Rhodopsin–Detergent Micelles Aggregate upon Activation of Cyclic Guanosine Monophosphate Phosphodiesterase

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ABSTRACT: In the presence of G protein and phosphodiesterase, GTP induces aggregation of phospholipid-free rhodopsin–detergent micelles or rhodopsin reconstituted in phospholipid vesicles. The net electrical charge of the vesicle is not critical to the aggregation process since this phenomenon is not altered by reconstitution with phospholipids with different charge. The aggregation process is observed by monitoring changes in the light-scattering properties of the detergent micelles or vesicle suspension and by phase-contrast microscopy. The lowest light intensity which triggers the aggregation process and concomitant light-scattering changes in a rhodopsin–detergent micellar suspension bleaches 6% rhodopsin. Under these conditions, the signal saturates at 30% rhodopsin bleaching. The aggregation process appears likely to depend on the protein–protein interaction, and the presence of a disk membrane is not necessary for this process.

In disk membrane suspensions, light-dependent changes in turbidity have been reported. Most of these turbidity changes require the presence of nucleotides, G protein,[1] and PDE and appear to be related to changes in the state of activation of G protein or PDE (Bignetti et al., 1980; Kuhn et al., 1981; Lewis et al., 1984; Kamps et al., 1985; Wagner et al., 1987; Bennet & Sytaramaya, 1988).

In previous reports (Caretta & Stein, 1985, 1986, 1987), we described reversible aggregation of photoreceptor disks elicited by rhodopsin photoisomerization. The signal studied required G protein, PDE, GTP, and bleached rhodopsin and was enhanced by cGMP. The change in the aggregation state of disk vesicles resulted in a light-scattering increase of the disk vesicle suspension, thus allowing us to follow the kinetics of this process.

In our earlier work, it was not possible to determine the physical mechanism responsible for the aggregation process. The studies reported below were designed to reveal the contribution of the membrane surface to the aggregation process and thus to allow greater understanding of the molecular basis of the aggregation process.

The data we obtained show that the aggregation process can be obtained regardless of the membrane charge and structure.

We were able to observe turbidity changes and formation of aggregates when purified rhodopsin in detergent micelles (without any phospholipid) was reconstituted with G protein, PDE, and the appropriate nucleotides.

For these reasons, we suggest that the disk aggregation process is the result of specific protein interactions and does not require (although kinetics are enhanced by) the presence of disk membrane.

Materials and Methods

Disk membranes were prepared from fresh bovine retinas as previously described (Caretta & Stein, 1985).

G protein and PDE were extracted with low ionic strength washings according to Kuhn (1980). G protein was washed free of GTP (or free of GTPγS) and concentrated by pressure on an Amicon filter to a final concentration of 15 μM. G–GTPγS was prepared by extracting G protein with GTPγS. PDE was similarly concentrated on an Amicon filter to a final concentration of 2.5 μM. No further purification of PDE was made. After concentration on the Amicon filter, the enzyme suspensions were centrifuged at 250000*g* for 1 h on a Beckman SW 65 rotor, 2 times to remove every possible contamination from disk membrane vesicles. At the end of the second centrifugation, no pellet could be detected in the tube.

Rhodopsin was purified by affinity chromatography on a Pharmacia concanavalin–Sepharose column. Disk membranes (0.8 mg of rhodopsin/mL) were solubilized in a solution containing (30 mM), Tris-HCl, pH 7.8, ammonium sulfate (195 mM), sodium cholate (35 mM), and phenylmethanesulfonyl fluoride (100 μM) (solubilization solution). The solution was centrifuged at 200000*g* for 1 h on an SW 65 rotor. No rhodopsin could be detected in the pellet. The supernatant was collected, and the absorption spectrum was recorded against the solubilization solution. The ratio A_{280}/A_{500} was 1.95 ± 0.1 (five preparations); the A_{256} was consistently lower than A_{500}. Rhodopsin was absorbed on a concanavalin A column (internal diameter = 1 cm, height = 4 cm, 5 mg of rhodopsin/column) at room temperature. Flux rate through the column was 200 μL/min. After absorption of rhodopsin, the column was washed with 15 mL of solubilization solution, and rhodopsin was eluted with 8 mL of solubilization solution containing 200 mM methyl mannoside. Concanavalin-purified rhodopsin was concentrated on an Amicon CF 25 filter at a final concentration of 10 mg/mL. After purification, the A_{280}/A_{500} ratio was 1.7 ± 0.08 (six preparations).

Organic phosphorus measurements on concanavalin A purified rhodopsin were made according to Bartlett (1959).

