Structure and function in rhodopsin: Asymmetric reconstitution of rhodopsin in liposomes

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Contributed by H. Gobind Khorana, August 27, 2002

We report on preparation of rhodopsin proteoliposomes with the cytoplasmic domain of rhodopsin facing the exterior of the proteoliposomes. Rhodopsin purified from rod outer segments of bovine retinae by immunoaffinity chromatography in octyl glucoside was reconstituted into liposomes prepared from soybean phospholipids by detergent dialysis. The orientation of rhodopsin in the liposomes was determined by susceptibility of its C terminus to papain and the endoproteinase, Asp-N, followed by SDS/PAGE, which showed that the cytoplasmic domain in at least 90% of rhodopsin faced the exterior of the proteoliposomes. By using escape of ^{32}P-KP$_i$ encapsulated in the proteoliposomes as the assay, the half-life of the proteasomes was ≈8 days. After light activation, rhodopsin in proteoliposomes showed the rate of decay of metarhodopsin II and the initial rate of transducin activation comparable with the rates of rhodopsin in rod outer segment membranes. This finding demonstrates the functional capability of rhodopsin in proteoliposomes for kinetic studies of protein–protein interactions.

Light initiates two biochemical cascades mediated by rhodopsin, one leading to sensitization (amplification) involving transducin (G_T) and the other to desensitization (quenching) involving rhodopsin kinase (1). Competition between G_T and rhodopsin kinase for binding to the light-activated rhodopsin must be central to the progression of the two cascades in vision. Therefore, studies of the kinetics of interaction of the two proteins with light-activated rhodopsin are of interest. The kinetic technique based on surface plasmon resonance has been applied fruitfully in several kinetic studies (2, 3) and is promising for the study of the protein–protein interactions in the visual system. To apply this technique, immobilization of an interactive protein on the sensor chip surface would be required. In our approach, immobilization of rhodopsin would require, first, reconstitution of rhodopsin into liposomes asymmetrically such that the cytoplasmic domain, where the protein–protein interactions occur, is oriented to the exterior of the liposome.

Here, we report reconstitution of rhodopsin into liposomes with the desired orientation. Reconstitution of rhodopsin into liposomes has been reported from many laboratories (4–9). The orientation of rhodopsin in the proteoliposome preparation was investigated carefully by DeGrip *et al*. (5) and, in particular, by Fung and Hubbell (4). Both groups found rhodopsin in a random orientation in the proteoliposomes after careful characterization by proteolytic studies. Because we obtained an essentially uniform orientation in the present work, we repeated the previous experiments of Fung and Hubbell and were able to confirm the results obtained by them. We offer possible interpretations for the difference between our results and those previously reported.‡

Materials and Methods

Materials. Frozen bovine retinae were obtained from W. L. Lawson (Lincoln, NE). Anti-rhodopsin monoclonal antibody rho-1D4 was purified from myeloma cell lines provided by R. S. Molday (University of British Columbia, Vancouver), and was coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia) as described (11). The rho-1D4 antibody epitope nonapeptide that has rhodopsin C-terminal sequence 339–348 was prepared by the Biopolymer Laboratory at the Massachusetts Institute of Technology. The rhodopsin-binding capacity of the 1D4-Sepharose beads was 1.2 mg/ml. Octyl glucoside (OG) and sodium cholate were from Anatrace (Maumee, OH). Crude soybean phospholipids (asolectin), egg phosphatidylcholine, and egg phosphatidylethanolamine were from Sigma. The proteases, endoproteinase Asp-N and papain, and iodoacetamide used as the papain inhibitor, were also from Sigma. The dialysis sack (molecular mass cutoff, 14 kDa) was from Spectrum Medical Industries. Protein molecular weight markers were from Amersham Pharmacia. All other chemicals were from Aldrich-Sigma.

The buffers used were buffer A [137 mM NaCl/8 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4 (pH 7.2)] and buffer B [10 mM Tris/2 mM $MgCl_2$/100 mM NaCl (pH 7.2)].

