (Godchaux & Zimmerman, 1979; Kuhn, 1980; Fung & Stryer, 1980). The activated G-protein–GTP complex dissociates from excited rhodopsin (Kuhn, 1980, 1984) and activates the phosphodiesterase (Yee & Liebman, 1978; Fung et al., 1981). The slow GTPase activity exhibited by G-protein (Godchaux & Zimmerman, 1979; Kuhn, 1980) returns it to the resting state.

Retinal G-protein consists of three subunits α, β, and γ, with apparent M_r of 39000, 35000, and 8000, respectively (Kuhn, 1980). All three subunits are necessary for the expression of GTP binding and GTPase activity (Shinozawa et al., 1980; Fung, 1983; Kuhn, 1984). α is the catalytic subunit and contains the nucleotide binding site (Fung et al., 1981; Fung, 1983), the GTPase activity (Fung, 1983; Fung & Nash, 1983), and the sites that are specifically ADP-ribosylated by both cholera and pertussis toxin (Abood et al., 1982; Van Dop et al., 1984b; Watkins et al., 1984; Navon & Fung, 1984, 1988). β and γ exist as a stable complex and participate in the association of G-protein with membranes and its interaction with photoexcited rhodopsin (Shinozawa et al., 1980; Fung, 1983; Kuhn, 1984). The retinal G-protein shows striking structural and functional resemblance to the regulatory G-proteins from other signal transducing systems [Shinozawa et al., 1979; Manning & Gilman, 1983; Tanabe et al., 1982; Medynski et al., 1982; Yatsunami et al., 1985; Hurley et al., 1984a,b; Sugimoto et al., 1985; Yatsunami & Khorana, 1985; Van Dop et al., 1984a; Ovchinikov et al., 1985; Sibley & Lefkowitz,

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[1] Abbreviations: ROS, rod outer segments; Rho, rhodopsin; G, G-protein (GTP binding protein); P, *N*-(1-pyrenyl)maleimide; C, 7-(diethylamino)-3-(4-maleimidylphenyl)-4-methylcoumarin; B, monobromobimane; A, *N*-(anilino-1-naphthyl)maleimide; P–Rho and C–Rho, rhodopsin labeled with P and C, respectively; P–G, C–G, B–G, and A–G, G-protein labeled with P, C, B, and A, respectively; DTT, dithiothreitol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P–PE, PE labeled with pyrene; PI, phosphatidylinositol; CL, cardiolipin; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; NaP_i, sodium phosphate; LDAO, lauryldimethylamine oxide.

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1985; reviewed by Stryer and Bourne (1986) and Gilman (1987)]. Moreover, bovine rhodopsin shows significant amino acid sequence homology with the mammalian β-adrenergic receptor (Dixon et al., 1986).

The interactions between G-protein and rhodopsin were studied by a variety of techniques [for reviews, see Stryer (1983), Kuhn (1984), and Chabre (1985)]. Information on structure/function relationships was derived from studies involving limited proteolysis (Kuhn & Hargrave, 1981; Fung & Nash, 1983; Halliday et al., 1984), ADP-ribosylation with toxins (Abood et al., 1982; Van Dop et al., 1984b; Navon & Fung, 1984), and -SH modification (Ho & Fung, 1984; Reichert & Hoffmann, 1985). Kinetic parameters of the interactions were indirectly assessed by light-scattering measurements (Kuhn et al., 1981; Lewis et al., 1984; Vuong et al., 1984) and rhodopsin spectroscopy (Emies et al., 1982; Bennett et al., 1982; Pfister et al., 1983).

Although much was revealed about the biochemical aspects of the interactions between rhodopsin and G-protein, a direct and detailed characterization of their structural parameters (e.g., the stoichiometry in the functional complex, the distances between unique sites on the interacting proteins) is missing. Resonance energy transfer provides an excellent tool to investigate structural and kinetic aspects of protein–protein interactions [for reviews, see Stryer (1978) and Fairclough and Cantor (1978)]. We report here an experimental system designed to perform resonance energy transfer measurements to directly monitor rhodopsin–G-protein interactions. We describe the preparation of functionally intact fluorescent derivatives of rhodopsin and G-protein and their purification and reconstitution. Resonance energy transfer measurements on recombinants of the purified components resolved the binding of G-protein to membranes and its specific dissociation in the presence of excess GTP or GTP analogues. A preliminary account of this work was presented elsewhere (Borochov-Neori, 1985).

Materials and Methods

Materials. Frozen dark-adapted retinas were purchased from J. A. Lawson Co. *N*-(1-Pyrenyl)maleimide (P) and 7-(diethylamino)-3-(4-maleimidylphenyl)-4-methylcoumarin (C) were from Molecular Probes. Dithiothreitol (DTT) and monobromobimane (B) were from Behring Diagnostics. *N*-(4-Anilino-1-naphthyl)maleimide (A) was from Fluka Chemical Corp. Egg phosphatidylcholine (PC) and bovine serum albumin (BSA, essentially fatty acid free) were from Sigma Chemical Co. Diphytanoyl-PC and bacterial phosphatidylethanolamine (PE) were obtained from Avanti-Biochemicals and Supelco, respectively. Pyrene-labeled PE (P-PE) was from Molecular Probes. Nucleotides were purchased from the following sources: GMP, GDP, GTP, and GppNHp were from Pharmacia P-L Biochemicals; GTPγS was from Boehringer Mannheim Biochemicals; [^{3}H]GppNHp was from ICN Biomedicals. All other chemicals were of analytical grade, and the organic solvents were of spectroscopic grade.