For reconstitution experiments, a chloroform–methanol (1/1) phospholipid solution (100 mg of phospholipids/mL) was dried on a Rotavapor. The phospholipids were resuspended at 15 mg/mL in a solution containing 2.5% sodium cholate, ammonium sulfate (192 mM), and Tris-HCl (30

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[1] Abbreviations: G protein, three polypeptide subunits of molecular masses 39, 37, and 6 kDa which comprise the GTPase; PDE, three polypeptide subunits of 94, 92, and 13 kDa which comprise phosphodiesterase; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium doecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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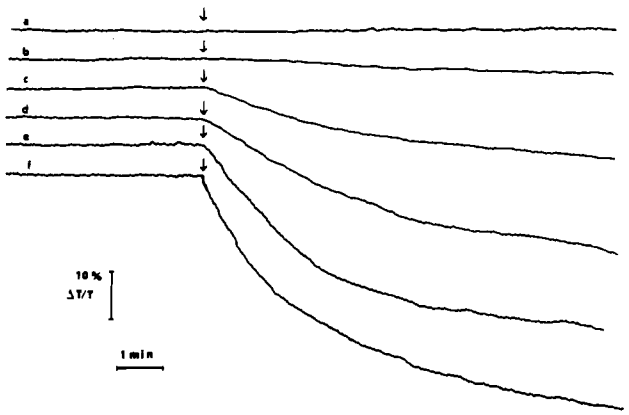

FIGURE 1: Turbidity changes in a micellar solution of rhodopsin reconstituted with different amounts of G protein and PDE. [Rhodopsin] = 5 μM; [cholic acid] = 0.5%. (Trace a) [G protein] = 5 μM, no PDE; (trace b) no G protein, [PDE] = 1 μM; (trace c) [G protein] = 5 μM, [PDE] = 400 nM; (trace d) [G protein] = 5 μM, [PDE] = 600 nM; (trace e) [G protein] = 5 μM, [PDE] = 1 μM; (trace f) [G protein] = 5 μM, [PDE] = 1.3 μM. Recording beam L = 308 nm; [GTPγS] = 10 μM (addition indicated by the arrow). Rhodopsin was fully bleached before the experiment was started.

mM), pH 7.7. The phospholipid suspension was mixed with concanavalin A purified rhodopsin at the appropriate ratio, and the mixture was dialyzed for 24 h at 4 °C (Visking tubing) against two changes of a 100-fold volume excess of Tris-HCl (30 mM), pH 7.7.

In most experiments, soluble proteins were reconstituted with rhodopsin–detergent micelles or phospholipid–rhodopsin vesicles. Final composition of the solution was as follows: Tris-HCl, 100 mM, pH 7.7; $MgCl_2$, 1 mM; EGTA, 100 μM.

Light-scatter measurements were made with a Beckman DB-GT spectrophotometer at 0° light-scatter angle, while stirring the solution in the cuvette.

PDE hydrolytic activity was measured with [^{3}H]cGMP and thin-layer chromatography as previously described (Bignetti et al., 1978). To determine rhodopsin partitioning between the supernatant and the pellet in centrifugation experiments, the supernatants (3 mL of each) were made 1% in SDS, and the pellets were resuspended in 1 mL of SDS (1%) and Tris-HCl (100 mM), pH 7.8, and the absorbance at 380 nM was immediately measured.

Negative staining of phospholipid vesicles was made with grids coated with carbon film. The vesicles were stained with a solution containing 2% phosphotungstic acid, adjusted to pH 7.4 with NaOH. Light microscopic observations of aggregation were made with a phase-contrast microscope. Aliquots of membrane suspensions were removed from the cuvette while turbidity changes were recorded. The samples were applied to a Burker chamber. The aggregation–disaggregation phenomenon was observed and photographed with a 20× phase objective.

Disk phospholipids were extracted from lyophilized membranes. Membranes (50 mg of rhodopsin) were stirred with 25 mL of chloroform–methanol, 1/1, for 30 min and then centrifuged at 8000g for 10 min. The supernatant was recovered and dried on a Rotavapor. About 35 mg of phospholipids was recovered under these conditions.

Phosphatidylcholine was from Sigma; phosphatidylserine was from Koch and Light or Sigma. Stearylamide was from Fluka.

RESULTS

We observed that addition of GTP (or its hydrolysis-resistant analogue) to disk membranes solubilized in detergent, in the presence of G protein and PDE, could still cause an increase in turbidity. Figure 1 shows that both G protein and PDE are necessary for activating this process and in the presence of a saturating concentration of G protein the amplitude and kinetics of the signal depend on the amount of PDE. In the presence of detergent (either cholic acid or Triton), the signal was slower than in native membranes, and its amplitude was reduced by increasing detergent concentration (data not shown).