Purification of Rhodopsin from Rod Outer Segments (ROS). ROS were prepared from frozen bovine retinae as described by Papermaster (12). For purification of rhodopsin, ROS were solubilized in buffer A containing 4% OG and 0.1% asolectin at 4°C for 2 h. The solubilized rhodopsin was bound to rho-1D4-Sepharose column (11) and the column was washed with buffer A containing 1.46% OG and 0.1% asolectin. Rhodopsin was eluted with the same buffer containing the epitope nonapeptide (100 μM). The ratio of the absorbance at 280 and 500 nm wavelength, respectively, for the eluted rhodopsin was 1.6. The molar extinction coefficient, E_{max}, at 500 nm used for calculating rhodopsin concentration was 42,600.

Purification of Transducin. G_T was purified from frozen bovine retina according to Baehr *et al.* (13), and stored in 50% glycerol at −80°C until use (14).

Preparation of Proteoliposomes. Two methods were used to prepare proteoliposomes.

Method A: Preparation of proteoliposomes from Rho-1D4-Sepharose-purified ROS rhodopsin. A solution of commercially available soybean phospholipids (asolectin) in buffer A (1 mg/ml) containing 1.46% OG was mixed with the purified rhodopsin. The molar ratio of asolectin (assumed molecular weight, 760) (8) to rhodopsin was 100–500:1 (15). The total solution was dialyzed in the dialysis sack against buffer A (1,350-fold excess) at 4°C, in the dark for 24 h with four buffer exchanges. The proteoliposome suspension was pelleted at 140,000 × *g* at 4°C for 1 h in a Beckman TL-100 ultracentrifuge, and the pellet was resuspended in buffer B for G_T assay or in buffer A for storage over argon at 4°C. (The volume of the buffer for storage was 2–5 μl/μg rhodopsin.)

Abbreviations: ROS, rod outer segments; G_T, transducin; OG, octyl glucoside; Meta II, metarhodopsin II.

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‡This is paper 51 in the series ''Structure–Function in Rhodopsin.'' The preceding paper is ref. 10.

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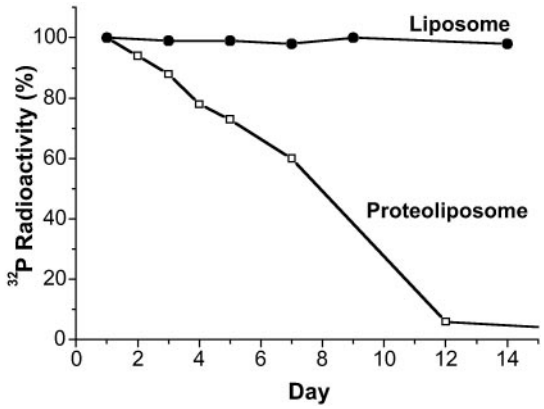

Fig. 1. Percentage of ^{32}P radioactivity in proteoliposomes (□) and in liposomes (●) as a function of time (day). Each point was an average from duplicate measurements.

Method B: Preparation of proteoliposomes containing ROS rhodopsin by Fung and Hubbell procedure (4). The procedure of Fung and Hubbell was used exactly except that egg phosphatidylcholine, egg phosphatidylethanolamine, and sodium cholate were purchased commercially without purification.

Stability Studies with Proteoliposomes Containing Encapsulated ^{32}P-KP$_i$. Proteoliposomes were prepared by detergent dialysis, by using method A, except that ^{32}P-KP$_i$ was included in the solution before the start of dialysis. In this experiment, the molar ratio of phospholipids to rhodopsin was 300:1. The free ^{32}P-KP$_i$ was removed by ultracentrifugation until the radioactivity in the supernatant was below background (<500 cpm). An aliquot of 25 μl of proteoliposomes containing ≈10 μg of rhodopsin (≈13,000 cpm radioactivity at the start of the procedure) was diluted to 100 μl in buffer A. The supernatant after centrifugation represented 65% of the volume, large enough so as not to disturb the pellet. Aliquots of the proteoliposomes were taken over days for ^{32}P radioactivity in both the supernatant and pellet. Radioactivity was measured by Cerenkov counting.