Buffer A (moderate ionic strength) contained 70 mM sodium phosphate (NaP_i), 2 mM DTT, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.0. Buffer B (low ionic strength) consisted of 5 mM Tris, 0.5 mM $MgCl_2$, 2 mM DTT, and 0.1 mM PMSF, pH 7.5. Buffer C (labeling medium) was 70 mM NaP_i, 1 mM $MgCl_2$, and 0.1 mM EDTA, pH 6.5 (for labeling with P, C, and A) or 8.0 (for labeling with B).

Assays. G-Protein concentration was determined by the method of Coomassie Blue binding (Bradford, 1976) using γ-globulin (Bio-Rad) as the standard. Rhodopsin concentration was determined spectroscopically from the light-sensitive absorbance at 498 nm with $\epsilon_{498} = 42\,700\ M^{-1}\ cm^{-1}$ (Hong & Hubbell, 1973). Fluorophore concentrations were measured spectroscopically with $\epsilon_{355}(A) = 13\,000\ M^{-1}\ cm^{-1}$ (Kanoaka et al., 1973), $\epsilon_{387}(C) = 29\,700\ M^{-1}\ cm^{-1}$ (Sippel, 1981), $\epsilon_{347}(P) = 34\,000\ M^{-1}\ cm^{-1}$ (Betcher-Lange & Lehrer, 1978), and $\epsilon_{383}(B) = 4500\ M^{-1}\ cm^{-1}$ (Kosower et al., 1979). These values were used to calculate the number of modified -SH groups per mole of protein.

SDS–polyacrylamide gel electrophoresis was performed with continuous 2.5–16% gradient gels (Isolab). For gels designated to resolve fluorescent protein bands, samples were dissolved at room temperature for 15 min prior to application to the gel. Following electrophoresis, the gel was photographed without further treatment. Gels were illuminated with a Hg lamp and photographed through a yellow filter (Corning 3-73) with Polaroid 667 film. The gels were then fixed and stained with Coomassie Blue to identify the fluorescent protein bands. When gels were used only to resolve protein bands, samples were dissolved by immersing them in boiling water for 3 min. Following electrophoresis, the gels were immediately fixed and stained.

[^{3}H]GppNHp binding was measured in triplicate in buffer A under the conditions detailed in the legends to figure 1 and Table I. A stock solution of 0.1 mM [^{3}H]GppNHp, 60 000 cpm/μL, was used. Depending on assay conditions, between 60 000 and 600 000 cpm was used per assay. The reaction was carried out for periods sufficiently long to reach saturation and stopped by a 10-fold dilution in ice-cold buffer A. The suspensions were, then, filtered through a 0.45-μm type HA Millipore filter. Filters were washed three times with ice-cold buffer B, and radioactivity was counted. Background count (e.g., nonspecific binding to the filter or to heat-inactivated ROS or to G-protein in the absence of rhodopsin) was always ≤10% of total counts, amounted to 0.6–1.0 pmol of [^{3}H]-GppNHp, and was subtracted to calculate specific nucleotide binding.

Membrane Preparations. All steps were carried out under dim red light at 4 °C. Rod outer segments (ROS) were purified from frozen dark-adapted retinas on a sucrose gradient following the protocol of McDowell and Kuhn (1977). The yield was typically 0.8 mg of rhodopsin per retina. Membranes had an absorption ratio (278 nm/498 nm) of 2.7–2.9 and exhibited a light-enhanced binding of [^{3}H]GppNHp. Light activation of GppNHp binding was 8-fold when ROS (0.9 μM rhodopsin) were incubated with [^{3}H]GppNHp (1 μM) for 10 min on ice. Binding was 1.6 and 12.4 mmol of GppNHp/mol of rhodopsin in dark-kept and in bleached membranes, respectively. GppNHp binding saturated at 36 mmol of bound nucleotide per mole of rhodopsin (10 μM rhodopsin, 5 μM [^{3}H]GppNHp, 30 min at room temperature). ROS were stripped of peripheral proteins by extensive washings with buffer B. Both ROS preparations were stored as pellets under N_2 at −70 °C until further use.

Preparation, Purification, and Reconstitution of Unmodified and Fluorescent Derivatives of Rhodopsin. All steps were carried out under dim red light at 4 °C. Stripped ROS were washed extensively in buffer C, pH 6.5. Labeling with P and C, rhodopsin purification, and reconstitution were performed as previously described (Borochov-Neori & Montal, 1983). Unless otherwise stated, rhodopsin was reconstituted at a 1:100 mole ratio with a mixture of diphytanoyl-PC and bacterial PE (1:1 w/w).

