Modulation of Arrestin Release in the Light-Driven Regeneration of Rh1 *Drosophila* Rhodopsin†

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ABSTRACT: We report studies of the in vitro regeneration of Rh1 *Drosophila* rhodopsin using immunochemical and spectroscopic probes for the release of arrestin (49 kDa). Upon illumination of metarhodopsin-containing membrane suspensions isolated from homogenized Drosophila heads, arrestin was released into the aqueous medium. In contrast, no release of arrestin was observed upon illumination of metarhodopsin in lipid/detergent micellar extracts. The spectroscopic changes associated with the transition from metarhodopsin to rhodopsin were, however, similar in membrane suspensions and in micellar extracts. The light-driven release of arrestin was restored in reconstituted liposomes formed by dialysis of detergent from the micellar extracts. We conclude that micellar solubilization of membranes decouples the light-driven release of arrestin from rhodopsin structural changes which are responsible for altering the λ_{max} of the chromophore. The finding that arrestin release from rhodopsin can be modulated by changes in the local membrane environment provides an opportunity to further characterize the nature of rhodopsin conformational changes during regeneration.

In vertebrate photoreceptors, light absorption by rhodopsin triggers the isomerization of the covalently attached retinal chromophore from the 11-*cis* to the *all*-*trans* configuration (Wald, 1968). The resulting changes in protein conformation culminate in the formation of the intermediate metarhodopsin II, which activates photoreceptor-specific G-proteins (Hamm, 1991; Hargrave & McDowell, 1992). Phosphorylation of metarhodopsin and arrestin binding are two key steps in the deactivation of metarhodopsin II, which eventually dissociates into *all*-*trans*-retinal and opsin (Wilden et al., 1986; Hofmann, 1986; Palczweski et al., 1989, Schleicher et al., 1989). Regeneration of rhodopsin, a process which involves arrestin release and rhodopsin dephosphorylation, requires binding of a newly synthesized molecule of 11-*cis*-retinal to opsin (Hubbard & Wald, 1952−53; Hofmann et al., 1992). Although steps in the invertebrate visual cycle are not as well characterized, a similar series of events appears to be involved in the activation of rhodopsin and the deactivation of metarhodopsin in insect, cephalopod, and Limulus photoreceptors (Blumenfeld et al., 1985; Tsuda, 1987; Dolph et al., 1993; Lee et al., 1994; Yarfitz et al., 1994). However, unlike vertebrate metarhodopsin II, metarhodopsin in invertebrate photoreceptors is thermally stable. Rhodopsin regeneration is triggered by the absorption of a second photon (Bentrop & Paulsen, 1986; Byk et al., 1993), and some evidence for short-lived optical intermediates in the regeneration pathway has been reported (Roebroek et al., 1989). Because invertebrate rhodopsins can be regenerated by light, they are particularly attractive model systems for studies of molecular mechanisms involved in visual pigment regeneration. We are studying rhodopsin regeneration in the fruit fly *Drosophila melanogaster* because it is uniquely suited to investigate steps in visual transduction using a combination of biochemical, spectroscopic, electrophysiological, and molecular genetic methods.

To understand the structural basis of the light-driven interactions of rhodopsin with key photoreceptor proteins, we are developing methods to trap and biochemically characterize intermediates generated by light absorption. Previously, we presented evidence for at least three biochemically distinct intermediates in the visual cycle (Figure 1; Kiselev & Subramaniam, 1994). Light absorption by rhodopsin generates a "thermally unstable" form of metarhodopsin (M^a) which either decays into retinal and opsin or is converted to a "thermally stable" form of metarhodopsin (M^b) following arrestin binding. Light-driven regeneration of the initial rhodopsin state occurs in at least two stages, first with the formation of an "inactive" rhodopsin-like intermediate (R^b) which is then converted to rhodopsin (R^a) in a process that involves arrestin release and rhodopsin dephosphorylation. Here, we present biochemical and spectroscopic experiments which provide further insights into the molecular mechanisms underlying the light-driven release of arrestin from Rh1 Drosophila rhodopsin. A principal finding is that in lipid/detergent micellar extracts, the light-driven regeneration process does not progress beyond the formation of R^b, i.e., arrestin is not released into the aqueous phase. These observations imply that the conformational changes initiated upon light absorption by metarhodopsin can be decoupled from conformational changes which are required for the release of arrestin.

MATERIALS AND METHODS

Chemicals. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids. 3-[(3-

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DSS, disuccinimidyl suberate; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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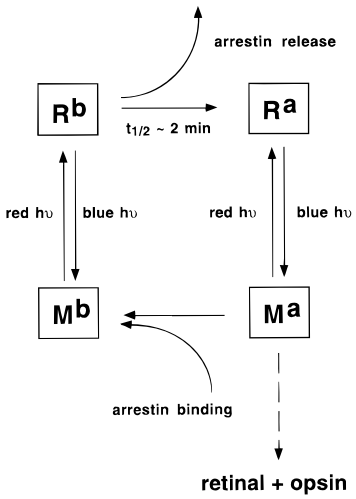

FIGURE 1: Model for the key stages in the *Drosophila* visual cycle [adapted from Kiselev & Subramaniam (1994)]. Light absorption by rhodopsin (R^a, λ_{max} ~480 nm; Schwemer & Langer, 1982, and references cited therein) generates a thermally unstable metarhodopsin (M^a, λ_{max} ~570 nm) which activates photoreceptor G-proteins efficiently. The thermally unstable metarhodopsin is either rapidly modified to a more thermally stable form of metarhodopsin (M^b), which has the same λ_{max}, or eventually decays into retinal and opsin. The binding of arrestin appears to be responsible for the increase in thermal stability and lowering of the efficiency of G-protein activation by metarhodopsin. Light absorption by arrestin-bound metarhodopsin first generates an intermediate (R^b) which has a λ_{max} indistinguishable from that of rhodopsin. Re-excitation of this intermediate, however, generates thermally stable metarhodopsin (M^b), which activates G-proteins poorly. The inactive rhodopsin (R^b) is gradually converted into rhodopsin (R^a) in a light-independent process which involves the release of arrestin.

Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and the protease inhibitors leupeptin and pepstatin A were from Calbiochem. Dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), protein A−Sepharose 4B, and Tween-20 were from Sigma. The cross-linking agent disuccinimidyl suberate (DSS) was from Pierce.

Preparation of Metarhodopsin-Containing Membranes. Wild-type, white-eyed *w1118* flies and the *ninaE ^{I17}* mutant (in a white-eyed background), which contains a deletion in the gene for Rh1 rhodopsin, were raised in the dark at 23 °C. With the exception of the controls shown in Figure 2A, all experiments reported here were carried out with wild-type flies. Adult flies were illuminated with blue light at 22 °C for 20 min, and metarhodopsin-containing membranes were isolated from fly heads as described (Kiselev & Subramaniam, 1994). For spectroscopic measurements and immunoblot experiments on the light-induced release of arrestin, membranes were suspended in buffer A (250 mM sucrose, 120 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, 1 mM DTT, 10 μg/mL leupeptin, 1.2 μg/mL pepstatin A, and 1 mM PMSF, at pH 7.0). For immunoprecipitation and cross-linking experiments, the membranes were suspended in buffer B [phosphate-buffered saline (PBS), 10 μg/mL leupeptin, 1.2 μg/mL pepstatin A, and 1 mM PMSF at pH 7.8].

Spectroscopy and Conditions of Illumination. All spectra were recorded in a UV2101 Shimadzu spectrophotometer equipped with an integrating sphere for measurements on scattering samples. A 150-W fiber optic illuminator (Cole-Parmer, Chicago, IL) equipped with colored glass filters

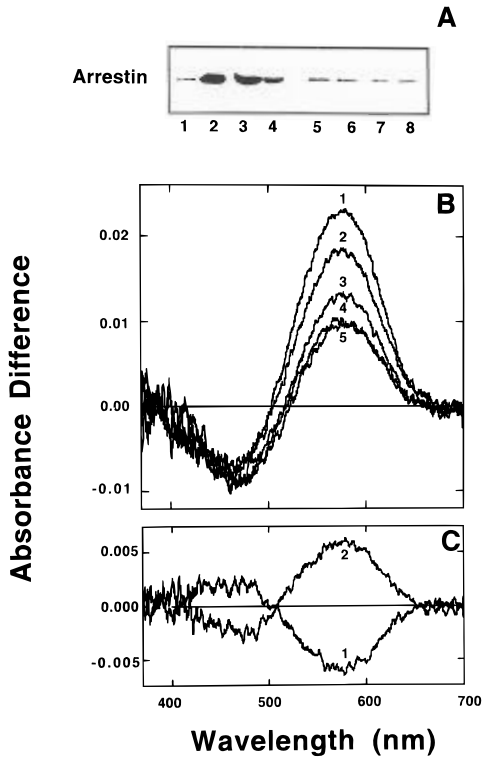

FIGURE 2: Immunochemical and spectroscopic probes for arrestin release from membranes. (A) Membrane suspensions containing metarhodopsin (generated in vivo) were isolated from the homogenized heads of illuminated flies. Lanes 1 and 3, supernatant and pellet fractions from wild-type *w1118* membranes centrifuged after being stored in the dark for 20 min; lanes 2 and 4, supernatant and pellet fractions from wild-type *w1118* membranes centrifuged after being illuminated by red light for 1 min and then stored in the dark for 20 min; lanes 5 and 7, supernatant and pellet fractions from *ninaE^{I17}* membranes centrifuged after being stored in the dark for 20 min; lanes 6 and 8, supernatant and pellet fractions from *ninaE^{I17}* membranes centrifuged after being illuminated by red light for 1 min and then stored in the dark for 20 min. All incubations were carried out at 22 °C, and centrifugations were carried out at 4 °C for 30 min. Each lane contained the equivalent of metarhodopsin from 50 fly heads. (B) Metarhodopsin-containing membrane suspensions were divided equally into sample and reference spectrophotometric cuvettes. Difference spectrum 1 was recorded following red illumination of the reference cuvette to convert metarhodopsin into rhodopsin. This protocol allowed determination of the amount of metarhodopsin present in the sample cuvette without having to directly illuminate the sample. Next, the sample cuvette was also illuminated with red light, resulting in conversion of metarhodopsin in the sample cuvette to rhodopsin. Following a wait period of 1 min, rhodopsin in the sample cuvette was reilluminated with blue light for 1 min to generate metarhodopsin. Spectra were then recorded continuously until no further decrease of the absorbance at 580 nm was observed (typically about 10 min). The final difference spectrum obtained is shown (spectrum 2). Ratio of the absorbance at 580 nm in spectrum 2 vs spectrum 1 provides a measure of the ratio $R^b/(R^a + R^b)$ at $t = 1$ min after red illumination, i.e., the ratio of rhodopsin from which arrestin has not yet been released to the total rhodopsin generated by red illumination (see text for details). A similar series of spectra was recorded following wait periods of 5 min (for spectrum 3), 20 min (for spectrum 4), or 60 min (for spectrum 5). A different sample was used for each wait period indicated, and each cuvette contained membranes from ~2500 homogenized fly heads resuspended in 1 mL. (C) Difference spectra obtained from membrane suspensions processed as in spectrum 5 above showing reversible interconversions between the residual rhodopsin and metarhodopsin upon illumination with red light (spectrum 1) and blue (spectrum 2) light. The time period between recording the two spectra was 10 min. No release of arrestin was observed upon photoconversion of the residual metarhodopsin to rhodopsin (data not shown). Each cuvette contained the equivalent of ~2000 homogenized fly heads suspended in 1 mL of buffer.