Orientation of Rhodopsin in Proteoliposomes as Determined by Proteolysis. Two proteases able to digest rhodopsin were used as follows.

Cleavage by endoprotease Asp-N (16). Asp-N, which specifically cleaves between Gly-329 and Asp-330 (17) in the C terminus, was used as follows. A total of 100 μl of proteoliposome containing 25 μg of rhodopsin in buffer A was subject to proteolysis. Aliquots of the sample were taken at various times. When completed (2–3 h), OG was added (1.46% final concentration) to solubilize the proteoliposomes for gel loading, or to allow those previously uncleaved rhodopsin molecules to be digested before gel loading. SDS/PAGE loading buffer was used to stop the proteolysis.

Cleavage by papain. The procedure described by Fung and Hubbell (4) was followed exactly. Proteolysis was performed at room temperature in the dark by using a molar ratio of protease to rhodopsin of 1:25–100. The proteolytically cleaved rhodopsin fragments were resolved by SDS/PAGE (12% gel).

Metarhodopsin II (Meta II) Decay Rates for Rhodopsin in ROS Membrane and for Rhodopsin in Proteoliposomes. The Meta II decay rate was determined as described by Farrens and Khorana (18) by using a PSEX 212X Fluorolog Instrument (Spex Industries, Edison, NJ). The excitation and emission wavelengths were, respectively, at 295 nm with 2-nm slit width and 330 nm with 12-nm slit width. A proteoliposome sample containing 2 μg of rhodopsin in 200 μl of buffer A was used with equal amount of rhodopsin in ROS membranes in the same buffer being as control. The samples were irradiated with a light source at >495 nm for 30 s (when conversion to Meta II was complete).

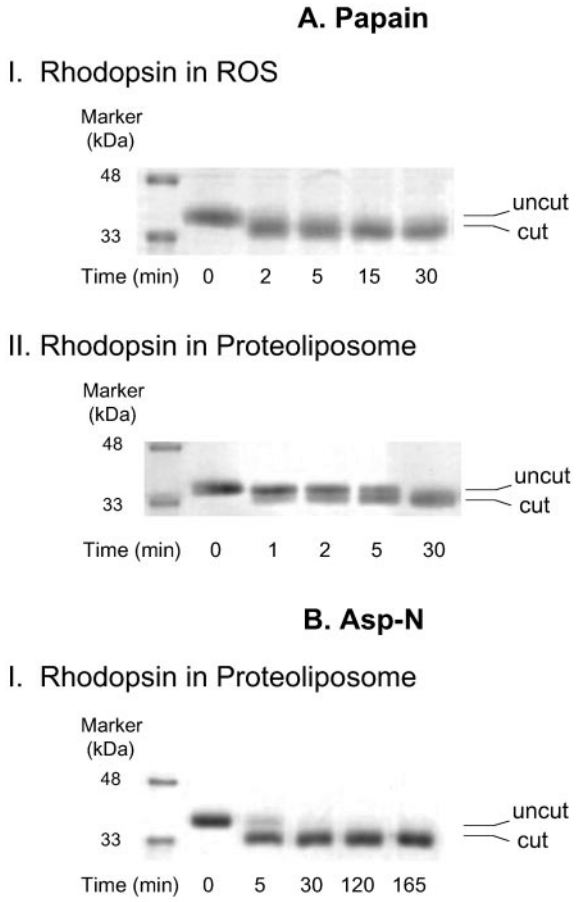

Fig. 2. Proteolysis of rhodopsin in proteoliposomes prepared by method A (see *Materials and Methods*). Aliquots of the rhodopsin samples were removed by the time specified below each panel and the protease activity was stopped as described in *Materials and Methods*. (*A*) SDS/PAGE for papain digestion of the rhodopsin in ROS membrane (*I*) and of rhodopsin in proteoliposomes (*II*). (*B*) SDS/PAGE for proteolysis by Asp-N for the proteoliposomes prepared by the same method.