Preparation and Purification of Fluorescent Derivatives of G-Protein. ROS were resuspended and washed twice in buffer

A (0.5 mg of rhodopsin/mL) under dim red light at 4 °C. Membranes were then resuspended in buffer B (0.5 mg of rhodopsin/mL) and bleached on ice in room light for 10 min. ROS were sedimented (40000g, 20 min) and washed twice with buffer B. ROS pellets were resuspended in buffer A (1 mg of rhodopsin/mL) and recentrifuged. Special care was taken to remove as much supernatant as possible to minimize the content of DTT during the subsequent labeling step. (It is worth noting that extensive removal of DTT by washing ROS in the absence of DTT decreased significantly the yield of extracted G-protein). ROS were resuspended in buffer C (0.3–0.5 mg of rhodopsin/mL), and a stock solution of fluorescent reagent (5–10 mM in acetonitrile or dimethylformamide) was added (final solvent content was less than 1%). The reaction proceeded for 15 min at room temperature in the dark and was terminated with 5 mM DTT. Labeled ROS were sedimented and washed three times by centrifugation in buffer B (0.5 mg of rhodopsin/mL). G-Protein was extracted by incubation with 0.1 mM GTP in buffer B (1.5 mg of rhodopsin/mL) followed by centrifugation. The extraction was repeated three times, and the supernatants were combined. The extract was concentrated, and residual-free fluorescent reagent as well as GTP was removed with Centricon-10 microconcentrators (Amicon). Purified G-protein (2 mg/mL) was supplemented with glycerol (25–40%) and stored in liquid N_2 until further use (1–10 days). Purification of unlabeled G-protein followed the same protocol with the omission of the labeling steps. The extraction yield was typically 50 μg of G-protein/mg of rhodopsin.

Steady-State Fluorescence Measurements. All fluorescence measurements were carried out in a Perkin-Elmer, MPF-4, spectrofluorometer equipped with a differential corrected spectra unit, polarization attachement, and thermostated cell holder with a magnetic stirrer. Recombinants of unlabeled rhodopsin-containing membranes and G-protein were used to correct for scattering and background contributions to the measured emission intensities. Small cuvettes of (3×3 mm^2) were used to reduce the optical density to ≤0.06 and, thus, eliminate inner-filter effects. All spectra were recorded at 15 °C. Quantum yields of bound chromophores were determined by comparison with the quantum yield of quinine sulfate (0.70 in 0.1 N H_2SO_4; Scott et al., 1970).

Resonance Energy Transfer—Preparation of Recombinants and Measurements. All solutions and suspensions were supplemented with freshly prepared 1 mM DTT and made 2 mM in $MgCl_2$ and 0.1 mM in EGTA. Unless otherwise specified, stripped ROS (10 μg of rhodopsin) or reconstituted vesicles (20 μg of rhodopsin) were incubated with 20 μg of G-protein for 1 h on ice in the smallest volume possible (0.5–3 mg of total protein/mL). BSA (1 mg/mL) was included to reduce nonspecific absorption of G-protein to membranes. Salt concentration was adjusted to the desired level with 1 M KCl. Samples were activated by exposure to room light for 10 min (on ice) and then diluted with >5-fold buffer B and centrifuged. After being washed twice with buffer B to remove unbound G-protein, recombinants were resuspended and their fluorescence spectra recorded. Recombinants in which either rhodopsin or G-protein was labeled were used to evaluate the fluorescence spectra expected in the absence of resonance energy transfer. Protein content and composition of recombinants were estimated from the corresponding fluorescence intensities measured following solubilization with 3% lauryldimethylamine oxide (LDAO). Fluorescence intensities measured for known concentrations of labeled proteins were used for calibration. Energy transfer was determined either by fluorescence excitation spectra (to detect sensitized fluorescence of the acceptor) or by fluorescence emission spectra (to detect donor quenching) [for reviews, see Stryer (1978) and Fairclough and Cantor (1978)].

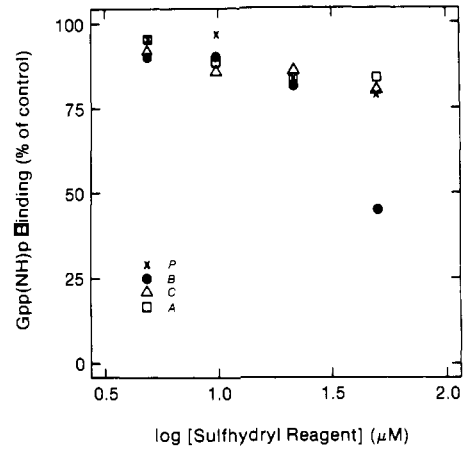

FIGURE 1: Effect of ROS labeling on GppNHp binding. ROS were labeled, washed, and used immediately. Membranes (12.5 μM rhodopsin) were supplemented with [^{3}H]GppNHp (10 μM) and incubated on ice for 40 min. In control ROS, the labeling steps were omitted.

Results and Discussion

Preparation of Fluorescent Protein Conjugates

Labeling Conditions. Labeling conditions were optimized to obtain functional G-protein with less than a single –SH group modified (on the average). Figure 1 describes the effect of reacting ROS with several sulfhydryl reagents on the expression of the light-activated GppNHp binding. Incubation of membranes with up to 50 μM reagent reduced the nucleotide binding by <20%, with the exception of mBB. Note that the membrane suspensions contained residual DTT, which determined the effective concentration of the sulfhydryl reagent. Purification of rhodopsin and G-protein from the modified ROS revealed that reaction with 50 μM reagent resulted in the labeling of 0.4–1.0 –SH group per G-protein molecule and less than 0.2 –SH residue per rhodopsin molecule, on the average. The extraction yield of G-protein labeled with <1.0 fluorophore per protein molecule was equivalent to that of unlabeled G-protein. Thus, in the extractable G-protein, the modification did not impair the specific tight binding of G-protein to light-activated rhodopsin, the GTP/GDP exchange step, and the subsequent release of G-protein in soluble form. More extensive modification of –SH groups reduced significantly both GppNHp binding by labeled membranes and the extraction yield of G-protein. Reacting dark-kept, instead of bleached, ROS with up to 50 μM fluorescent reagents (results not shown) did not affect the ionic-strength-dependent association of G-protein with the membranes but impaired the interaction with bleached rhodopsin, in agreement with Hoffman and Reichert (1985).