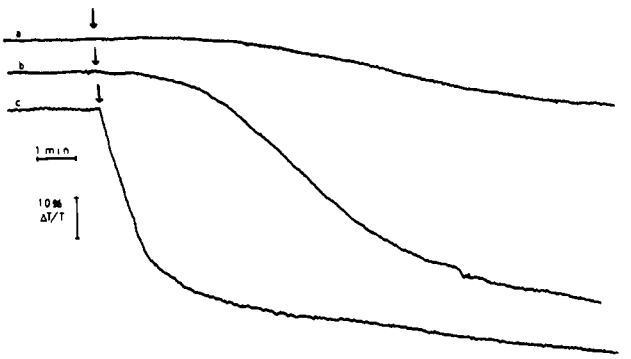

FIGURE 2: Turbidity change of detergent-solubilized disk membranes recorded at different wavelengths. [Rhodopsin] = 5 μM; [G protein] = 5 μM; [PDE] = 1 μM; [cholic acid] = 0.5%. (Trace a) Recording beam L = 640 nm; (trace b) recording beam L = 480 nm; (trace c) recording beam L = 310 nm. [GTP] = 200 μM (indicated by the arrow). Rhodopsin was fully bleached before the experiment was started.

The turbidity change in micellar suspensions of disk membranes was better monitored at shorter wavelengths than those previously employed (Figure 2).

Figure 3A shows that this effect is not modified when rhodopsin is purified by chromatography on concanavalin A–Sepharose. After this purification step, rhodopsin micelles are almost completely devoid of organic phosphorus (in five membrane preparations the organic P to rhodopsin ratio before and after the concanavalin A column was 85 and 2, 93 and 0, 81 and 0, 91 and 2, 90 and 1, respectively), thus suggesting that nearly all phospholipids have been removed. No other intrinsic membrane proteins can be detected on SDS–PAGE gels (Figure 3B), thus suggesting that rhodopsin is the only intrinsic membrane protein involved in this effect. When GTP was used instead of GTPγS, the turbidity change was fully reversible, upon completion of GTP hydrolysis either in detergent-solubilized disk membranes or in concanavalin-purified rhodopsin–detergent micelles (data not shown). Figure 3C shows that the presence of cholate reduces PDE hydrolytic activity about 6-fold in comparison to control membranes (note the change of scale and different enzyme concentration in Figure 3C), although GTPγS activation is still detectable. Purification of rhodopsin by concanavalin A–Sepharose (that is, delipidation) causes no further decrease in PDE activity.

Figure 3E shows that under the same experimental conditions, micelle–micelle aggregation occurs. These aggregates are smaller than those observed when native membranes are employed (see Figure 5B) and can be detected with visible light phase-contrast microscopy only after a considerable delay (30 min).

Under the same conditions in which micelle aggregation occurs, centrifugation experiments show that almost 90% bleached rhodopsin can now be pelleted with relatively mild centrifugation (46000g, 15 min, on a Beckman SW 65 rotor). Centrifugation of control samples did not sediment bleached rhodopsin which remained in the supernatant. The absorbance at 380 nm of the supernatant (3 mL) in the absence of GTPγS was =0.11 OD, while in the presence of GTPγS it was =0.01

