Studies of Rh1 Metarhodopsin Stabilization in Wild-Type *Drosophila* and in Mutants Lacking One or Both Arrestins†

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ABSTRACT: We have used *Drosophila* mutants which are deficient in one or both of the arrestins present in photoreceptor cells to critically test the requirements for arrestin in the stabilization of Rh1 metarhodopsin under in vitro and in vivo conditions. Heads from flies illuminated with blue light were homogenized to obtain membranes or micellar extracts, and the amount of metarhodopsin present was quantitated by spectroscopic methods. Compared to wild-type, ∼64% Rh1 metarhodopsin was recovered in flies deficient in arrestin-1 (*arr1*^{1} mutant), ∼38% in flies deficient in arrestin-2 (*arr2*^{3} mutant), and ∼6% in flies deficient in both arrestin-1 and arrestin-2 (*arr1*^{1}, *arr2*^{3} double mutant). In contrast, no decrease was observed in the amounts of Rh1 metarhodopsin recovered from illuminated flies which were deficient either in the eye-specific phosphatase (*rdgC* mutant) or in the eye-specific phospholipase C (*norpA* ^{H24} and *norpA*^{H52} mutants). Further, reconstitution experiments in total head homogenates showed that metarhodopsin produced in the *arr1*^{1}, *arr2*^{3} double mutant could be stabilized upon the addition of exogenous arrestin-2. These studies provide definitive evidence that arrestin binding stabilizes Rh1 metarhodopsin under in vitro conditions. To test whether arrestin was also required to stabilize metarhodopsin in intact photoreceptor cells, metarhodopsin was generated in *arr1*^{1}, *arr2*^{3} double mutant flies by in vivo illumination, and after a wait period of 20 min, converted back into rhodopsin by further illumination with red light. Quantitation of the regenerated rhodopsin in extracts from *Drosophila* heads showed no significant change in the level of rhodopsin recovered by this illumination protocol. Together, these experiments demonstrate that in disrupted photoreceptor cells, metarhodopsin is not stabilized unless arrestin is present, but in intact photoreceptor cells, significant metarhodopsin stabilization occurs even in the absence of bound arrestin.

Vertebrate and invertebrate photoreceptors provide attractive model systems (Hargrave & McDowell, 1992; Zuker, 1996) for the study of signal transduction by receptors which belong to the family of seven-helix, G-protein-coupled receptors (Baldwin, 1994). In both vertebrate and invertebrate photoreceptors, light absorption by rhodopsin isomerizes retinal from the 11-*cis* to the *all-trans* form, and initiates protein conformational changes which result in the formation of a series of photointermediates (Hofmann, 1986). In vertebrates, there is considerable evidence that the intermediate known as metarhodopsin II (Mathews et al., 1963) activates G-proteins in the photoreceptor cell (Bennett et al., 1982; Kibelbek et al., 1991). Phosphorylation of rhodopsin in its C-terminal region, as well as arrestin binding, plays an important role in the termination of G-protein activation by metarhodopsin (Kuhn & Wilden, 1987; Palczewski, 1994; Palczewski et al., 1992; Wilden et al., 1986). In vertebrate photoreceptors, metarhodopsin eventually decays into *all-trans* retinal and opsin (Baumann, 1972; Cone & Brown, 1969), and rhodopsin is regenerated by the recombination of opsin with 11-*cis*-retinal (Cone & Brown, 1969; Hubbard & Wald, 1952−53). However, in invertebrate photoreceptors, metarhodopsin is considerably more stable than in vertebrate photoreceptors, and rhodopsin is regenerated from metarhodopsin by further light absorption, resulting in a two-photon visual cycle (Hamdorf, 1979; Hamdorf et al., 1973; Hillman et al., 1983; Paulsen, 1984; Schwemer, 1984).

We are studying steps in the photocycle of invertebrate visual pigments using Rh1 rhodopsin, the most abundant visual pigment in the eye of *Drosophila melanogaster*, as a model system. In previous work (Kiselev & Subramaniam, 1994), we reported the surprising finding that Rh1 metarhodopsin generated by in vitro illumination of washed membranes isolated from dark-reared flies decayed into retinal and opsin rapidly ($t_{1/2}$ <2 min at 22 °C). However, membranes isolated from illuminated flies contained Rh1 metarhodopsin with greatly enhanced thermal stability ($t_{1/2}$ ∼300 min at 22 °C). A number of lines of evidence indirectly implicated arrestin binding as an important factor in the stabilization of Rh1 metarhodopsin (Kiselev & Subramaniam, 1994, 1996): (i) Arrestin, one of the most abundant rhabdomeric proteins, was present in membranes from illuminated flies but not in membranes from dark-reared flies. (ii) The amount of thermally stable Rh1 metarhodopsin recovered in illuminated flies was ∼30−40% of the total rhodopsin present before illumination, consistent with the high estimated abundance of arrestin in photoreceptor cells (Matsumoto & Yamada, 1991). (iii) Thermally unstable Rh1 metarhodopsin generated by in vitro illumination of washed membranes isolated from dark-reared flies activated G-proteins with a much greater efficiency than thermally stable Rh1 metarhodopsin isolated from illuminated flies. This observation suggests similarity to the related finding that arrestin binding inhibits the ability of vertebrate metarhodop-

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sin to activate G-proteins (Wilden et al., 1986). (iv) Following light absorption by metarhodopsin, arrestin was released from membranes with a time course that was similar to that required for the conversion of an "inactive" rhodopsin-like intermediate to "active" rhodopsin. Together, these experiments established a strong correlation between the stabilization of Rh1 metarhodopsin and the presence of bound arrestin under in vitro conditions.