Rate of G_T Activation. The method used was that of Fahmy and Sakmar (19), which monitors the fluorescence increase by formation of Gα·GTP-γ-S complex after G_T activation by Meta II. The excitation wavelength was 295 nm with 2-nm slit width and emission wavelength was 340 nm with 12-nm slit width. G_T and rhodopsin-containing proteoliposomes were premixed with stirring. The final concentrations of transducin, rhodopsin, and GTP-γ-S being 250 nM, 5 nM, and 5 μM, respectively, in a total volume of 700 μl of buffer B. The sample was irradiated with a light source at >495 nm for 30 s.

Data Collection and Analysis. Meta II decay rate and G_T activation were measured at 20°C. All other measurements were at room temperature. The data were analyzed by using SIGMA PLOT and ORIGIN programs.

Results

Stability of Liposomes and of Rhodopsin-Containing Proteoliposomes. Liposomes and proteoliposomes containing ^{32}P-KP$_i$ were prepared and the release of ^{32}P-KP$_i$ into the supernatant was

followed as a function of time as in *Materials and Methods*. Because of ^{32}P decay in the period of many days, the rate of ^{32}P escape from the liposomes or proteoliposomes was determined by directly measuring in each sample the radioactivity in both the supernatant and pellet in the same aliquot. The percentage of radioactivity remaining in the liposome or proteoliposome as a function of time (day) is shown in Fig. 1. As is seen, liposomes were stable for the period of 14 days tested. However, the proteoliposome released the radioactivity into the supernatant with a half-life of ≈8 days. Within 3 days, however, close to 90% of the proteoliposomes still remained intact and therefore are quite useable.

Orientation of Rhodopsin in Proteoliposomes Prepared by Method A. The orientation of rhodopsin in proteoliposomes was determined by proteolysis with papain and endoproteinase Asp-N.

Method A: Digestion with papain. Previously, proteolysis by papain was used by Fung and Hubbell (4) to ascertain the orientation of proteoliposomes prepared by method B (see below). The result of our experiments with this enzyme on proteoliposomes prepared by method A are shown in Fig. 2*A*. Fig 2*AI* shows results of a control experiment performed with rhodopsin as present in ROS membrane. As seen, ROS rhodopsin was converted completely in 30 min to a slightly faster-moving product in SDS/PAGE, as found by Fung and Hubbell (4). Fig. 2*AII* shows the results, by using the same enzyme, with rhodopsin in proteoliposomes prepared by method A. Thus, conversion to the faster-moving band was essentially complete in 30 min, showing that >90% of rhodopsin was susceptible to papain.

Method B: Digestion with endoproteinase Asp-N. The orientation of rhodopsin in proteoliposomes prepared by method A was further explored by using the endoproteinase Asp-N. Previously, the enzyme has been shown to cleave rhodopsin specifically between Gly-329 and Asp-330 in the C-terminal tail (10, 17, 20). The results, in Fig. 2*B*, showed essentially a complete conversion, in 30 min, to a single faster-moving product.

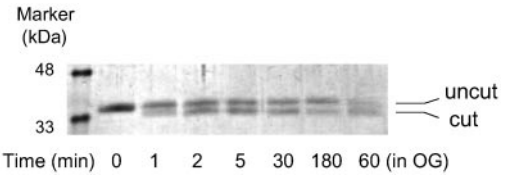

Fig. 3. Proteolysis of rhodopsin by papain in proteoliposomes prepared by method B (see *Materials and Methods*). Aliquots of rhodopsin proteoliposomes were removed by the intervals indicated. Lane 1, from the right, corresponds to the digestion for additional 60 min by papain in the presence of 1.46% OG (final concentration); the OG was added after 180 min of digestion of proteoliposomes.

Orientation of Rhodopsin in Proteoliposomes Prepared by Method B. Because of the significant difference between results obtained above with the proteoliposomes prepared by method A and the results reported previously by Fung and Hubbell (4) with proteoliposomes prepared by their method or method B, we repeated the papain digestion with the proteoliposomes prepared by method B of Fung and Hubbell. The results, in Fig. 3, show that the cleavage of rhodopsin from this preparation was partial even over prolonged digestion, confirming what Fung and Hubbell had found.