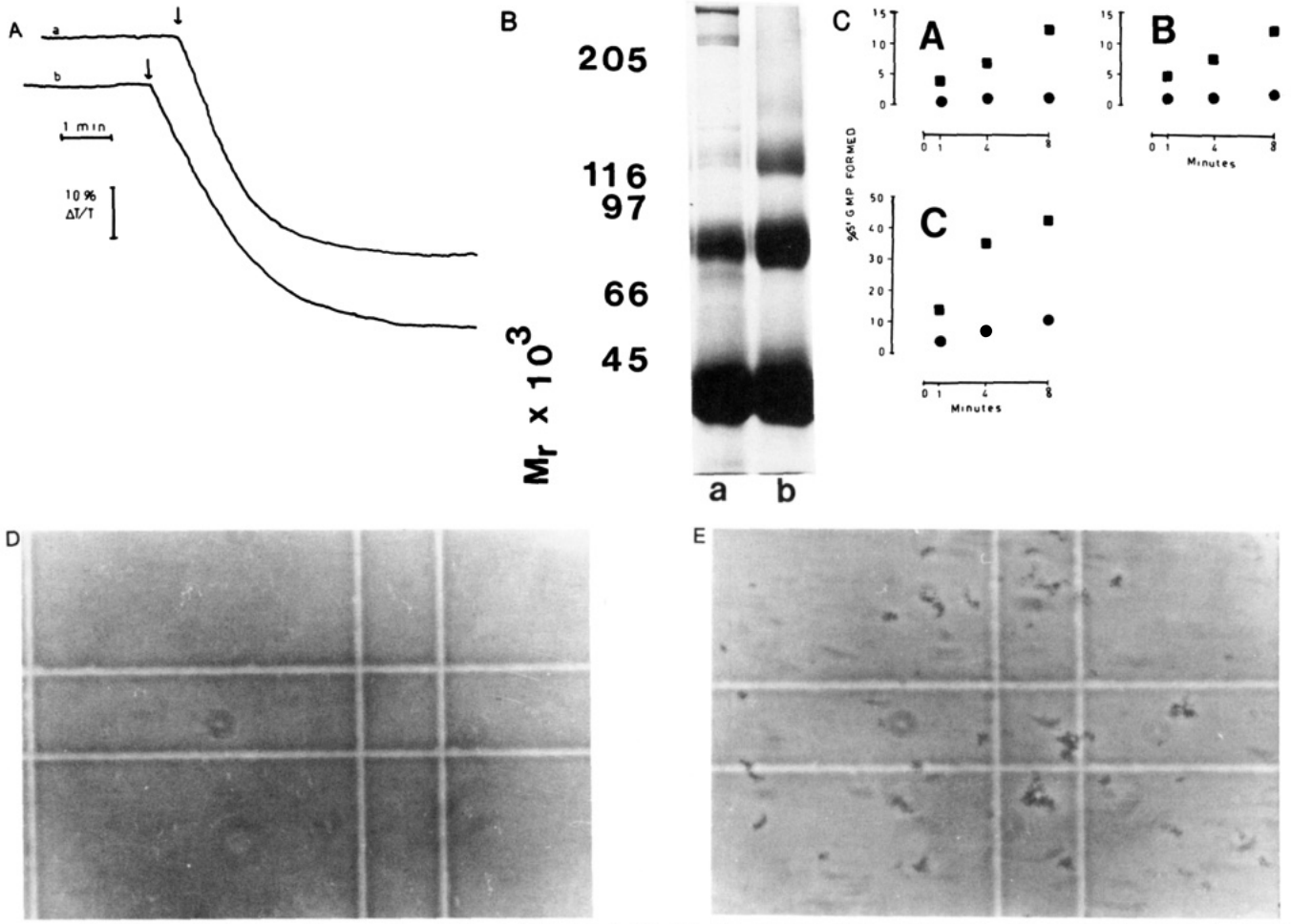

FIGURE 3: (A) Turbidity change in a solution of bleached rhodopsin (5 μM), G protein (5 μM), and PDE (1 μM). Recording beam L = 308 nm. (Trace a) Disks were solubilized in the presence of Triton (0.5%). (Trace b) Same amount of rhodopsin, purified by concanavalin A–Sepharose chromatography as described under Materials and Methods. In trace b, [cholic acid] = 0.5%. G protein and PDE, same concentrations as before. [GTPγS] = 10 μM (indicated by the arrow). (B) SDS–7% PAGE gel of native low ionic strength washed membranes (lane a) and concanavalin A purified rhodopsin (lane b). The only protein left in lane b is rhodopsin (and its dimers). (C) PDE hydrolytic activity in the presence (filled squares) and in the absence (filled circles) of GTPγS. [cGMP] = 1 mM, [GTPγS] = 10 μM, [Tris-HCl] = 100 mM, [$MgCl_2$] = 200 μM, [EGTA] = 50 μM. (Panel A) Detergent-solubilized disk membranes, as described under Materials and Methods. In the test, [rhodopsin] = 1 μM, [cholic acid] = 0.5%, [G protein] = 200 nM, and [PDE] = 50 nM. (Panel B) Concanavalin A–Sepharose-purified rhodopsin; in the test, [rhodopsin] = 1 μM, [G protein] = 100 nM, and [PDE] = 50 nM. (Panel C) Reconstituted native membranes; [rhodopsin] = 1 μM, [G protein] = 100 nM, and [PDE] = 25 nM. (D) Phase-contrast photograph of concanavalin A purified rhodopsin micelles reconstituted with PDE and G protein [same conditions as in (A), trace b]. The sample was incubated for 40 min in the absence of GTPγS. (The side of a small square in a Burker chamber is 50 μm long). (E) Phase-contrast photograph of concanavalin A purified rhodopsin micelles reconstituted with G protein and PDE [same conditions as in (A), trace b]. The sample was incubated for 40 min in the presence of 10 μM GTPγS.

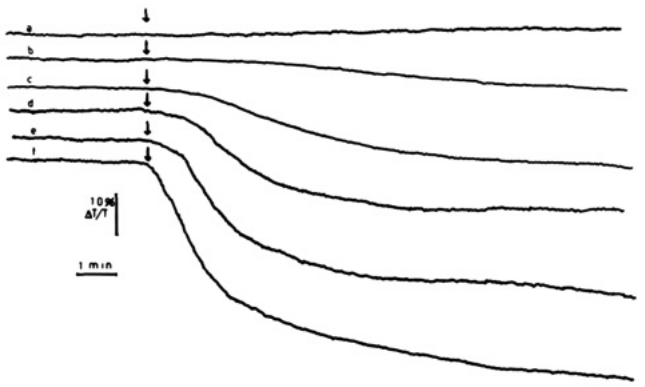

FIGURE 4: Effect of increasing bleaching levels on GTPγS-dependent turbidity increase. To avoid photomultiplier artifact, concanavalin-purified rhodopsin was bleached before beginning the experiment. [Rhodopsin] = 5 μM; [G protein] = 5 μM; [PDE] = 1 μM; [cholic acid] = 0.5%. Bleaching from the recording beam was 0.5% every 2 min. Recording beam L = 308 nm. (Trace a) Bleaching = 1%; (trace b) bleaching = 6%; (trace c) bleaching = 15%; (trace d) bleaching = 21%; (trace e) bleaching = 30%; (trace f) bleaching = 100%. [GTPγS] = 10 μM (indicated by the arrow).