To directly compare the requirement for arrestin binding in metarhodopsin stabilization under in vitro and in vivo conditions, we have now carried out spectroscopic studies of the stabilization of Rh1 metarhodopsin in *Drosophila* mutants (Dolph et al., 1993) deficient in arrestin-1 ($arr1^1$ mutant), arrestin-2, ($arr2^3$ mutant), or both arrestins ($arr1^1$, $arr2^3$ double mutant). The amount of thermally stable Rh1 metarhodopsin that could be recovered from illuminated flies of each of these mutants was quantitated both in membranes and in detergent extracts. As controls, measurements were also carried out with wild-type flies, and in three other mutants: the *rdgC* mutant (Steele et al., 1992), which lacks a phosphatase that interacts with rhodopsin; and two *norpA* mutants (Deland & Pak, 1973), which have defects in the phospholipase C (Bloomquist et al., 1988) that is activated by G-proteins in the photoreceptor cell following light absorption. The results presented here demonstrate that arrestin binding dramatically increases the stability of Rh1 *Drosophila* metarhodopsin under in vitro conditions, but that it can be stabilized in vivo even in the absence of bound arrestin.

EXPERIMENTAL PROCEDURES

Chemicals. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)[1] was purchased from Avanti Polar Lipids. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and the protease inhibitors leupeptin and pepstatin A were from Calbiochem. Dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), and 3-(*N*-morpholino)-propanesulfonic acid (MOPS) were from Sigma.

Fly Stocks. Wild-type *w1118* flies and mutant stocks in white-eyed backgrounds ($arr1^1$; $arr2^3$; $arr1^1$, $arr2^3$ double mutant; *rdgC*; $norpA^{H24}$; $norpA^{H52}$ and $ninaE^{I17}$) were raised in the dark at 23 °C.

Preparation of Metarhodopsin-Containing Membranes. 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, membranes were suspended in buffer containing 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.

Preparation of Detergent Extracts from Drosophila Heads. Heads from wild-type or mutant flies were homogenized on ice in a solution containing 1% DMPC, 1% CHAPS, 250 mM sucrose, 120 mM KCl, 10 mM MOPS, 5 mM $MgCl_2$, 1 mM DTT, 10 μg/mL leupeptin, 1.2 μg/mL pepstatin A, and 1 mM PMSF, pH 7.0. The suspension was incubated overnight at 4 °C, and then centrifuged twice at 250000*g* for 20 min at 4 °C to remove insoluble material. The supernatants were used for absorption spectroscopic measurements.

Isolation of Arrestin-2. Metarhodopsin-containing membranes from illuminated wild-type flies were suspended in 1 mL of buffer, illuminated with red light for 2 min, and kept at room temperature for 20 min. Release of arrestin from the membranes was complete over this time period (Kiselev & Subramaniam, 1994). After centrifugation of the membrane suspension at 100000*g* for 20 min at 4 °C, arrestin-2-containing supernatants were used for the reconstitution experiments described in Figure 5. Analysis of the supernatant by SDS−PAGE, followed by Coomassie stain, showed a single band corresponding to arrestin-2; no significant amount of arrestin-1 was detected (data not shown).

Immunochemical Procedures. Anti-Rh1 rhodopsin, anti-arrestin-2, and anti-arrestin-1 antibodies, raised against peptide determinants within the C-terminal region, were obtained as described (Dolph et al., 1993; Kiselev & Subramaniam, 1994). SDS−PAGE analysis and immunoblot analysis were carried out as described in Kiselev and Subramaniam (1996).

Spectroscopy and Conditions of Illumination. All spectra were recorded in a UV2101 Shimadzu spectrophotometer as previously described (Kiselev & Subramaniam, 1996). 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 Rh1 metarhodopsin into rhodopsin, samples were illuminated with red light (λ > 610 nm, obtained using an RG 610 filter). Typical illumination times to convert Rh1 metarhodopsin into rhodopsin (or vice versa) were about 1 min. For dim red illumination and other darkroom operations, a Kodak safety lamp with a GBX-2 red filter was used.

Quantitation of Rhodopsin and Metarhodopsin. The amounts of Rh1 rhodopsin and Rh1 metarhodopsin in dark-reared and in illuminated flies were determined using spectra such as those shown in Figure 3. All of the absolute absorption spectra display a peak at 412 nm whose intensity was not affected by either red or blue illumination and was proportional to the number of flies used for homogenization. Further, this 412 nm peak is near the isosbestic point for the conversion of Rh1 rhodopsin into retinal and opsin. We have used the amplitude of this peak as an internal marker for the number of fly equivalents present in the extract. Thus, the ratio x1/N1 (Figure 3A, inset) is the normalized amount of Rh1 rhodopsin present in the extract from dark-reared flies, the ratio x2/N2) (Figure 3B, inset) is the normalized amount of Rh1 metarhodopsin present in the extract from pre-illuminated flies, and the ratio x3/N2 (Figure 3B, inset) is the normalized amount of Rh1 metarhodopsin which includes contributions from the Rh1 metarhodopsin initially present in the extracts (x2/N2) and the additional Rh1 metarhodopsin generated by excitation of the fraction of visual pigment present in the rhodopsin state. From these ratios, two quantities were determined for each mutant, taking into account the 1.6-fold higher extinction coefficient of Rh1 metarhodopsin compared to Rh1 rhodopsin (Ostroy, 1978): (1) (x2/N2)/(x1/N1)/1.6, which is the scaled amount of Rh1 metarhodopsin recovered from flies illuminated with blue light; and (2) [(x3 − x2)/N2]/(x1/N1)/1.6, which is the