(Oriel, Stamford, CT) was used as the light source. To convert rhodopsin into metarhodopsin, samples were illuminated with blue light (405 nm < λ < 458 nm, obtained using a combination of BG-12 and GG 420 filters), and to convert metarhodopsin into rhodopsin, samples were illuminated with red light (λ > 610 nm, obtained using an RG 610 filter) as previously described (Kiselev & Subramaniam, 1994). The wavelengths for blue illumination were selected to optimally excite Rh1 rhodopsin and to minimize excitation of minor rhodopsins which absorb in the near ultraviolet region. Typical illumination times to convert metarhodopsin into rhodopsin (or vice versa) were ~1 min. This time period was chosen because longer illumination times did not result in any further light-driven changes in the absorption spectrum, whereas much shorter times resulted in lower extents of conversion. For dim red illumination and other darkroom operations, a Kodak safety lamp with a GBX-2 red filter was used.

Solubilization of Membranes. Metarhodopsin-containing membranes prepared as described above were centrifuged, and the pellet was homogenized using a Brinkmann Polytron homogenizer either in buffer A (for the spectroscopic studies) or in buffer B (for the immunoprecipitation and cross-linking experiments), each of which was supplemented with 1% DMPC, 1% CHAPS. Following overnight incubation, at 4 °C in the dark, the extracts were centrifuged 3 times (250000*g* for 20 min at 4 °C) to remove insoluble material. The supernatants were used without further processing.

Preparation of Liposomes. Detergent extracts were dialyzed in the dark at 4 °C for 96 h against PBS at pH 7.0 supplemented with 0.01 mM DTT and 0.02 mM PMSF. The resulting suspension was centrifuged (300000*g* for 30 min at 4 °C) to collect the liposomes in the pellet fraction.

Immunochemical Procedures. Polyclonal antibodies were raised against the 358−373 residue fragment of Rh1 rhodopsin and the 370−379 residue fragment of arrestin (49 kDa). Antibodies were purified from rabbit antisera with Sulfolink Gel (Pierce) coupled with the corresponding peptide fragment. The antibodies were eluted by the addition of 0.1 M glycine hydrochloride, pH 1.75, neutralized, and dialyzed against PBS at pH 7.4. Concentrations of purified anti-arrestin and anti-rhodopsin antibodies were 0.75 mg/mL and 0.45 mg/mL, respectively. For all immunoprecipitation experiments described except those presented in Figure 4B, the required amounts of membrane suspensions or detergent extracts (see legend to Figure 4) were mixed with 23 μL of anti-arrestin antibodies and incubated 10 min at 22 °C, followed by addition of 30 μL of protein A−Sepharose (6 mg of ligand/mL) and further incubation for 30 min at 4 °C. For the experiments reported in Figure 4B, 115 μL of anti-arrestin antibodies was used; all other conditions remained unchanged. All manipulations were done in the dark or under dim red illumination. Samples were analyzed by SDS−PAGE (8% gel). The buffer used to block the blots prior to incubation with anti-arrestin or anti-rhodopsin antibodies contained 5% nonfat dry milk in 20 mM Tris, 137 mM NaCl, pH 7.6, with 0.1% Tween-20. Western blots were probed using an enhanced chemiluminescence kit from Amersham.

Cross-Linking Experiments. Membrane suspensions and detergent extracts were mixed with the cross-linking reagent disuccinimidyl suberate (DSS) (final concentration 1 mM) and incubated for 10 min at 22 °C. The reaction was quenched after addition of Tris-HCl, pH 8.8, to a final concentration of 50 mM. All manipulations were carried out under dim red illumination.

RESULTS

To study regeneration under in vitro conditions, flies were illuminated in vivo with blue light to produce thermally stable metarhodopsin (M^b). Membranes were isolated from homogenized fly heads and then illuminated in vitro with red light to generate R^b, thus triggering the release of arrestin during the transition from R^b to R^a. The extent of arrestin release was followed using two different approaches. In one approach, arrestin release was determined by immunoblot analysis (with anti-arrestin antibodies) of the supernatant and pellet fractions of illuminated membrane suspensions. In the second approach, arrestin release was monitored using spectroscopic differences in the intermediates generated upon in vitro excitation of R^b and R^a. This method takes advantage of our previous result showing that whereas in vitro excitation of R^b generates thermally stable metarhodopsin (λ_{max} ~570 nm, with properties similar to M^b), the metarhodopsin produced by in vitro excitation of R^a (λ_{max} ~570 nm, with properties similar to M^a) is not thermally stable as determined by the time-dependent loss of absorbance at 580 nm. Previous studies have shown that the kinetics of arrestin release monitored by immunoblot analysis and by the spectroscopic methods are comparable (Kiselev & Subramaniam, 1994).