OD. After resuspension of the pellet in 1 mL, the absorbance at 380 nm was =0.29 OD on the tube with GTPγS, while it was =0.01 OD on the tube without GTPγS, where no pellet was visible by eye.

In a suspension of concanavalin A purified rhodopsin–detergent micelles, a turbidity increase is triggered only by bleaching a high percent of rhodopsin molecules (Figure 4).

Figure 5A shows the kinetics of light-scattering changes in the presence of vesicles reconstituted by dialysis with increasing ratios of phospholipids to concanavalin A purified rhodopsin. Because of the higher turbidity of the samples, recording was made at a longer wavelength than that employed for detergent–micelle suspensions. Negative-staining electron microscopy showed that phospholipid vesicles were of uniform size (average diameter = 0.15 ± 0.03 μm), independently of the rhodopsin concentration, except for the last condition (rhodopsin/lecithin 1:1 w/w, average diameter = 0.23 ± 0.05 μm). The presence of phosphatidylserine (a negatively charged lipid) or stearylamine (a positively charged substitute of lipids) does not affect the speed or the amplitude of the signal (traces

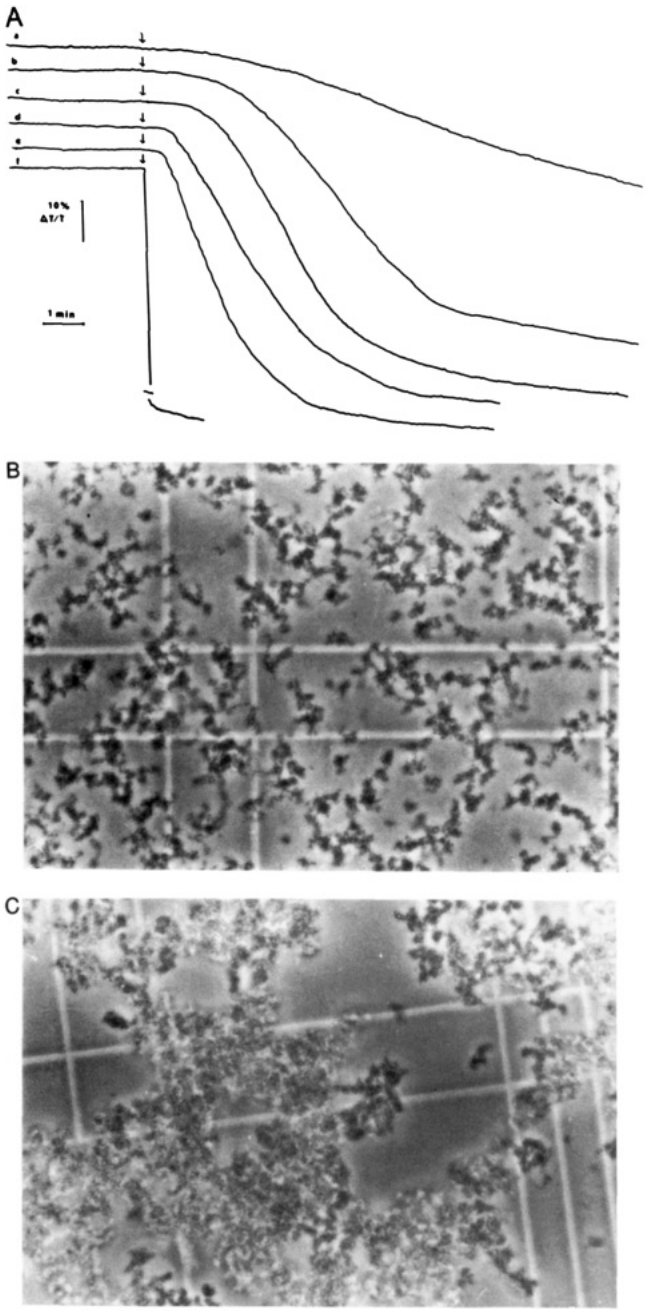

FIGURE 5: (A) Turbidity change in a suspension of rhodopsin–phospholipid vesicles reconstituted at different rhodopsin:phospholipid ratios. [Rhodopsin] = 1.5 μM (60 μg/mL, fully bleached before starting the experiment), [G] = 1.5 μM, [PDE] = 0.3 μM. Recording beam L = 640 nm. Phospholipid composition was different in the different traces: trace a = 13.2 mg of phosphatidylcholine/mL; trace b = 6 mg of phosphatidylcholine/mL; trace c = 4.5 mg of phosphatidylcholine/mL; trace d, phosphatidylcholine = 2.25 mg/mL, phosphatidylserine = 2.25 mg/mL; trace e, phosphatidylcholine = 3.33 mg/mL, stearylamine = 1.17 mg/mL; trace f = 60 μg of phosphatidylcholine/mL. [GTPγS] = 10 μM, indicated by the arrow. (B) Phase-contrast photograph of rhodopsin–phospholipid vesicles [the same as in (A), trace c] in the presence of 10 μM GTPγS. (In the absence of GTPγS, no structure could be detected; see Figure 3D). (The side of a small square = 50 μm). (C) Phase-contrast photograph of native membranes incubated under the same conditions as in (B), in the presence of 10 μM GTPγS.

c–e). Similar results are obtained when rhodopsin is reconstituted with disk phospholipids. No signal can be observed when disk phospholipid vesicles are tested without added rhodopsin (data not shown).