Functional Characterization of Rhodopsin Reconstituted into Liposomes. ***Decay of Meta II.*** The rate of the Meta II decay was measured as in *Materials and Methods*. Fig. 4*A* shows the results

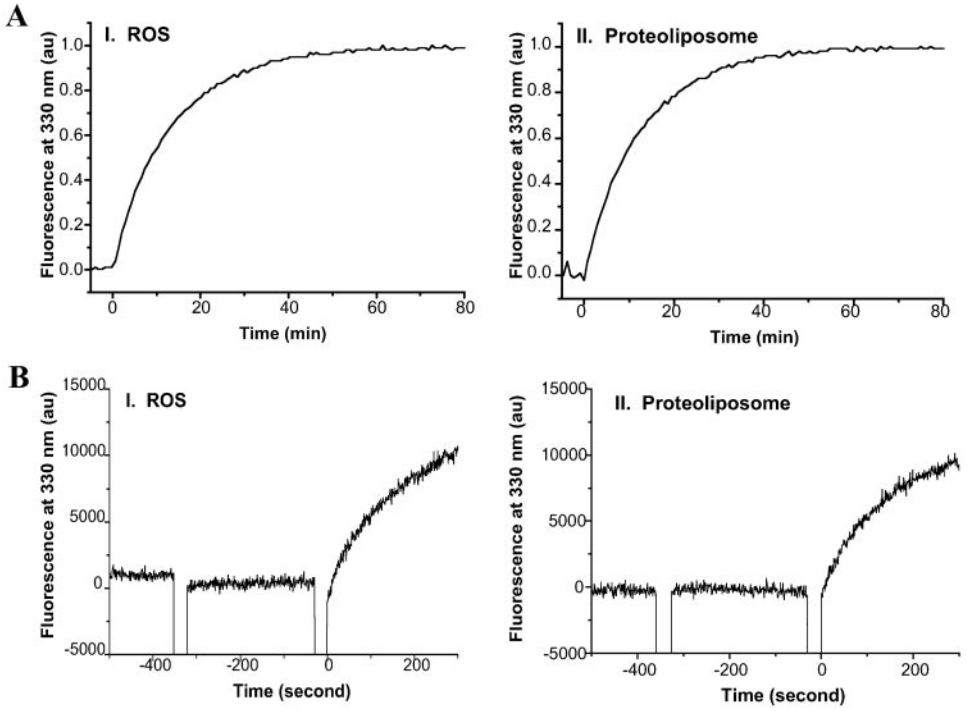

Fig. 4. (*A*) Rates of Meta II decay (*I*, ROS rhodopsin; *II*, rhodopsin in proteoliposomes) as monitored by fluorescence increase (see *Materials and Methods*). The maximum fluorescence, at the steady-state level, was used to normalize the progression of the signal. The ratio of the half-life of the Meta II decay for rhodopsin in proteoliposomes to that of the rhodopsin in ROS membrane was 1.16 ± 0.28 from three measurements. (*B*) G_T activation by light-activated rhodopsin in ROS membrane (*I*) and by rhodopsin in proteoliposomes (*II*). The first and the second negative-going spikes were the artifact of the light irradiation of the sample and the addition of GTP-γ-S, respectively. The rise of the fluorescence starting from time 0, in arbitrary units, indicated the formation of the Gα·GTP-γ-S (see *Materials and Methods*). The ratio of the initial rate of the reaction with the rhodopsin in proteoliposomes to that of rhodopsin in ROS membranes was 1.18 ± 0.24 from three measurements with equivalent amounts of rhodopsin.

for rhodopsin in proteoliposomes and in ROS membrane, as a control. As is seen, the rates, within experimental error, were identical.

G_T activation by activated rhodopsin in proteoliposomes and in ROS membrane. G_T activation was studied for both the proteoliposomes and ROS rhodopsin as in *Materials and Methods*. The kinetics of activation, as measured by the initial rate of formation of Gα·GTP-γ-S, are compared in Fig. 4*B*. Again, by using identical, calculated amounts of rhodopsin in the two side-by-side experiments, the rates were identical within experimental error.