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

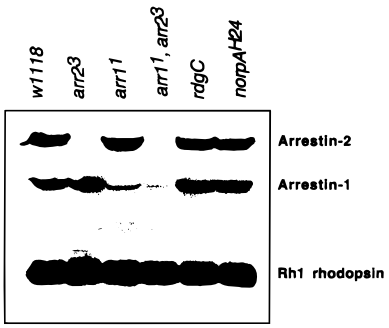

FIGURE 1: Expression levels of rhodopsin, arrestin-1, and arrestin-2 in *Drosophila* mutants defective in visual transduction. Each lane contained total homogenates from two fly heads. The immunoblot was developed with a mixture of polyclonal antibodies specific for rhodopsin, arrestin-1, and arrestin-2.

residual "regenerated" amount of the visual pigment present as rhodopsin in illuminated flies.

Since these quantities are scaled to x1/N1, which is the amount of Rh1 rhodopsin present in the flies prior to illumination, the estimates of Rh1 metarhodopsin, regenerated Rh1 rhodopsin, and total chromoprotein for each mutant can be directly compared.

RESULTS

Figure 1 shows a Western blot of heads from wild-type flies and the different mutants probed with antibodies against Rh1 rhodopsin and the two arrestins. Levels of Rh1 rhodopsin, arrestin-1, and arrestin-2 in the *rdgC* and *norpA*^{H24} mutants were similar to those in wild-type flies. As reported previously by Dolph et al. (1993), arrestin-2 was not present at a detectable level in *arr2*^{3} mutant flies, while the level of arrestin-1 was comparable to that detected in wild-type flies. Similarly, the amount of arrestin-1 in the *arr1*^{1} mutant flies was considerably lower than in wild-type flies, whereas the level of arrestin-2 was unaffected. As expected, in the *arr1*^{1}, *arr2*^{3} double mutant, neither arrestin-1 nor arrestin-2 was detected in significant amounts.

To determine whether the absence of one or both arrestins affected the amount of Rh1 metarhodopsin which could be recovered from illuminated flies, membranes were isolated by homogenizing heads from illuminated flies. The amount of Rh1 metarhodopsin present was then determined by measuring the depletion in absorbance at 580 nm following red illumination in vitro (Figure 2). In the absence of arrestin-1, only ~55% of the Rh1 metarhodopsin observed in wild-type flies was recovered. In the absence of arrestin-2, only ~19% was recovered, and in the absence of both arrestins, only ~7% of the Rh1 metarhodopsin observed in wild-type flies was recovered. The lower amounts of Rh1 metarhodopsin detected cannot be attributed to differences in the levels of rhodopsin because all mutants displayed rhodopsin levels comparable to that observed in wild-type flies (Figure 1). Together, these experiments show that the amounts of Rh1 metarhodopsin recovered in the isolated membranes are lower in the mutants lacking one arrestin and much lower in the mutant lacking both arrestins.

The spectra shown in Figure 2 were recorded using comparable amounts of flies from the different mutants as determined by their total weight. Since the average weight per fly in different mutants varied by as much as 20% (A.K. and S.S., unpublished results), we developed a method to

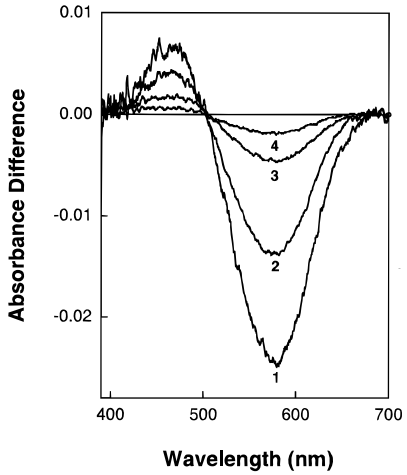

FIGURE 2: Difference absorption spectra comparing Rh1 metarhodopsin amounts recovered in membranes isolated from wild-type flies and in the *arr1*^{1}, *arr2*^{3} and *arr1*^{1}, *arr2*^{3} double mutants. In each case, approximately equal weights of flies were used to enable direct comparison of the traces. Membrane suspensions isolated from flies illuminated with blue light were divided equally into sample and reference spectrophotometric cuvettes. The sample cuvette was illuminated with red light to convert Rh1 metarhodopsin into rhodopsin, and a spectrum was recorded immediately afterward. Since Rh1 metarhodopsin is quantitatively converted into rhodopsin under these conditions, the magnitude of the negative absorbance peak at 580 nm provides a measure of the Rh1 metarhodopsin present in the membrane suspension. Each cuvette contained membranes obtained from ~1 g of flies resuspended in 1 mL of buffer.