Figure 5B shows that visible aggregation occurs in conditions which promote turbidity increases, in experiments which employed rhodopsin reconstituted into phospholipid vesicles. This micrograph shows vesicles taken from the sample which produced trace b in Figure 5A. Under these conditions, there is no appreciable delay between the generation of the light-scattering signal and the appearance of the aggregated vesicles. While the speed of the light-scattering changes varied at each different condition tested in Figure 5A, the aggregates detected at completion of the signal did not appear to differ. However, they are consistently smaller in size than those formed upon activation of native low ionic strength washed disk membranes reconstituted with G protein and PDE (Figure 5B).

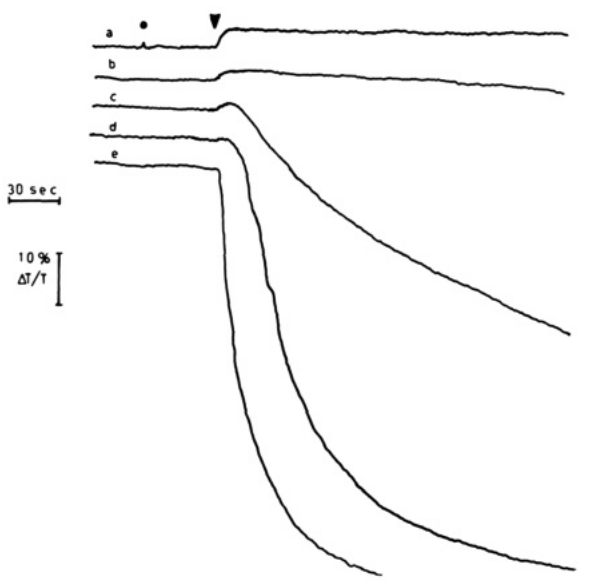

FIGURE 6: Effect of ionic strength on turbidity change in a disk suspension. Fully bleached low ionic strength washed disk membranes (5 μM rhodopsin) were reconstituted with 1 μM G protein and 100 nM PDE, in the presence of different concentrations of Tris-HCl buffer, final pH 7.7. Recording beam L = 640 nm. Trace a, [Tris-HCl] = 5 mM; trace b, [Tris-HCl] = 40 mM; trace c, [Tris-HCl] = 60 mM; trace d, [Tris-HCl] = 75 mM; trace e, [Tris-HCl] = 100 mM. Addition of GTP (100 μM final concentration, buffered with Tris base at pH 7.7, 3 μL/3 mL) indicated by the arrowhead. In trace a, the filled circle indicates the control addition of 3 μL of buffer.

The aggregation effect and turbidity increase reported above require the presence of bleached rhodopsin, G protein, PDE, and normal ionic strength (100 mM Tris-HCl). At low ionic strength (5–40 mM Tris-HCl), only a fast decrease in turbidity [presumably related to the so-called dissociation signal (Kuhn et al., 1981)] is observed upon addition of GTP (Figure 6, traces a and b), and no aggregates appear. This signal is not modified when reconstitution is made only with G protein, that is, in the absence of PDE, so it is presumably related to the so-called G signal (Kuhn et al., 1981). At higher ionic strengths (60–100 mM Tris-HCl), in the presence of G protein and PDE, turbidity decreases can be observed, and vesicle aggregation occurs, until at very high ionic strength (600 mM Tris-HCl) no turbidity change or vesicle aggregation is detected. A similar ionic strength sensitivity is observed for PDE binding to photoreceptor membranes and for GTP-dependent activation of PDE hydrolytic activity (data not shown).