Discussion

Reconstitution of integral membrane proteins into liposomes, pioneered and studied extensively by Racker and his coworkers (15, 21, 22), has been used in a variety of *in vitro* studies of membrane proteins. In general, reconstitution results in proteins adopting both orientations, outside-out and inside-out, in the proteoliposomes. Exceptions, however, exist. For example, bacteriorhodopsin reconstituted into vesicles, starting with a purple membrane suspension or bacteriorhodopsin completely freed from endogenous lipids, adopts mostly an inverted orientation as determined by the direction of proton pumping (23).

In the previous experiments on reconstitution of rhodopsin in proteoliposomes, mixed orientations were found (4, 5). The procedure used in the present work, however, gave proteoliposomes with essentially an asymmetric orientation. One possible explanation for this important difference between the present results and the results obtained by the previous workers is the purification of rhodopsin by extensive detergent washing in the presence of soybean phospholipids before reconstitution. Thus, the rhodopsin samples used in all of the previous experiments may have retained the "coat" of native ROS lipids when they were treated for reconstitution with phospholipid-detergent mixtures. In contrast, rhodopsin used in the present reconstitution experiments had been solubilized in 4% octyl glucoside, bound to the rho 1D4 column, and washed excessively, before elution, with the same detergent (1.46%) containing 0.1% asolectin. Rhodopsin thus purified may have lost essentially all of the endogenous ROS lipids and the new phospholipid "coat" formed in rhodopsin subsequently eluted consisted of asolectin phospholipids. Proteoliposomes prepared from rhodopsin by octyl glucoside dialysis then contained rhodopsin in an essentially one uniform orientation. A similar explanation was advanced in the reconstitution of bacteriorhodopsin with asymmetric orientation (23).

In the stability test, we showed that the half-life of the escape of the ^{32}P-KP$_i$ radioactivity from the proteoliposomes was about 8 days, which may be due to breakup of the proteoliposomes or fusion of proteoliposomes during the storage. Nevertheless, within 3 days, close to 90% of the radioactivity still remained, indicating that the proteoliposomes are useable. Further, within several hours as in the orientation assay (see Figs. 2 and 3), any breakup of proteoliposomes, thus rendering rhodopsin completely accessible to proteases, could not have caused any detectable difference in the percentage of orientation in our experiments. In addition, during this short period, the proteases used had no effect on the stability of proteoliposomes, as tested with ^{32}P-containing proteoliposomes (data not shown).

The method we presented here now permits the making of proteoliposomes harboring asymmetrically oriented rhodopsin with the cytoplasmic domain accessible for protein–protein interactions, which can be studied, for instance, by surface plasmon resonance. To use the surface plasmon resonance technique, one of the interactive proteins must be immobilized on a sensor chip surface. Rhodopsin in proteoliposomes as now prepared is poised for this application. The reconstitution of rhodopsin into liposomes, as shown in this study, satisfied three requirements. First, the immobilized rhodopsin molecules in proteoliposomes are expected to be free from any constraint in undergoing conformational changes during both light activation and subsequent protein–protein interactions. Second, the membrane environment around rhodopsin is as close as possible to the native ROS membrane environment where rhodopsin normally functions. Last, nearly all of the rhodopsin molecules are aligned asymmetrically, as they are in ROS membrane, making this configuration uniquely suitable for our projected kinetic studies.

We thank Professors Wayne Hubbell at the University of California, Los Angeles, Uttam RajBhandary at the Massachusetts Institute of Technology, and Dr. Kiweon Cha of this laboratory for valuable discussions. We thank Judy Carlin for sustained assistance in preparation of this manuscript. This work has been supported by National Institutes of Health Grant GM28289 (to H.G.K.). L.N. was supported as a postdoctoral trainee by Massachusetts Institute of Technology Department of Chemistry Cancer Training Grant CA 09112.

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