more reliably quantitate the amounts of rhodopsin and Rh1 metarhodopsin isolated from the illuminated flies. The principle of the quantitation is outlined below, while details are presented in the legend to Figure 3 and under Experimental Procedures. In membranes isolated from dark-reared flies, all of the visual pigment is in the rhodopsin state. However, in membranes isolated from flies illuminated with blue light, the Rh1 visual pigment is present as a mixture of metarhodopsin, regenerated rhodopsin, and opsin. Metarhodopsin is present because it is generated from rhodopsin by light absorption. Due to the partial overlap between the absorption spectrum of Rh1 metarhodopsin and the wavelength range of the blue illumination used, some of the metarhodopsin absorbs light to produce regenerated rhodopsin. A fraction of the metarhodopsin also decays thermally to generate opsin and retinal. To quantitate the amounts of Rh1 metarhodopsin, rhodopsin, and opsin present in preparations from each of the mutants, flies were reared in the dark and divided into two sets. One set was illuminated with blue light, while the other was kept in the dark. Heads from both sets were homogenized, and the visual pigments were extracted with mixed micelles containing 1% DMPC, 1% CHAPS. The amount of Rh1 rhodopsin present prior to illumination was determined using extracts from dark-reared flies (Figure 3A). The amounts of Rh1 metarhodopsin and regenerated Rh1 rhodopsin present in illuminated flies were determined using extracts from the blue light-illuminated flies (Figure 3B), and the amount present as opsin in illuminated flies was deduced from these two sets of measurements. Both rhodopsin and metarhodopsin determinations were scaled to a pigment present in extracts from both dark-reared and illuminated flies with a λ_{max} at 412 nm (Figure 3), whose absorbance did not change with either blue or red illumination.

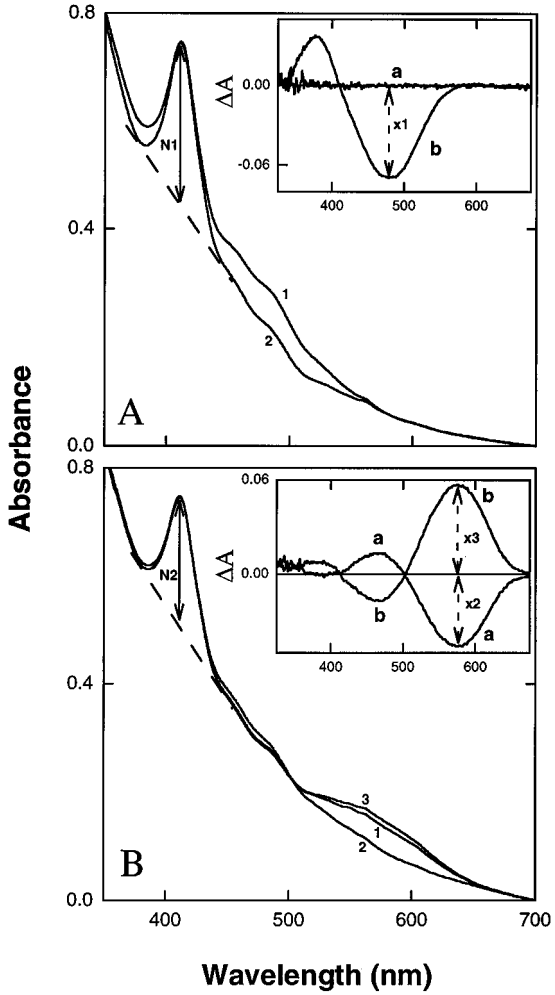

FIGURE 3: Absorption spectra of extracts of homogenized wild-type *Drosophila* heads recorded at 20 °C. (A) Spectrum 1 is the absorption spectrum of an extract from dark-reared flies. Illumination of the samples with red light did not change the absorption spectrum, demonstrating that there was no Rh1 metarhodopsin initially present (difference spectrum is spectrum a of inset). Spectrum 2 was recorded after illumination of the extract with blue light for 1 min and a wait period of 30 min at 24 °C. The inset shows the difference between spectra 2 and 1 (spectrum b), indicating the depletion of rhodopsin (λ_{max} ~480 nm) and the production of retinal (λ_{max} ~380 nm) resulting from the decay of Rh1 metarhodopsin (λ_{max} ~580 nm). (B) Spectrum 1 is the absorption spectrum of an extract from illuminated flies (same number of flies as used in panel A). Spectrum 2 was recorded following illumination of the extract with red light for 1 min, which resulted in conversion of Rh1 metarhodopsin into rhodopsin. Spectrum 3 was recorded immediately after further illumination of the extract with blue light for 1 min, which converts both the newly generated rhodopsin and also the regenerated rhodopsin already present in extract into metarhodopsin. The inset is a plot of two difference spectra: a, which is the difference between spectrum 2 and spectrum 1, showing the red light-driven conversion of Rh1 metarhodopsin into rhodopsin; and b, which is the difference between spectrum 3 and spectrum 2, showing the blue light-driven conversion of rhodopsin into metarhodopsin. All spectra were recorded at 20 °C.