Fluorescence Properties of G-Protein and Rhodopsin Conjugates. Table I summarizes the steady-state fluorescence properties of the fluorescent derivatives of G-protein and rhodopsin. The fluorescence spectra of the P, B, and C derivatives of G-protein and rhodopsin are similar to those of other protein conjugates (Sippel, 1981; Betcher-Lange & Lehrer, 1978; Kosower et al., 1979). The emission maximum exhibited by A–G-protein is indicative of the highly hydrophobic nature of the immediate environment of the modified –SH groups (Kanaoka et al., 1973).

Table I: Spectral and Biochemical Properties of the Fluorescent Derivatives of Rhodopsin and G-Protein

protein derivative	GppNHp binding[a]	spectral properties			
		exc (nm)[b]	em (nm)[c]	*P* free	*P* bound
unlabeled G	0.50[d]				
C–G	0.40	390[e]	465	0.27[f]	0.30[g]
B–G	0.38	390	465	0.12	0.15
P–G	0.45	342	373	0.09	0.16
A–G	0.46	355	430	0.24	0.27
Rho vesicles	0.43[h]				
C–Rho vesicles	0.48	390	465	0.30[i]	0.35[j]
P–Rho vesicles	0.46	342	373	0.25	0.25
C–ROS	0.50	390	465	0.27	0.27
P–ROS	0.48	342	373	0.17	0.18

[a] Expressed in mole of bound nucleotide per mole of G-protein. [b] Wavelength of maximal excitation. [c] Wavelength of maximal emission. [d] Binding assay: Stripped ROS (6.25 μM Rho) and purified G (1.6 μM) were incubated for 2 h in room light on ice (0.2 M NaCl, 14 mM Tris, 2 mM $MgCl_2$, 2 mM DTT, 5% glycerol, pH 7.5); 10 μM [^{3}H]GppNHp was added, and binding was allowed to proceed for 20 min at room temperature. [e] Excitation and emission maxima were not significantly affected by recombination. [f] Fluorescence polarization (*P*) of 0.1 mg/mL purified G derivative in 5 mM Tris, 0.5 mM $MgCl_2$, 2 mM DTT, 1 mg/mL BSA, and 5% glycerol, pH 7.5, room temperature. [g] *P* after incubation with bleached ROS (0.15 mg of rhodopsin/mL) for 2 h on ice. [h] Binding assay was as in footnote *d*. Unlabeled G was used. [i] Measured in bleached membrane preparations (0.1 mg of rhodopsin/mL). Other conditions were as in footnote *f*. [j] As in footnote *i* but preparations were first incubated with purified G for 2 h on ice.

The degree of fluorescence polarization of the fluorophores attached to G-protein increased in the presence of membranes, suggesting a more restricted motion (presumably arising from the binding of G-protein to membranes) since the fluorescence spectra and quantum yields were not affected. The degree of polarization of the fluorophores attached to rhodopsin did not change upon addition of G-protein.

Label Distribution among Subunits of G-Protein. Figure 2 shows SDS–polyacrylamide gels of purified C–G-protein: Lane a presents a typical protein-band resolution in a gel which was fixed and stained with Coomassie Blue immediately following electrophoresis. Lane b shows the fluorescence intensities associated with the coresponding protein bands of lane a. Unlike lane a, the bands in lane b appear diffuse. This is most probably the outcome of the conditions experienced by the gel when photographed under UV illumination. Indeed, Coomassie Blue staining of the gel (lane c and molecular weight standards on the right-hand side of the figure) revealed diffuse bands. The band corresponding to the γ subunit was hardly detectable. Since the same preparation was analyzed in lanes a, b, and c, γ subunit was clearly present in all three lanes. The fluorescence emission associated with it was intense and detectable even when the band was highly diffuse (lane b). Coomassie Blue binding, on the other hand, was not as sensitive, and it was hard to detect γ in lane c. Gels, similar to those presented in Figure 2, were obtained for the other G-protein derivatives. It is clear that labeling was not limited to a single site. Half of the fluorescence intensity was associated with the γ subunit and the other half distributed between α and β. Previous studies on the limited modification of G-protein –SH groups showed that labeling was restricted to α and led to inhibition of the light activation of G-protein by rhodopsin (Ho & Fung, 1984; Hoffman & Reichert, 1985). Labeling was performed on either soluble purified G-protein (Ho & Fung, 1984) or dark-kept ROS (Hoffman & Reichert, 1985). The accessibility of the γ subunit to –SH modification, observed only in our studies, may, thus, reflect a specific conformation of G-protein when bound to bleached rhodopsin.