The experiments reported here were motivated by the finding that during in vitro regeneration, although metarhodopsin was completely converted to rhodopsin as judged by absorption difference spectroscopy, arrestin release from rhodopsin was not quantitative. This finding is summarized in Figure 2, which shows that illumination of metarhodopsin-containing membranes resulted in the release of arrestin into the aqueous medium (Figure 2A, lanes 1 and 2). However, a significant fraction of arrestin remained associated with the membranes as seen by comparing the initial membrane pellet (Figure 2A, lane 3) to the pellet remaining after illumination (Figure 2A, lane 4). Illumination with red light for longer times did not increase the amount of arrestin released. Two possible origins for the existence of this unreleased fraction are that (i) it reflects arrestin bound to proteins other than Rh1 rhodopsin and/or that (ii) the conversion from R^b to R^a is incomplete under our experimental conditions. Control experiments (Figure 2A, lanes 5−8) with *ninaE*^{I17} flies which lack Rh1 rhodopsin show that the background binding of arrestin to other membrane proteins is insignificant under our experimental conditions, thus excluding the first possibility.

Spectroscopic analysis of the illuminated membrane suspensions (Figure 2B,C) strongly supports the hypothesis that the incomplete release of arrestin is due, at least in part, to the incomplete conversion of R^b to R^a. When the regenerated rhodopsin suspensions were re-excited with blue light immediately after the red illumination, most of the metarhodopsin recovered was thermally stable. However, re-excitation of the regenerated membrane suspensions with blue light at different times after the initial illumination resulted in a progressive decrease in the amount of thermally stable metarhodopsin formed from 100% at t=0 to a plateau value of ~40% for t > 20 min, (Figure 2B). The stable metarhodopsin generated by excitation at the later times (t

> 20 min) could be reversibly inter converted to rhodopsin (Figure 2C). Since the stable metarhodopsin is generated from rhodopsin in the R^b state, the ratio of this plateau value to the initial amount of metarhodopsin provides a measure of the ratio of $R^b/(R^a + R^b)$.

Spectroscopic Transitions and Inhibition of Arrestin Release in Detergent-Solubilized Membranes. To test whether changes in the physical state of the membrane can modulate arrestin release, we solubilized metarhodopsin-containing membranes in mixed micelles containing 1% DMPC, 1% CHAPS. Under these conditions, more than 95% of the total visual pigment (i.e., the sum of rhodopsin, metarhodopsin, and opsin) and arrestin were routinely solubilized as judged by immunoblot analysis with anti-rhodopsin and anti-arrestin antibodies (data not shown).

Illumination of the solubilized metarhodopsin with red light produced rhodopsin (Figure 3A). However, in contrast to what was observed with membrane suspensions (Figure 2B), all of the rhodopsin produced had R^b-like properties; i.e., the amount of metarhodopsin recovered upon re-excitation with blue light was independent of the time elapsed between red and blue illumination, and could be quantitatively converted to rhodopsin (Figure 3A, see also Figure 5B). This light-driven inter conversion in micellar extracts could be carried out for pH values in the range 7.7−9.8 (Figure 3A−D), and the spectroscopic features of the transition were essentially similar to those reported previously for digitonin-solubilized Calliphora rhodopsin (Paulsen, 1984). With increasing pH, two absorption maxima (at ∼580 nm and ∼400 nm) were observed for metarhodopsin due to contributions from species containing protonated (acidic form) and deprotonated (alkaline form) Schiff bases. A pK of ∼8.5 was determined for the inter conversion between the two species from a plot of the pH dependence of the variation in absorbance at 580 and 400 nm. The data demonstrate that the two species are in equilibrium because illumination with red light resulted in conversion of both the 400 and 580 nm absorbing peaks to rhodopsin. Similar results were obtained upon solubilization of membranes in 1% dodecyl maltoside (A. Kiselev and S. Subramaniam, unpublished observations).

Next, we determined whether light absorption by metarhodopsin in micellar extracts resulted in release of arrestin into the aqueous medium. In the case of membrane suspensions, arrestin release into the aqueous medium was monitored by pelleting the membrane fraction, and analyzing the supernatant (Figure 2A). However, a similar protocol could not be followed with micelles, because their small size prevented pelleting by centrifugation. We reasoned that if arrestin binding to rhodopsin (and metarhodopsin) sterically interfered with its binding to anti-arrestin antibodies, one could measure the extent of arrestin release by preferentially immunoprecipitating the unbound arrestin. Determination of the amount of unprecipitated arrestin in the micellar supernatant would therefore provide an assay for the loss of arrestin from the bound state into the aqueous medium. Several control experiments were first carried out to establish that free arrestin could be immunoprecipitated under our experimental conditions. Comparison of lanes 2 and 3 of Figure 4A demonstrates that arrestin was immunoprecipitated by addition of anti-arrestin antibodies + protein A−Sepharose as shown by loss of arrestin in the supernatant. Similarly, comparison of lanes 1, 2, and 3 (Figure 4A) shows that