Using the method outlined above, we quantitated the amounts of Rh1 rhodopsin and metarhodopsin recovered from illuminated wild-type flies and the different arrestin mutants (Figure 4A). In wild-type flies, the amount of metarhodopsin recovered was ~47% of the rhodopsin initially present. Of the remaining 53%, ~13% was present as regenerated rhodopsin, whereas ~40% was converted into opsin and retinal (Table 1). In contrast, the amounts of Rh1

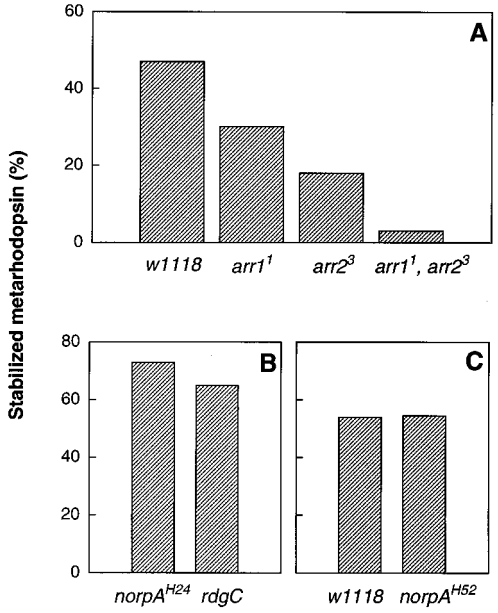

FIGURE 4: Normalized relative amounts of stabilized Rh1 metarhodopsin extracted from illuminated wild-type and mutant flies using the protocol described in Figure 3. (A) Comparison of amounts of thermally stable Rh1 metarhodopsin in wild-type and mutants deficient in arrestin-1 (*arr1*[1] mutant), arrestin-2 (*arr2*[3] mutant), or both (*arr1*[1], *arr2*[3] mutant). The values represent averages of 3−5 experiments, and the maximal deviation from the average was <5% in each case. (B) Comparison of amounts of thermally stable Rh1 metarhodopsin recovered in mutants deficient in phospholipase C (*norpA*^{H24}) or the eye-specific rhodopsin phosphatase (*rdgC*). (C) Comparison of thermally stable Rh1 metarhodopsin amounts recovered following heat shock (20 min at 37 °C) in wild-type and *norpA*^{H52} mutant flies. Under these conditions, the phospholipase C is irreversibly inactivated in the *norpA*^{H52} mutant, but not in wild-type flies (Deland & Pak, 1973).

Table 1: Normalized Amounts of Metarhodopsin, Regenerated Rhodopsin, and Opsin in Micellar Extracts from Illuminated Flies[a]

type of flies	Rh1 metarhodopsin recovered (%)	regenerated rhodopsin (%)	opsin (%)	decay of metarhodopsin in 1 h (%)
wild type (*w1118*)	47	13	40	29
arr1[1]	30	12	58	32
arr2[3]	18	10	72	31
arr1[1], *arr2*[3]	3	11	86	28
rdgC	65	8	27	15
norpA^{H24}	73	9	18	20

[a] The last column shows the fraction of Rh1 metarhodopsin which decays thermally over the first hour after warming the samples to 20 °C, and provides a measure of the relative thermal stability of Rh1 metarhodopsin in the different mutants. Note that the overall stabilities in the detergent extracts are lower than the values reported for membrane suspensions (Kiselev & Subramaniam, 1994). The values determined here for regenerated rhodopsin are also about 2-fold lower than estimates for the in vivo steady-state levels of rhodopsin after blue illumination (Bentrop & Paulsen, 1986).

metarhodopsin recovered in the *arr1*[1] single mutant, *arr2*[3] single mutant, and *arr1*[1], *arr2*[3] double mutants were ~30%, ~18%, and ~3%, respectively of the rhodopsin initially present in each case; i.e., the amounts of Rh1 metarhodopsin recovered were ~ 64%, ~38%, and ~6%, respectively, of the amount recovered in wild-type flies. In all mutants, the measured thermal stabilities of Rh1 appear to be similar, and amount of the visual pigment that was recovered as regenerated rhodopsin was ~11−12%, similar to that recovered from illuminated wild-type flies (Table 1). These experiments,

together with the results in Figure 2, strongly suggest that the majority of the Rh1 metarhodopsin recovered in wild-type flies corresponds to the population of metarhodopsin to which arrestin is bound, and support the hypothesis that the fraction of the visual pigment which is present as retinal and opsin must be derived from arrestin-unbound Rh1 metarhodopsin (Kiselev & Subramaniam, 1994). They also imply that arrestin-2 is only about 1.8 times more abundant than arrestin-1 in terms of the requirements for metarhodopsin stabilization, and that the overall abundance of arrestin relative to rhodopsin may be as high as ∼44%. This estimate for arrestin abundance is higher than the ratios based on mRNA levels (LeVine et al., 1990), but lower than the 1:1 stoichiometry suggested from metarhodopsin−arrestin binding experiments (Plangger et al., 1994).

To verify that the stabilization of metarhodopsin by arrestin binding was specific, control experiments were carried out with three other mutants which are defective in other proteins essential for light transduction. As shown in Figure 4B, the absence of the eye-specific phosphatase (*rdgC* mutant) or the eye-specific phospholipase C (*norpA*^{H24} mutant) did not lead to a reduction in the amount of Rh1 metarhodopsin recovered from illuminated flies. Interestingly, in both mutants, the amounts of metarhodopsin recovered (∼65% and ∼73% of the rhodopsin initially present) were higher than that observed in wild-type flies. Figure 4C shows another control using the temperature-sensitive *norpA*^{H52} mutant after heat shock under conditions (37 °C for 20 min) known to irreversibly denature the *norpA* phospholipase C (Deland & Pak, 1973). It can be seen that similar amounts of Rh1 metarhodopsin are recovered in wild-type and in *norpA*^{H52} mutant flies subjected to heat shock. These experiments exclude the possibility that light-triggered events requiring the *norpA* phospholipase (such as calcium influx or phosphorylation by the calcium/calmodulin-dependent kinase) are required for Rh1 metarhodopsin stabilization.