Addition of a limited amount of trypsin to disk vesicle aggregates causes reversal of the turbidity increase. Microscopic observations show that aggregates disappear after trypsin treatment (data not shown). Under these conditions, we have observed by centrifugation partitoning of PDE between the pellet and the supernatant that only 8% of the initial PDE hydrolytic activity is found in the pellet while the rest (92%) is in the supernatant. On an SDS–PAGE gel, no PDE is

detected in the pellet. In this experiment, PDE hydrolytic activity after trypsin treatment was 2.6 times higher than that after GTP activation. Addition of trypsin-activated PDE to a fully bleached disk suspension caused no change in turbidity or in the state of aggregation of the vesicles. Similarly, addition of G protein–GTPγS to dark-adapted membranes reconstituted with PDE in 100 mM Tris-HCl buffer causes no change in turbidity (data not shown).

Discussion

In our previous studies, we documented that aggregation of disk membrane vesicles required PDE activation and correlated with increased PDE binding to disk membranes (Caretta & Stein, 1986; Liebman & Zimmerman, 1989). The objective of the present study was to determine the mechanism responsible for the aggregation process. By performing experiments in the absence of membrane structure (i.e., in detergent suspensions), we determined that the presence of a membrane is not essential for the aggregation process while it is necessary for the G signal (Figure 1). In the critical experiments described above, virtually all phospholipids have been extracted from the purified rhodopsin. Since the aggregation phenomenon also occurred in detergent micelles of different charge (Triton or cholic acid) and in reconstituted vesicles containing phospholipids of different charge, direct interactions between bleached rhodopsin and the proteins of the cyclic nucleotide cascade were responsible for vesicle or micelle aggregation and the consequent turbidity changes observed. The delay between light-scatter changes and microscopical observation of aggregates is likely to depend on the time required for the rhodopsin–detergent micelles to grow from aggregates which interact with a short-wavelength recording beam (presumably some nanometers) to aggregates which can be observed with a 20× objective (some micrometers; see Figure 3D). However, we cannot rule out that other unidentified processes take part in this phenomenon.

Bleached rhodopsin is an absolute requirement for the aggregation process and the scattering signal since chemical activation of PDE hydrolytic activity by trypsin or by G–GTPγS does not produce the aggregation process or the scattering signal. An association of PDE with bleached rhodopsin (considering the slow time course of the aggregation process, a stable form, either meta III or opsin, is likely to be involved) is a necessary intermediate in the aggregation process.

The light threshold of this signal in the presence of detergent is very different from that in native membranes (6 bleached rhodopsins every 100 molecules instead of 1 every 10^7 rhodopsins or 1 every 10^4 rhodopsins, respectively, in fully dark-adapted toad membranes and bovine membranes; Caretta & Stein, 1985, 1986). In the presence of detergent, bleached rhodopsin undergoes structural changes (Osborne & Nabedryk-Viala, 1978) which may reduce its ability to activate the light-sensitive enzymatic cascade (Figure 3B). Moreover, since in every rhodopsin–detergent micelle much fewer rhodopsin molecules are present than in a native disk (an exact number cannot be given because the state of aggregation of rhodopsin molecules in detergent micelles changes with bleaching; Osborne et al., 1974), a high percent of rhodopsin molecules must be bleached in order that most micelles contain bleached rhodopsin molecules and therefore can aggregate.

On the other hand, after removal of detergent, few bleached rhodopsin molecules are sufficient to drive the aggregation of phospholipid vesicles (Figure 5A,B); in the experiments with vesicles reconstituted with different phospholipid to rhodopsin ratios, at the highest phospholipid/rhodopsin dilution, only seven to eight rhodopsin molecules are estimated to be present for every vesicle.

The physiological meaning of the structural changes correlated to the in vitro activation of the cyclic nucleotide cascade (Caretta & Stein, 1985, 1986, 1987) is still unclear. Binding of light-activated PDE to bleached rhodopsins (and the consequent aggregation of the latter) can reduce the diffusion rate of both molecules in the interdiskal space or within the disk membrane, respectively. This phenomenon may play an important role in activating the cyclic nucleotide cascade, thus regulating the cGMP cation channel (Caretta et al., 1979; Fesenko et al., 1985), whose molecular characterization is still controversial (Cook et al., 1987; Matesic & Liebman, 1987; Clack & Stein, 1989).

The interdiskal distance in the intact cell is 150 Å. A complex formed by rhodopsin–PDE–PDE–rhodopsin is large enough to span this distance (assuming 70-Å length for PDE and 10 Å for the extradiskal portion of rhodopsin). In the vertebrate retina, a reduction of the interdiskal distance (Chabre & Cavaggioni, 1973) and a change in the optical properties of the photoreceptors (Harari et al., 1978) affected by PDE competitive inhibitors (Stein & Caretta, 1986) have been observed after a flash of visible light; we suggest that the molecular interactions we have described (Caretta & Stein, 1986; this paper) are responsible for the structural changes observed in vivo.