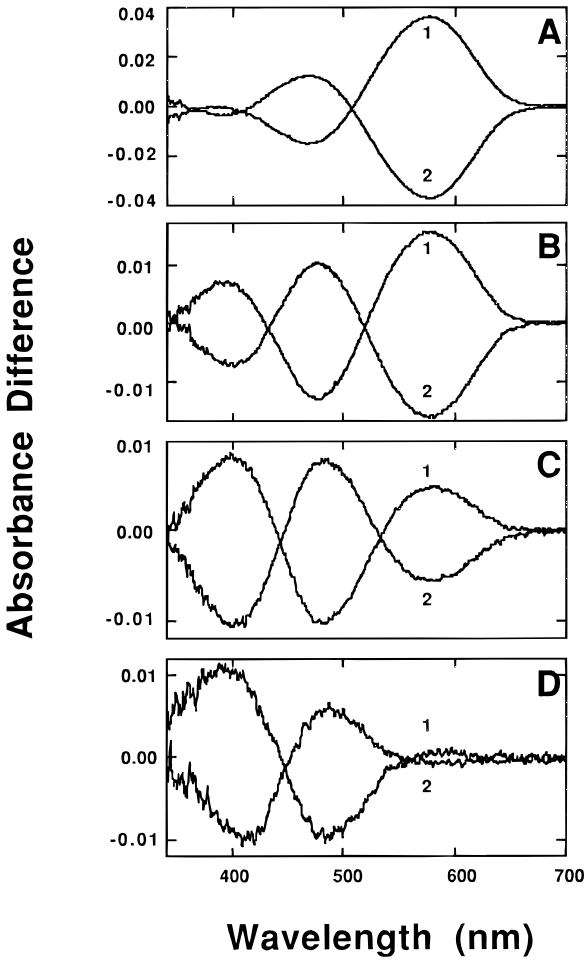

FIGURE 3: Light-driven inter conversion between rhodopsin and metarhodopsin in lipid/detergent micelles at different pH values. Metarhodopsin-containing membrane suspensions were isolated from 4000 illuminated flies, and extracted with 1 mL of 1% DMPC, 1% CHAPS dissolved in buffer A. Solubilized membranes (see Materials and Methods) were illuminated with blue light for 1 min, stored in the dark for about 1 h at 14 °C, and illuminated with red light to regenerate rhodopsin. The pH was then adjusted (within 5 −7 min) successively to 7.7 (A), 8.5 (B), 9.0 (C), and 9.8 (D), and spectra were recorded. At each pH value, an absolute spectrum was first recorded using a solution of 1% DMPC, 1% CHAPS as a reference. The sample was illuminated with blue light for 1 min, and a second spectrum was recorded. The sample was then illuminated with red light for 1 min, and a third spectrum was recorded. Spectra labeled 1 were obtained by taking the difference between absolute spectra recorded after and before blue illumination. Spectra labeled 2 were obtained by taking the difference between absolute spectra recorded after and before red illumination. All spectra were recorded at 14 °C.

immunoprecipitation of arrestin by anti-arrestin antibodies + protein A−Sepharose was not inhibited in the presence of 1% DMPC, 1% CHAPS (compare similar amounts of arrestin in lanes 1 and 2, both of which are substantially lower than the amount in lane 3).

Since arrestin can be specifically immunoprecipitated in micelles, we then carried out immunoprecipitation with micellar extracts containing metarhodopsin under identical antibody concentrations. Comparison of lanes 4 and 6 (Figure 4A) shows that similar amounts of arrestin remain in the supernatant whether the immunoprecipitation is carried out before (lane 4) or after (lane 6) illumination. Thus, no arrestin is released into the aqueous medium upon red illumination of metarhodopsin in micellar extracts, which is

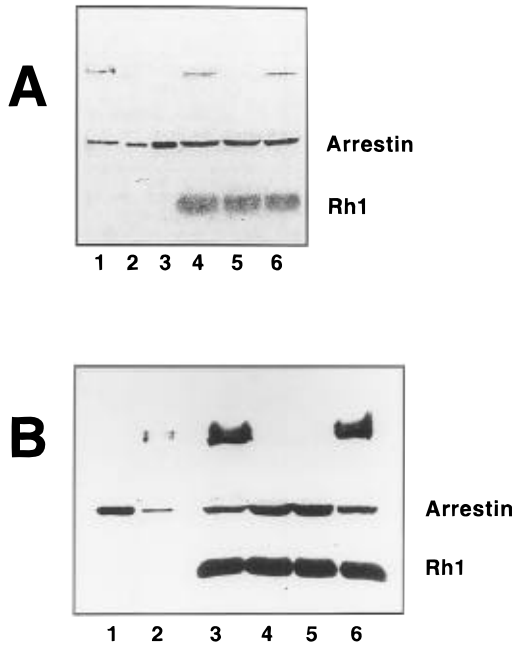

FIGURE 4: Immunochemical evidence for inhibition of light-induced dissociation of arrestin in micellar extracts. (A) (Lanes 1−3) Metarhodopsin-containing membranes from 4000 illuminated flies were suspended in 400 μL of buffer B, illuminated with red light for 1 min, stored for 20 min in the dark, and centrifuged. The resulting supernatant, which contained arrestin, was aliquoted into three equal portions. The first (lane 1) was diluted with an equal volume of buffer B containing 2% DMPC, 2% CHAPS, incubated with anti-arrestin antibodies and protein A−Sepharose and centrifuged. The second portion (lane 2) was incubated with anti-arrestin antibodies and protein A−Sepharose and centrifuged. The third portion (lane 3) was directly incubated with protein A−Sepharose and centrifuged. In each case, the supernatant containing the equivalent of 50 fly heads was analyzed. (Lanes 4−6). Metarhodopsin-containing membranes from 4000 illuminated flies were incubated with 400 μL of 1% DMPC/CHAPS, and the resulting micellar extract was divided into three equal portions. The first was stored in the dark for 10 min, incubated first with anti-arrestin antibodies (for 10 min) and then with protein A−Sepharose. After centrifugation, the supernatant was analyzed (lane 4). The second portion was illuminated with red light, stored in the dark for 20 min, incubated with protein A−Sepharose, and centrifuged, and the resulting supernatant was analyzed (lane 5). The third portion of the extract was illuminated with red light, stored for 10 min in the dark, and incubated first with anti-arrestin antibodies (for 10 min) and then with protein A−Sepharose. After centrifugation, the supernatant was analyzed (lane 6). All samples in (A) and (B) were probed with a mixture of anti-arrestin and anti-Rh1 rhodopsin antibodies. (B) Immunoprecipitation of arrestin carried out as described in (A), except that antibody concentrations used were 5-fold higher. Lanes 3, 4, and 6 correspond respectively to lanes 4, 5, and 6 of Figure 4A. Lane 5 shows as an additional control supernatant from a sample to which neither antibodies nor protein A−Sepharose were added. The similar band intensities in lanes 4 and 5 exclude that protein A−Sepharose causes any nonspecific precipitation in the absence of added antibodies. Lanes 1 and 2 correspond respectively to lanes 3 and 2 of Figure 4A. The higher molecular weight bands visible in the lanes with added antibodies are due to nonspecific cross-reactivity with the antibody, and were unaffected by the illumination protocols used.