Our experiments establish that arrestin binding is required for the thermal stability of Rh1 metarhodopsin generated in vivo, and measured in vitro. We next tested whether arrestin binding also resulted in stabilization of Rh1 metarhodopsin when generated in vitro. In previous work (Kiselev & Subramaniam, 1994), we had shown that illumination of Rh1 rhodopsin in washed, arrestin-free membranes generated metarhodopsin which decayed rapidly into retinal and opsin. Here, we show that in total homogenates obtained from heads of wild-type flies, illumination of rhodopsin generates Rh1 metarhodopsin whose thermal stability is greatly enhanced in comparison to the Rh1 metarhodopsin observed in washed membranes (Figure 5A). When the same experiment was carried out with total head homogenates isolated from the *arr1*^{1}, *arr2*^{3} double mutant flies, almost all of the newly generated Rh1 metarhodopsin decayed within 20 min (Figure 5B). However, when homogenates from the *arr1*^{1}, *arr2*^{3} double mutant flies were illuminated in the presence of exogenously added arrestin-2, a majority of the Rh1 metarhodopsin was stabilized (Figure 5C). These experiments demonstrate that Rh1 metarhodopsin generated in vitro is stabilized by the addition of arrestin.

We next determined whether arrestin binding alone was *sufficient* for stabilizing metarhodopsin. Washed, arrestin-free membranes from dark-reared wild-type flies were illuminated in the presence or absence of exogenously added arrestin-2. In the absence of arrestin-2, Rh1 metarhodopsin

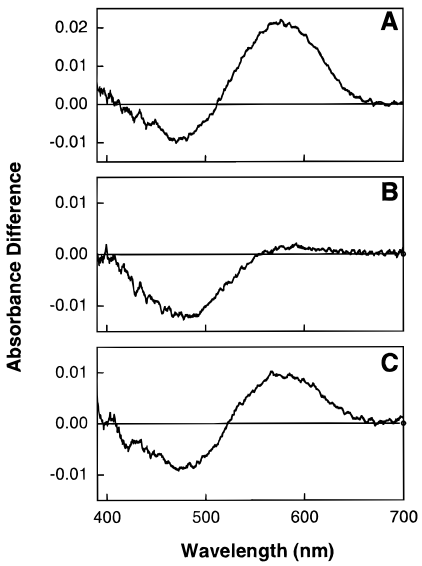

FIGURE 5: Formation of thermally stable Rh1 metarhodopsin in total head homogenates of wild-type and *arr1*^{1}, *arr2*^{3} double mutant flies. Heads from 4000 dark-reared flies were gently homogenized in 4 mL of buffer containing 250 mM sucrose, 120 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, 10 μg/mL leupeptin, and 1.2 μg/mL pepstatin A at pH 7, filtered through glass wool, and divided equally into reference and sample cuvettes. The sample cuvette was illuminated with blue light for 1 min The spectra shown were recorded 20 min after illumination at room temperature. (A) Homogenate from wild-type flies. (B) Homogenate from *arr1*^{1}, *arr2*^{3} mutant flies. (C) Homogenate from *arr1*^{1}, *arr2*^{3} mutant flies to which arrestin-2 isolated from 4000 illuminated wild-type flies was added.

generated upon illumination of washed membranes decayed rapidly into opsin and retinal within 20 min (spectrum 1, Figure 6A). Immunoblot analysis confirmed that, as expected, these membranes did not contain significant amounts of residual arrestin-2 (lane 1, Figure 6B). Upon illumination of an equivalent amount of membranes in the presence of added arrestin-2, the newly generated Rh1 metarhodopsin was stabilized to only a small extent as judged by the amount of Rh1 metarhodopsin present 20 min after illumination of the membranes. Thus, in contrast to the stabilization achieved by illuminating total homogenates of *arr1*^{1}, *arr2*^{3} double mutant flies in the presence of arrestin (Figure 5C), addition of arrestin to washed membranes stabilized Rh1 metarhodopsin rather poorly. Despite the poor stabilization, significant arrestin binding was observed to the membranes, as detected by immunoblot analysis (lane 2, Figure 6B). This amount of bound arrestin was comparable to the amount of arrestin which was bound to Rh1 metarhodopsin isolated from illuminated flies (lane 3, Figure 6B, and spectrum 3, Figure 6A). We conclude that arrestin can bind to metarhodopsin generated in washed membranes, but in contrast to total head homogenates, this binding is not sufficient to stabilize metarhodopsin.