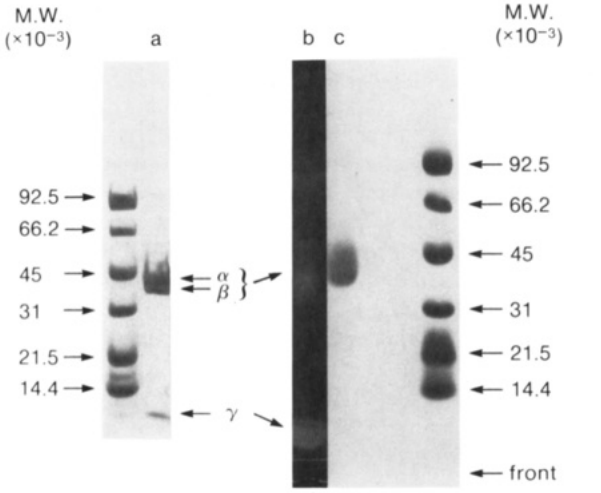

FIGURE 2: SDS–polyacrylamide gels of purified C–G-protein. Gels presented are continuous (2.5–16%) gradient gels. Lane a (30 μg of C–G-protein) and the molecular weight standards (Bio-Rad) on the left were taken from a gel which was fixed and stained with Coomassie Blue immediately following electrophoresis. Lanes b and c (60 μg of C–G-protein, each) as well as the molecular weight standards on the right were taken from a gel which was first photographed under UV excitation (lane b) and then stained with Coomassie Blue (lane c).

Light-Activated GppNHp Binding with the Fluorescent Conjugates of Rhodopsin and G-Protein. (i) Rhodopsin in reconstituted vesicles recombined with purified G-protein: As shown in Table I, recombination of rhodopsin-containing vesicles with purified G-protein restored the specific binding of GppNHp. GppNHp binding was light activated. When rhodopsin vesicles (6 μM) were recombined with purified G-protein (1 μM) and incubated with GppNHp (1 μM) on ice for 10 min, 70 nmol of GppNHp was bound per mole of G-protein in dark-kept recombinants, whereas 350 nmol of GppNHp was bound per mole of G-protein in bleached recombinants, i.e., a 5-fold increase in GppNHp binding upon bleaching. Similar light dependence was obtained with labeled purified G-protein (results are not shown). Labeling rhodopsin with –SH reagents had no effect on the extent of binding in bleached recombinants (approximately 0.45 mol of GppNHp/mol of G-protein, see Table I). The reconstitution efficiency was independent of vesicle lipid composition [e.g., partially purified asolectin (Kagawa & Racker, 1971), equimolar mixtures of diphytanoyl-PC or egg PC and bacterial PE, diphytanoyl-PC or egg PC only]. (ii) Stripped ROS combined with purified G-protein: Recombination of stripped ROS with purified G-protein resulted in the binding of 0.5 mol of GppNHp per mol of G-protein (see Table I). In the range studied, of 0.1–0.4 mole ratio of G-protein to rhodopsin, [^{3}H]GppNHp binding per G-protein was constant (i.e., nucleotide binding was proportional to the amount of G-protein added). The recombinants exhibited light activation of GppNHp binding similar in magnitude to that measured with reconstituted rhodopsin vesicles. Modification of ROS with sulfhydryl reagents prior to recombination (resulting in the blockade of up to two –SH groups per rhodopsin) did not affect nucleotide binding. This is in agreement with results obtained with reconstituted purified rhodopsin derivatives (Table I) and with Kohnken et al. (1981) and Hoffman and Reichert (1985). As shown in Table I, purified fluorescent conjugates of G-protein were 15–25% less effective in restoring GppNHp binding.

To summarize, limited modification of –SH groups in rhodopsin and G-protein did not impair their specific, light-

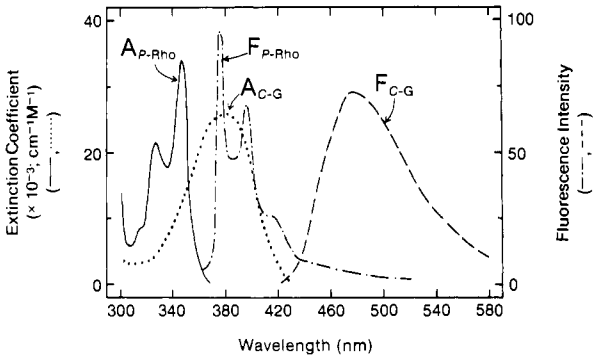

FIGURE 3: Pyrene and coumarin conjugates of rhodopsin and G-protein are suitable donor/acceptor couples for resonance energy transfer measurements. Absorption (—) (···) and fluorescence emission (–·–) (– –) spectra of pyrene and coumarin, respectively, conjugated to rhodopsin (P–Rho) and G-protein (C–G). Spectra of extracted G-protein conjugates and of bleached purified rhodopsin conjugates in octyl glucoside were recorded. The absorption spectrum of an analogous preparation of bleached purified unlabeled rhodopsin was subtracted to produce the net absorption spectrum of bound pyrene. Emission spectra were obtained with excitation at 342 and 380 nm for pyrene and coumarin, respectively, slits of 4 nm being used.

dependent interactions in situ. Clearly, one or more reactive –SH groups in G-protein are not involved directly in the specific interactions with rhodopsin. One of these groups is located on the γ subunit. The specific modification of the latter required preexposure of membranes to light. The affinity of modified G-protein for bleached rhodopsin was comparable to that of the unlabeled protein, as shown by the resistance to removal by low ionic strength and the similar extraction yields in the presence of excess GTP. Rhodopsin modification did not affect the interactions of the purified proteins. However, restoration of the light-activated GppNHp binding with purified labeled G-protein was incomplete.