in contrast to what was observed in membrane suspensions (Figure 2A). As expected, addition of protein A−Sepharose in the absence of anti-arrestin antibodies (lane 5, Figure 4A) did not result in loss of arrestin from the supernatant (compare arrestin amounts in lanes 4 and 5, Figure 4A). These experiments show that in DMPC/CHAPS micellar extracts, light-driven arrestin release following illumination of metarhodopsin is inhibited but the spectroscopic inter conversions between metarhodopsin and rhodopsin remain unaffected (Figure 3).

To further establish that the lack of arrestin immunoprecipitation in the micellar extracts was because binding to rhodopsin (metarhodopsin) competed with antibody accessibility, we repeated the same experiment with a 5-fold higher concentration of anti-arrestin antibodies (Figure 4B). Comparison of lane 3 with lanes 4 or 5 shows that arrestin, but not Rh1 rhodopsin, was immunoprecipitated at the higher antibody concentration. Importantly, the amount of arrestin present in the supernatant was similar before and after red illumination (compare lane 3 vs lane 6, Figure 4B), consistent with the results obtained in Figure 4A. Control experiments show that essentially all free arrestin present in solution could be specifically immunoprecipitated (see lanes 1 and 2, Figure 4B). These experiments strongly support the previous result that arrestin release is inhibited in the DMPC/CHAPS micellar extracts.

Light-Induced Arrestin Release in Reconstituted Liposomes. To test whether the observed inhibition of arrestin release in the DMPC/CHAPS micellar extracts could be reversed by detergent removal, metarhodopsin-containing extracts were dialyzed in the dark. The spectroscopic properties of rhodopsin regenerated by illumination of the resulting liposomes were then compared with those in membrane suspensions obtained from homogenized heads and in the micellar extracts. As shown in Figure 5, the conversion from R^b to R^a was observed both in membrane suspensions and in liposomes, but not in the micellar extracts, demonstrating that the spectroscopic effects of detergent solubilization were reversed by detergent removal.

To confirm whether detergent removal also restored the light-driven release of arrestin observed in the membrane suspensions, the reconstituted liposomes were illuminated with red light and centrifuged. The amount of arrestin released in the supernatant was then analyzed by immunoblots with anti-arrestin antibodies. Comparison of the amounts of arrestin released in the supernatants of liposomes stored in the dark (lane 1, Figure 6) vs liposomes illuminated with red light (lane 2, Figure 6) shows that removal of detergent restored the light-dependent release of arrestin. As in the case of the homogenized membrane suspensions from fly heads (Figure 2A), complete release of arrestin was not observed from the liposomal membranes (compare lanes 3 and 4, Figure 6).

Cross-Linking of Arrestin in Membrane Suspensions and in Micellar Extracts. The above experiments show that disruption of metarhodopsin-containing membranes by detergent solubilization inhibits the light-driven release of arrestin. One possible explanation is that arrestin release from membranes may require an abundant membrane-associated factor that is lost upon solubilization into micelles, and regained upon formation of liposomes. Washing of the metarhodopsin-containing membranes with 2 M NaCl or with 0.1% dodecyl maltoside did not significantly change the amount of thermally stable metarhodopsin recovered upon re-excitation (data not shown), arguing against regulation of arrestin release by a loosely bound peripheral factor. To determine whether the detergent solubilization indeed disrupted the packing of bound arrestin in the membrane suspensions, membrane suspensions and micellar extracts were incubated with disuccinimidyl suberate, a homobifunc-