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Subunit Stoichiometry of Retinal Rod cGMP Phosphodiesterase†

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ABSTRACT: The cyclic GMP phosphodiesterase of the retinal rod is composed of three distinct types of polypeptides: α (90 kDa), β (86 kDa), and γ (10 kDa). The γ subunit has been shown to inhibit phosphodiesterase activity associated with α and β. To investigate the subunit stoichiometry of the retinal phosphodiesterase, we have developed a panel of monoclonal and peptide antibodies that recognize individual phosphodiesterase subunits. By quantitative and immunochemical analysis of the purified subunits, we have shown that each phosphodiesterase molecule contains one copy each of α and β subunit and two copies of γ subunit. Moreover, γ can be chemically cross-linked to both α and β, but not to itself, suggesting that α and β may each bind one γ. The phosphodiesterase is fully activated when both copies of γ were removed by proteolysis with trypsin. Upon recombination of the purified γ subunit with the trypsin-activated phosphodiesterase containing $\alpha\beta$, the $\alpha\beta\gamma_2$ stoichiometry is once again restored, with concomitant total inhibition of activity. Our results suggest that at least two activated transducin molecules are required to fully activate one molecule of phosphodiesterase in retinal rods.

Retinal phosphodiesterase (PDE),[1] which serves to regulate the cytosolic cGMP concentration of vertebrate retinal rods, is one of several key proteins involved in visual excitation (Chabre, 1985; Fung, 1986; Stryer, 1986; Hurley, 1987; Liebman et al., 1987). It is a peripheral membrane protein consisting of α (90 kDa), β (86 kDa), and γ (10 kDa) polypeptides (Baehr et al., 1979). In this multimeric form, the hydrolytic activity associated with $\alpha\beta$ is inhibited by γ (Hurley & Stryer, 1982). The inhibition is relieved when the γ subunit is removed by the GTP-bound form of the retinal G-protein transducin (Fung et al., 1981; Wensel & Stryer, 1986; Deterre et al., 1986; Fung & Griswold-Prenner, 1989), leading to a transient reduction of the intracellular level of cGMP in ROS (Miki et al., 1975; Yee & Liebman, 1978; Woodruff & Bownds, 1979; Blazynski & Cohen, 1986; Cote et al., 1986) and the closure of many cGMP-sensitive cation channels at the plasma membranes (Fesenko et al., 1985; Yau & Nakatani, 1985). As a result, the influx of Na^+ through the plasma membrane decreases (Hagins et al., 1970; Baylor et al., 1979), and the rod hyperpolarizes (Tomita, 1970).

Although rod PDE has been purified to homogeneity (Miki et al., 1975; Baehr et al., 1979) and the amino acid sequences of both α- and γ-polypeptides have been deduced from their corresponding cDNA (Ovchinnikov et al., 1986, 1987), the relative stoichiometry of α, β, and γ is still not well-defined. PDE has always been assumed to be composed of α- and β-polypeptides containing one to two copies of γ. Evidence supporting this stoichiometry comes from the observations that the molecular weight of PDE determined by gel filtration or by sucrose density centrifugation is approximately 170000 and that the Coomassie Blue staining patterns of the PDE α and β chains separated by SDS-PAGE are consistently equally intense (Baehr et al., 1979). These findings, however, do not provide information on the number of copies of γ per PDE molecule, nor do they exclude the possibility that purified PDE is a mixture consisting of equal amounts of isozyme containing either α or β. In agreement with this latter possible interpretation, Hurwitz et al. (1985) have reported the chromatographic separation of the β subunit on a Mono-Q anion-exchange column.

In this paper, we demonstrate by immunoprecipitation of the PDE holoenzyme with α-specific monoclonal antibodies and by quantitative Western blot analyses that the stoichiometry of purified rod PDE is $\alpha\beta\gamma_2$. We further support this finding by showing that total inhibition of the hydrolytic activity of $\alpha\beta$ requires two γ per $\alpha\beta$. Finally, we study the topological organization of the α-, β-, and γ-polypeptides in a qualitative manner by specifically cross-linking the PDE subunits using the homobifunctional cross-linker dimethyl suberimidate, which results in the successive formation of a protein product containing first one and then two copies of γ cross-linked to each of the α and β subunits. While this work was in progress, Deterre et al. (1988) have reported the chromatographic separation of two populations of activated PDE, one with about 50% of the γ content of native PDE and the other totally devoid of γ. Whalen and Bitensky (1989) have also demonstrated two classes of γ binding sites per $\alpha\beta$

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MOPS, morpholinopropanesulfonic acid; mAb, monoclonal antibody; PDE, retinal cGMP phosphodiesterase; ROS, rod outer segment(s); TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; T_α, the α subunit of transducin; α, β, and γ, subunits of phosphodiesterase.

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