Resonance Energy Transfer Measurements

Monitoring Interactions in Recombinants. The fluorescent protein conjugates were used as donor/acceptor energy couples in the resonance energy transfer experiments. Absorption and fluorescence emission spectra of P–rhodopsin and C–G-protein are illustrated in Figure 3. The corresponding spectra of P–G-protein and C–rhodopsin are indistinguishable from those presented in the figure. Clearly, the fluorophores are suitable for resonance energy transfer measurements, with the pyrene derivatives as energy donors and the coumarin derivatives as acceptors. Using a quantum yield of 0.5 for the coumarin derivatives, we calculated $R_0(2/3)$ = 39 Å, where $R_0(2/3)$ is the distance of 50% energy transfer at random orientation. As we showed earlier (Borochov-Neori et al., 1983), pyrene and bimane are also a useful donor/acceptor couple for energy transfer with a calculated $R_0(2/3)$ = 30 Å. To summarize, pyrene-derived conjugates, P–rhodopsin in either reconstituted vesicles or labeled membranes, and P–G-protein were used as energy donors, whereas C– and B–G-protein as well as C–rhodopsin served as energy acceptors.

Fluorescence excitation spectra of recombinants of P–rhodopsin in reconstituted vesicles or stripped ROS and C–G-protein are shown in Figure 4. These spectra are compared to the calculated sum of the spectra of each derivative under analogous experimental conditions, but in the absence of the other derivative. The spectra of recombinants clearly deviated from the corresponding sums of individual spectra. When the emission of coumarin at 465 nm was monitored, increased intensities were recorded at excitation wavelengths coinciding with the absorption maxima of pyrene, i.e., 327 and 347 nm.

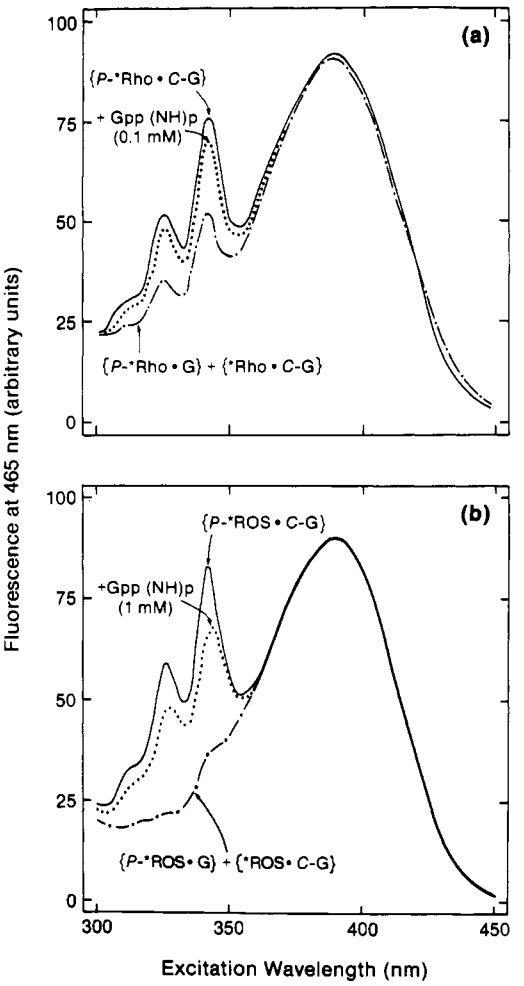

FIGURE 4: Fluorescence excitation spectra of recombinants of P–rhodopsin with C–G-rhodopsin. Spectra of recombinants in the absence (—) and in the presence (···) of GTP analogues. Calculated sum of individual spectra of P–rhodopsin (P-Rho) or P–ROS and of C–G-protein (C–G) (–·–). (a) P–rhodopsin vesicles + C–G-protein: P–rhodopsin, 0.15 mg of rhodopsin/mL, 1.5 pyrene moieties per protein; C–G-protein, 0.050 mg of protein/mL, 0.6 coumarin moiety per protein. (b) P–ROS + C–G-protein: P–ROS, 0.1 mg of rhodopsin/mL, 1.2 pyrene moieties per protein; C–G-protein, 0.025 mg of protein/mL, 0.8 coumarin moiety per protein.

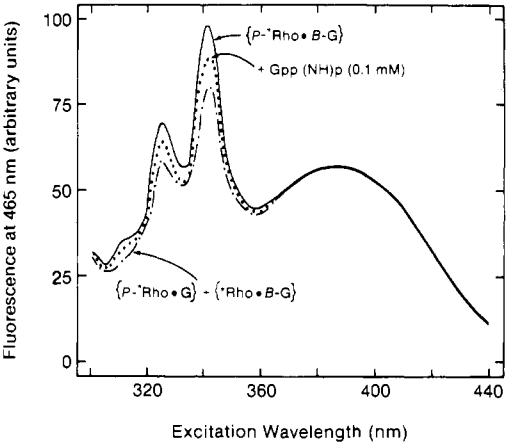

FIGURE 5: Fluorescence excitation spectra of recombinant of P–rhodopsin vesicles and B–G-protein. P–rhodopsin, 0.1 mg of rhodopsin/mL, 1 pyrene moiety per protein; B–G-protein (B–G), 0.050 mg of protein/mL, 1 bimane moiety per protein. Other conditions were as for Figure 4.

Similar spectra and deviations were obtained with recombinants of P–rhodopsin vesicles and B–G-protein (Figure 5).