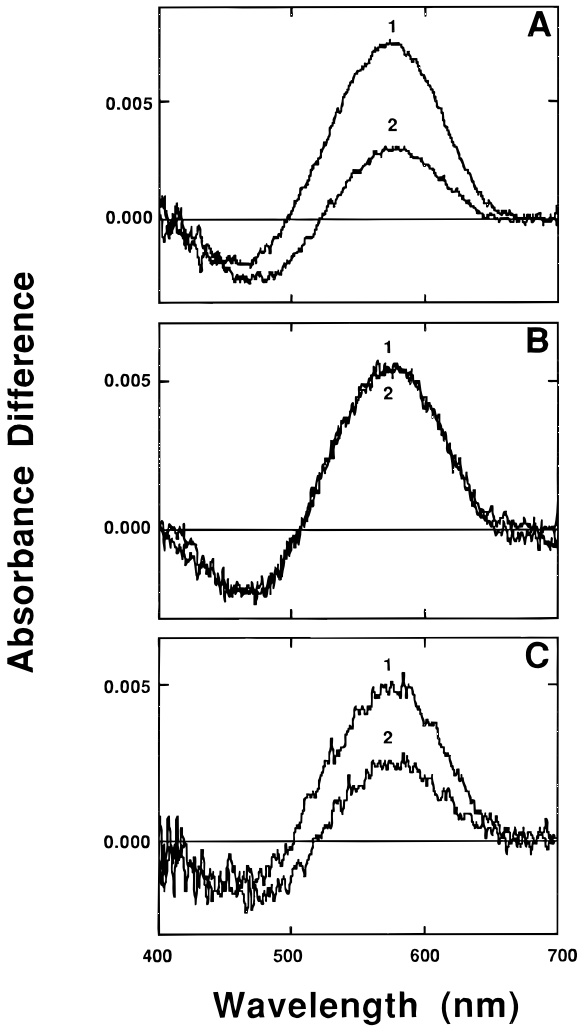

FIGURE 5: Spectroscopic transitions of rhodopsin in reconstituted liposomes. Membranes from illuminated flies were divided into three equal aliquots (each containing the equivalent of ∼1400 homogenized fly heads in 2 mL of buffer). Rhodopsin was regenerated in the first aliquot, and the amount of thermally stable metarhodopsin generated upon re-excitation of rhodopsin was determined as described in the legend to Figure 2B (panel A). In the second aliquot, the regeneration and re-excitation were carried out following solubilization with 1% DMPC, 1% CHAPS (panel B). The third aliquot was solubilized with 1% DMPC, 1% CHAPS, dialyzed to generate reconstituted liposomes, and then analyzed for the amount of thermally stable metarhodopsin (panel C). Spectra labeled 1: Difference spectrum recorded after red illumination of the reference cuvette; the absorbance at 580 nm is from the metarhodopsin present in the samples. Spectra labeled 2: Following regeneration of rhodopsin in the sample cuvette, it was then re-excited by blue light, and after a 10 min wait period, the difference spectrum was recorded to measure residual thermally stable metarhodopsin. All measurements were carried out at 22 °C. In the membrane suspensions (panel A), as well as in the reconstituted liposomes (panel C), ∼50% of the metarhodopsin produced by re-excitation of rhodopsin was thermally stable. In contrast, in the micellar extracts (panel B), essentially all of the metarhodopsin produced by re-excitation was thermally stable under our experimental conditions.

tional cross-linking agent (span 11.4 Å) specific for amino groups. Incubation of membrane suspensions with the cross-linking agent (lanes 1, 2, Figure 7) results in the formation of a ladder of bands which cross-reacted with anti-arrestin antibodies. The apparent molecular masses of the resolved lower mobility bands are ∼100 and ∼150 kDa, consistent with the expected mobilities for formation of dimers and

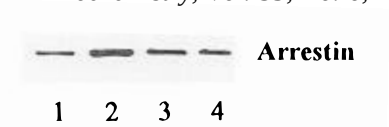

FIGURE 6: Immunoblot analysis of light-induced release of arrestin from metarhodopsin-containing liposomes. Lanes 1 and 3, supernatant and pellet fractions from liposomes centrifuged after being stored for 10 min in the dark; lanes 2 and 4: supernatant and pellet fractions from liposomes centrifuged after being illuminated by red light for 1 min and then stored in the dark for 10 min. Each lane contained the equivalent of metarhodopsin from 15 fly heads.

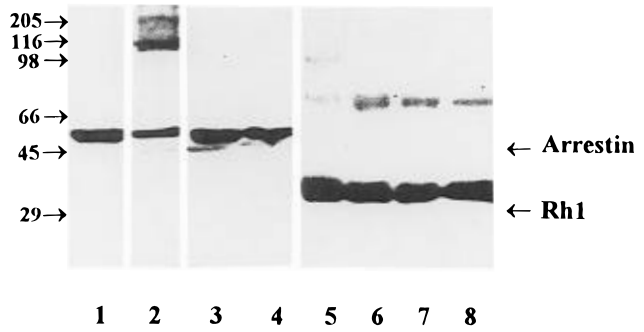

FIGURE 7: Immunoblot analysis of metarhodopsin-containing membranes and detergent extracts incubated with the cross-linking agent DSS. Membrane suspensions obtained from illuminated flies and micellar extracts of membranes in 1% DMPC, 1% CHAPS were incubated with DSS, and analyzed by SDS−PAGE (8%). Lanes 1−4 were probed with anti-arrestin antibodies, and lanes 5-8 were probed with anti-rhodopsin antibodies. Lanes 1 and 5, untreated membranes; lanes 2 and 6, membranes treated with DSS; lanes 3 and 7, untreated micellar extracts; lanes 4 and 8, micellar extracts treated with DSS. Each lane contained the equivalent of rhodopsin from 15 flies. The lower mobility bands observed in lanes 5−8 are aggregates of rhodopsin unrelated to the presence or absence of DSS.

trimers of arrestin. In contrast, no evidence for these high molecular mass bands was found when the cross-linking was carried out in the micellar extracts (lanes 3, 4, Figure 7). The higher molecular mass bands detected by immunoblot with anti-arrestin antibodies do not cross-react with anti-rhodopsin antibodies (lanes 5−8, Figure 7). These experiments imply that in membrane suspensions, a significant fraction of arrestin molecules are close enough to be cross-linked at a distance of ∼11.4 Å, a feature lost upon detergent solubilization.

DISCUSSION

The light-driven release of arrestin from rhodopsin in the visual cycle is a representative example of transmembrane signal transduction by G-protein-coupled receptors. In previous work, we showed that upon light absorption by metarhodopsin, arrestin is released from membranes with a time course that is similar to that required for the conversion of an inactive rhodopsin-like intermediate to active rhodopsin (Kiselev & Subramaniam, 1994). The new results suggest that this inactive intermediate can be trapped, and identify conditions under which spectroscopic transitions from metarhodopsin to rhodopsin can be observed without the release of arrestin. These experiments clearly identify the spectroscopic transitions and arrestin release/binding as separate molecular events which can be decoupled from each other.