The difference in Rh1 metarhodopsin stabilization observed between the total homogenates and washed membranes suggests that arrestin association with Rh1 metarhodopsin may be different in the two systems (i.e., homogenates and washed membranes). We have previously shown that arrestin-2 does not bind to washed membranes in the absence of illumination (Kiselev & Subramaniam, 1994). However, it is possible that arrestin is pre-associated with membranes in total homogenates. To test this hypothesis, we determined whether arrestin was prebound to

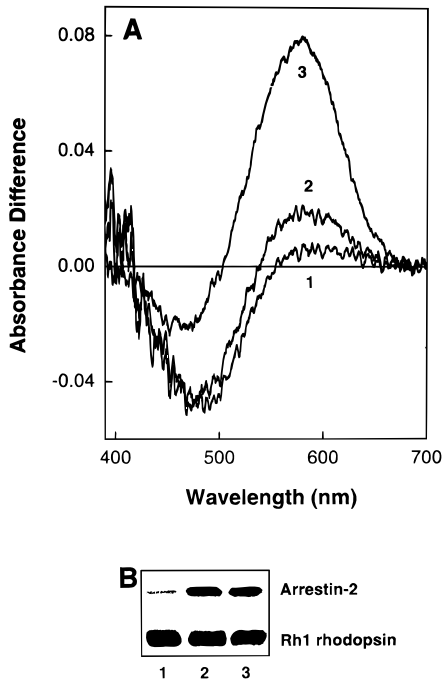

FIGURE 6: Arrestin binding and Rh1 metarhodopsin stabilization in washed membranes isolated from dark-reared flies. (A) Absorbance difference spectra recorded 20 min after blue illumination of washed membranes isolated from 4000 dark-reared flies resuspended in buffer alone (spectrum 1) or in buffer containing added arrestin-2 (spectrum 2) obtained as in Figure 5. To obtain an estimate of the amounts of thermally stable Rh1 metarhodopsin in spectra 1 and 2 as compared to that present in membranes isolated from illuminated flies, an equivalent number of flies were illuminated with blue light. Membranes were isolated, and an absorption spectrum was recorded following red illumination of the sample, which provides a measure of the maximum amount of Rh1 metarhodopsin that one may expect to obtain under the conditions of the experiment. For ease of comparison, the inverse of this spectrum is shown as spectrum 3. (B) Immunoblot analysis of the membranes whose spectra are shown above. Samples in lanes 1 (membranes from dark-reared flies; no added arrestin) and 2 (membranes from dark-reared flies; with added arrestin) are aliquots of the centrifuged membrane suspensions taken after blue illumination. The sample in lane 3 is an equivalent aliquot of metarhodopsin-containing membranes from illuminated flies.

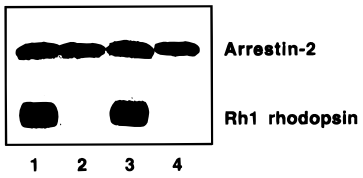

FIGURE 7: Prebinding of arrestin to membranes present in total head homogenates. Lanes 1 and 3, wild-type flies; lanes 2 and 4, *ninaE*^{I17} mutant flies. Total homogenates were prepared from dark-reared flies as described in the legend to Figure 5, and centrifuged at 50000*g* for 20 min to separate the membrane fraction. The pellet was resuspended in the same volume as the initial homogenate. Identical amounts of the initial homogenate (lanes 1 and 2) and the resuspended solution (lanes 3 and 4) were analyzed.

membranes in total homogenates isolated from dark-reared flies. The membrane fraction was separated from the supernatant by a brief, one-step centrifugation, and analyzed without further washing by immunoblotting (Figure 7). Immunoblot analysis showed that almost all of the arrestin-2 in the total homogenate was associated with the membrane fraction (compare lanes 1 and 3). Thus, in the total homogenate, there is a prebinding of arrestin to the membranes. Coomassie staining patterns of the total proteins in the supernatant indicated that a majority of the proteins present in the homogenate were not associated with the membrane pellet, arguing that the observed association of arrestin is not simply an artifact of the incomplete disruption of the photoreceptor cells. Since this property of the total homogenates is lost upon washing the membranes, it must be mediated by factors other than rhodopsin. To verify this, we determined whether arrestin prebinding was observed in *ninaE*^{I17} mutant flies that do not express Rh1 rhodopsin (lanes 2 and 4, Figure 7). As in wild-type flies, almost all of the arrestin-2 was associated with the membrane fraction. Together, these results suggest a model in which Rh1 metarhodopsin stabilization is achieved by the prebinding of arrestin to the rhabdomeric membrane, thus allowing rapid binding of arrestin to newly generated metarhodopsin.

The experiments reported here establish that arrestin binding is required for the thermal stability of Rh1 metarhodopsin as measured in vitro either in membranes or in micellar extracts. We next addressed the requirement of arrestin for metarhodopsin stabilization in the context of the intact photoreceptor cell. One reason for the absence of significant amounts of Rh1 metarhodopsin in the membranes and micellar extracts obtained from the *arr1*^{1}, *arr2*^{3} double mutant flies could be that it is unstable in vivo, and decays into retinal and opsin within the 20 min illumination period. Alternatively, it is possible that both arrestin-bound and arrestin-unbound Rh1 metarhodopsins are thermally stable in vivo over the 20 min illumination period, but that the arrestin-unbound form selectively decays into opsin and retinal upon disruption of the native environment of the photoreceptor cell. To discriminate between these two possibilities, we devised the following experiment: Wild-type or *arr1*^{1}, *arr2*^{3} double mutant flies were illuminated with blue light for 20 min to generate metarhodopsin. Immediately afterward, the flies were illuminated with red light for 10 min to convert all Rh1 metarhodopsin present into rhodopsin. The amounts of rhodopsin present after the (blue + red) illumination cycle were then compared to the rhodopsin levels present before illumination. If arrestin-unbound Rh1 metarhodopsin is thermally stable in vivo over the 20 min blue illumination period, then the subsequent red illumination is expected to result in recovery of most of the initial rhodopsin. However, if a significant amount of the arrestin-unbound Rh1 metarhodopsin decays thermally in vivo over the 20 min blue illumination period, a corresponding decrease should be observed in the amount of rhodopsin recovered by red illumination.