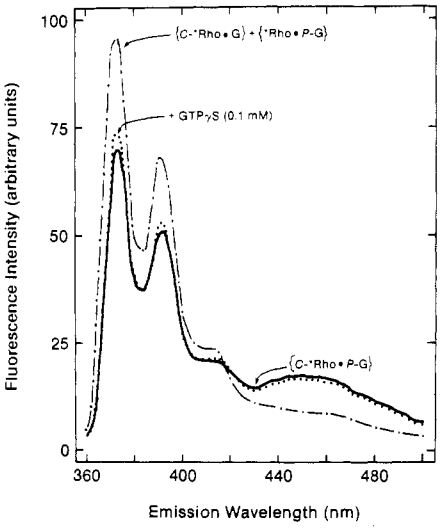

FIGURE 6: Fluorescence emission spectrum of recombinants of C–rhodopsin vesicles with P–G-protein. Emission spectra of recombinants in the absence (—) and the presence (···) of GTPγS. Calculated sum of emission spectra of C–rhodopsin (C–Rho) and P–G-protein (P–G) (–·–). C–rhodopsin, 0.1 mg of rhodopsin/mL, 0.8 coumarin moiety per protein; P–G-protein, 0.05 mg of protein/mL, 1 pyrene moiety per protein. Samples were excited at 342 nm with excitation and emission slits of 4 and 6 nm, respectively.

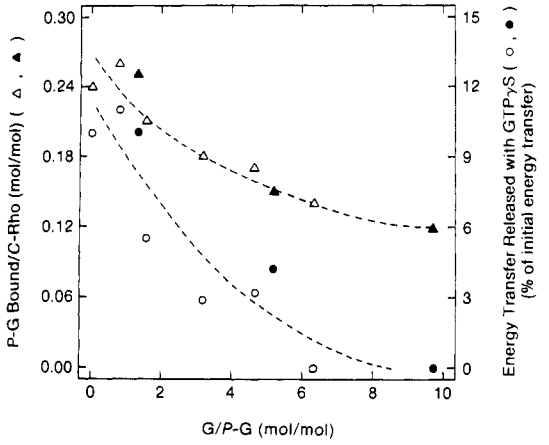

FIGURE 7: Competition of G-protein and P–G-protein for specific and nonspecific binding sites on C–rhodopsin vesicles. Quantitative assay of labeled proteins was done by measuring fluorescence intensities as described under Materials and Methods. Energy transfer was calculated from the quenching of pyrene fluorescence. Empty and full symbols refer to different preparations. Each experimental point is the average of a duplicate. Other conditions were as for Figure 6.

These results reflect the occurrence of energy transfer.

In analogous experiments, C–rhodopsin in reconstituted vesicles was supplemented with P–G-protein. Figure 6 illustrates the emission spectra. Energy transfer was detected, as manifested by the decrease in pyrene fluorescence intensity at 373 and 392 nm when the recombinant was excited at 347 nm. A concomitant increase in coumarin fluorescence at 465 nm was recorded.

The occurrence of energy transfer indicates that the added G-protein was bound to rhodopsin vesicles and to ROS and that a fraction of G-protein molecules were in close proximity to rhodopsin. The extent of energy transfer with P–rhodopsin vesicles (Figure 4a) was routinely lower than that measured with P–ROS (Figure 4b). Unlike ROS, rhodopsin in reconstituted vesicles is symmetrically oriented (Fung & Hubbell, 1978). Half of the rhodopsin molecules are, therefore, in the wrong orientation for the interaction with G-protein, with their fluorescent residues facing the inner surface of the membrane, effectively reducing the measured energy transfer. In addition, a significant fraction of the fluorescence intensity measured in P–ROS was associated with the lipid phase. Close proximity of the labeled residues of specifically bound G-protein to the membrane lipid phase and nonspecific adsorption of G-protein to lipid are expected to contribute to the observed energy transfer.

When P–rhodopsin vesicles (4 μM) were incubated with C–G-protein at a ratio of 0.05–1.5 mol of G-protein/mol of rhodopsin, the measured energy transfer increased in a saturable manner. Half of the maximal energy transfer was measured at a ratio of approximately 0.35 mol of G-protein/mol of rhodopsin. Maximal energy transfer was already approached at an equimolar ratio of the proteins in the mixture and corresponded to the actual binding of 0.25 and 0.34 mol of G-protein/mol of P–rhodopsin reconstituted with phospholipids at a mole ratio of 1:50 and 1:100, respectively. Maximal binding of purified unlabeled G-protein to bleached stripped ROS, as determined by protein assays, was 0.25 mol of G-protein/mol of rhodopsin, in agreement with Kohnken et al. (1981), who attributed this extent of binding to the available membrane surface. In the experiments shown in Figures 4–6, G-protein binding was below saturation (G-protein/rhodopsin mole ratio of 0.12 for ROS and 0.25 for rhodopsin reconstituted with lipid at a mole ratio of 1:100).

Specific Interactions between G-Protein and Rhodopsin. (*1*) *Effects of Guanosine Nucleotides.* A fraction of the observed energy transfer (10–40%) was reversed by addition of GppNHp (Figures 4–6), GTP, or GTPγS. The residual energy transfer was removed only by solubilizing the recombinants (3% LDAO), suggesting that it originated from nonspecific associations of G-protein to membranes. Alternatively, conformational modifications introduced to the labeled G-protein inhibited its fast dissociation from bleached membranes in the presence of excess GTP and analogues. Since energy transfer was observed in recombinants devoid of rhodopsin (using phospholipid vesicles containing P–PE as the energy donor), it is likely that in the recombinants of G-protein and rhodopsin a fraction of the measured energy transfer reflected nonspecific adsorption of G-protein to membranes. Attempts to reduce nonspecific binding by inclusion of BSA and by low ionic strength were partially successful (results are not shown).