The finding that arrestin release is not observed in micellar extracts suggests that changes in the physical state of the rhabdomeric membrane can modulate the light-induced release of arrestin. The effects of detergent solubilization

could be due to disruption of the membrane structure. Since rhodopsin and metarhodopsin can be reversibly photoconverted in the pH range 7−9.8 (Figures 3 and 5B), it is also unlikely that deprotonation of the Schiff's base is related to events that regulate arrestin release. The cross-linking experiments in Figure 7, which indicate that arrestin molecules interact more closely in membrane suspensions than in micellar extracts, raise the possibility that these interactions may also play some role in arrestin release. Interestingly, it has been suggested recently that the novel light-induced formation of 3-D crystalline domains in frog cone cells may be mediated by arrestin−arrestin interactions in the cone outer segments, and that this process may play a role in light adaptation (Corless et al., 1994).

It is important to note aspects of rhodopsin regeneration that are not addressed by the data presented here. First, the nature of changes in the interaction between rhodopsin and other less abundant photoreceptor components such as G-proteins, the minor arrestin (39 kDa), and rhodopsin kinase during regeneration remains to be determined. Second, the immunoprecipitation experiments (Figure 4) do not distinguish between complete inhibition of arrestin release by micellar solubilization vs a low-affinity interaction of arrestin with rhodopsin in the micelles that is sufficient to prevent immunoprecipitation. Third, we cannot exclude the possibility that the cross-linked bands in Figure 7 are simply a consequence of the close-packed arrangement of arrestin and other abundant photoreceptor components in the rhabdomeric membrane with molecular masses similar to arrestin. Further molecular insights into the mechanisms of rhodopsin regeneration will require the development of experimental conditions under which different rhodopsin−protein interactions can be reconstituted using purified components.

Inspection of the spectra shown in Figure 2B reveals a difference in the spectroscopic features of metarhodopsin decay observed here following in vitro re-excitation in comparison to the decay of metarhodopsin (M^a) observed in membranes isolated from dark-adapted flies (Kiselev & Subramaniam, 1994). Whereas metarhodopsin decay in the latter case results in the formation of free retinal, this is not apparent in the decay of metarhodopsin produced by illumination of rhodopsin (R^a) regenerated in vitro. One possibility is that the decay product of metarhodopsin has an absorption maximum close to that of metarhodopsin but with a lower extinction coefficient, thus masking its formation from metarhodopsin. An important chemical difference between rhodopsin isolated from dark-adapted flies and the rhodopsin regenerated in vitro is that the latter is expected to be phosphorylated under our experimental conditions. Thus, it is also possible that phosphorylation may have an influence on the nature of the decay product generated from metarhodopsin, either directly or by promoting interaction of metarhodopsin with other photoreceptor proteins. Because of the large scattering backgrounds observed in the spectra recorded from membranes, we have not carried out a more detailed analysis of the difference spectra to investigate the spectroscopic identity of the decay product of the metarhodopsin produced by in vitro re-excitation.

It is useful to compare the finding that the local membrane environment can modulate spectroscopic transitions in the Drosophila visual cycle with similar observations reported for the vertebrate visual cycle. The rate of formation of metarhodopsin II from metarhodopsin I is considerably accelerated upon solubilization in some detergents such as octyl glucoside, but not significantly affected in others such as dodecyl maltoside (Arnis & Hofmann 1993). Detergent solubilization also does not necessarily affect the ability of vertebrate metarhodopsin II to bind transducin (Konig et al., 1989). The extents of metarhodopsin II formation, as well as transducin activation, are much lower when rhodopsin is illuminated in solid phase membranes as compared to fluid phase membranes, arguing that steps in activation can be modulated by alterations in membrane fluidity (Baldwin & Hubbell, 1985). Since similar results were obtained upon solubilization in dodecyl maltoside micelles and DMPC/CHAPS micelles, disruption of the native structure of the *Drosophila* photoreceptor membranes appears to be a likely explanation for the absence of light-induced arrestin release in the micellar extracts.

The binding of arrestin to metarhodopsin initiates termination of signaling in the visual cascade. An interesting difference between vertebrate and invertebrate visual cycles is that whereas rhodopsin phosphorylation appears to be required for arrestin binding in vertebrates, arrestin binding is independent of rhodopsin phosphorylation in insect visual pigments (Bentrop et al., 1993; Plangger et al., 1994). A recently discovered splice variant of arrestin, p44, however, appears to bind both unphosphorylated and phosphorylated bovine metarhodopsin (Palczewski et al., 1994). There is also evidence that the phosphorylated region of bovine metarhodopsin is not required for arrestin binding, but that the phosphorylated C-terminal segment may be required to induce a conformational change in arrestin, leading to its subsequent binding to the cytoplasmic domain of metarhodopsin (Puig et al., 1995). Characterization of the specific interactions of rhodopsin and arrestin is thus clearly fundamental to an understanding of the molecular mechanisms of visual signaling. In particular, studies of the interaction of rhodopsin with the minor and major arrestins using Drosophila mutants lacking one or the other arrestin (Dolph et al., 1993) are certain to provide further comparative insights into similarities and differences between vertebrate and invertebrate visual cycles.

A central aspect of understanding transmembrane signal transduction is to define the mechanisms which underlie coupling between conformational changes induced by ligand binding and those directly involved in signaling. In the case of light-driven transitions in the rhodopsin−metarhodopsin system, solubilization may represent only one of several ways in which subtle alterations in the membrane environment can modulate coupling between structural changes in the transmembrane and cytoplasmic domains. Mutations in key regions of the transmembrane domain may also effectively result in such decoupling. The identification of in vitro biochemical conditions that can selectively inhibit arrestin release provides a useful tool to further investigate structural aspects of rhodopsin conformational changes in the visual cycle.

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