(*2*) *Effect of Unlabeled G-Protein.* To investigate if nonspecific binding of G-protein to membranes was limited to the modified, fluorescently labeled G-protein, the effect of supplementing increasing amounts of unlabeled protein in the recombination step was determined on (i) the extent of binding of P–G-protein to reconstituted C–rhodopsin vesicles and (ii) the fraction of the energy transfer sensitive to the addition of GTPγS. The results are summarized in Figure 7. Clearly, unlabeled G-protein competed with P–G-protein for both specific (GTPγS sensitive) and nonspecific binding sites on C–rhodopsin vesicles. However, unlabeled protein exhibited higher specificity, as it was more effective in replacing P–G-protein from specific than from nonspecific sites. Thus, following purification, modified G-protein was more labile than unlabeled protein. The structural origin and the biological relevance of such a labile conformation remain to be investigated, but it could be related to an additional mechanism of adaptation, as suggested for the G-protein of the hormone receptor/adenylate cyclase system (Sibley & Lefkowitz, 1985).

Experiments such as that presented in Figure 6 were used to estimate, to a first approximation, the distance between labeled sites of rhodopsin and G-protein in a functional complex. Addition of excess GTPγS, to remove all the specific

energy transfer (10–12% of the total energy transfer), was accompanied by the release of approximately 7% of the bound P–G-protein (as measured from the fluorescence of solubilized recombinants). Since the total energy transfer was 26%, the efficiency associated with specific binding was ~40%. Assuming 1:1 binding of P–G-protein to C–rhodopsin, uniform labeling of P–G-protein and of C–rhodopsin, and random orientation of the fluorophores, the labeled site of the specifically bound G-protein is estimated to be located approximately 42 Å away from the surface of rhodopsin. The residual energy transfer (GTPγS insensitive) may originate from a different mode of attachment of P–G-protein to C–rhodopsin and/or from P–G-protein bound to lipid and randomly distributed on the membrane surface (Fung & Stryer, 1978).

To summarize, specific interactions between rhodopsin and G-protein were investigated in recombinants of the purified proteins, employing fluorescence energy transfer measurements to monitor changes in proximity. Functional fluorescent derivatives of rhodopsin and G-protein were prepared, rhodopsin was reconstituted into lipid vesicles, and G-protein was added. Reconstitution of specific interactions between fluorescent conjugates of rhodopsin and G-protein was achieved. Energy transfer measurements were highly sensitive and reproducible. Specific binding was readily discerned by monitoring the effect of excess guanosine nucleotides. However, nonspecific binding hampered the quantitative evaluation of the structural parameters of rhodopsin–G-protein interactions. Refinement of the preparation procedures should allow the rigorous use of energy transfer measurements. Considering the close relationship between the retinal system and that of the hormone receptor/adenylate cyclase, the strategy described here may be of general use in studies of membrane signal transduction.

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Registry No. GppNHp, 34273-04-6; GTP, 86-01-1; GTPγS, 37589-80-3; diphytanoyl-PC, 64626-70-6.

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Selective Labeling of the Erythrocyte Hexose Carrier with a Maleimide Derivative of Glucosamine: Relationship of an Exofacial Sulfhydryl to Carrier Conformation and Structure†

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ABSTRACT: Sulfhydryl-reactive derivatives of glucosamine were synthesized as potentially transportable affinity labels of the human erythrocyte hexose carrier. *N*-Maleoylglycyl derivatives of either 6- or 2-amino-2-deoxy-D-glucopyranose were the most potent inhibitors of 3-*O*-methylglucose uptake, with concentrations of half-maximal irreversible inhibition of about 1 mM. Surprisingly, these derivatives were very poorly transported into erythrocytes. They reacted rather with an exofacial sulfhydryl on the carrier following a reversible binding step, the latter possibly to the exofacial substrate binding site. However, their reactivity was determined primarily by access to the exofacial sulfhydryl, which, as predicted by the one-site model of transport, required a carrier conformation with the exofacial substrate binding site exposed. Once reacted, the carrier was "locked" in a conformation unable to reorient inwardly and bind cytochalasin B. In intact erythrocytes the *N*-maleoylglycyl derivative of 2-[^{3}H]glucosamine labeled predominantly an M_r 45 000–66 000 protein on gel electrophoresis in a quantitative and cytochalasin B inhibitable fashion. By use of changes in carrier conformation induced by competitive transport inhibitors in a "double" differential labeling method, virtually complete selectivity of labeling of the carrier protein was achieved, the latter permitting localization of the reactive exofacial sulfhydryl to an M_r 18 000–20 000 tryptic fragment of the carrier.

A variety of poorly permeant (VanSteveninck et al., 1965; Krupka, 1985a) or impermeant (Batt et al., 1976; Roberts et al., 1982) sulfhydryl reagents have been shown to irreversibly inhibit hexose transport in the human erythrocyte, probably by reacting with an external or exofacial sulfhydryl group on the carrier protein (Abbott & Schachter, 1976; Roberts et al., 1982). The enhanced selectivity of such otherwise nonspecific reagents for this sulfhydryl results from their impermeant nature, from their progressive covalent chemical reactivity, and from the fact that their access to the exofacial sulfhydryl can be manipulated by modifying the conformation of the carrier with competitive transport inhibitors. With regard to

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