Figure 8 shows the implementation of the above experiment. Amounts of total "chromoprotein" (i.e., Rh1 rhodopsin + Rh1 metarhodopsin) recovered from either wild-type (Figure 8A) or *arr1*^{1}, *arr2*^{3} double mutant (Figure 8B) flies subjected to different illumination protocols were determined. Columns 1 in Figure 8A,B show measurements of the amounts of chromoprotein recovered from dark-reared flies, normalized to 100%. In column 2, the amounts of chromoprotein recovered after flies were illuminated only with blue light are shown. As expected from the results in Figure 4, upon extraction of pigment following blue illumination of wild-type flies, ~40% of the initial chromoprotein was lost. The remaining 60% is a sum of the steady-state populations of regenerated rhodopsin (~13%) and Rh1 metarhodopsin (~47%) present at the end of the 20 min period of blue illumination (see Figure 3 and Table 1).

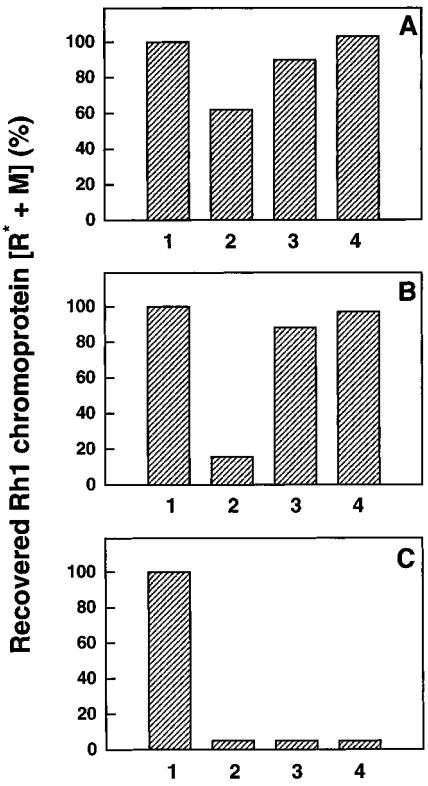

FIGURE 8: (A and B) Estimation of total Rh1 chromoprotein (rhodopsin plus metarhodopsin) amounts present in vivo in wild-type (A) and the *arr1*[1], *arr2*[3] mutant (B) flies. Approximately 2000 flies were used for each illumination protocol, and rhodopsin and Rh1 metarhodopsin levels were determined as described in Figure 3. 1, Amount from dark-reared flies (normalized to 100%), corresponding to R levels; 2, amount from flies illuminated for 20 min with blue light, corresponding to (R* + M) levels; 3, amount from flies illuminated for 20 min with blue light and then for 10 min with red light, corresponding to R* levels; 4, illuminated with two rounds of blue and red illumination as described in 3, also corresponding to R* levels. To ensure that the recovery of pigment occurred only as a consequence of red illumination, control experiments were carried out by determining the levels of visual pigment by storing the flies in the dark for 20 min after blue illumination. The amount of chromoprotein recovered was the same as in column 2, showing that the higher level of pigment recovered in column 3 is a consequence of red illumination. (C) Same illumination protocol as in panels A and B, except that the illumination was carried out on 1% DMPC/1% CHAPS extracts of washed, arrestin-free membranes isolated from dark-reared wild-type flies as in Figure 6. Identical results were obtained upon illumination of the washed membranes prior to solubilization, or upon illumination of total head homogenates obtained from *arr1*[1], *arr2*[3] double mutant flies.

Similarly, extraction of the total chromoprotein following blue illumination of the *arr1*[1], *arr2*[3] double mutant flies showed that ~86% of the initial chromoprotein was lost (column 2, Figure 8B). The remaining 14% is mostly from the steady-state amount of regenerated rhodopsin present at the end of the illumination with blue light. However, when wild-type flies were illuminated with red light following blue illumination (column 3, Figure 8A), most of the chromoprotein initially present was recovered. Even more strikingly, the same result was observed with the *arr1*[1], *arr2*[3] mutant flies (column 3, Figure 8B), indicating that most of the Rh1 metarhodopsin initially generated was converted back into rhodopsin by red illumination. In both wild-type and *arr1*[1], *arr2*[3] double mutant flies, almost all of the initial chromoprotein was recovered even following two cycles of (blue + red) illumination (column 4, Figures 8A,B). As expected, in control experiments (Figure 8C) with extracts from washed, arrestin-free membranes obtained from either wild-type or *arr1*[1], *arr2*[3] double mutant flies, no recovery of rhodopsin was observed after the cycle of (blue + red) illumination. These findings suggest that the arrestin-unbound Rh1 metarhodopsin must be thermally stable in the photoreceptor cell over at least a 40 min period, but decays rapidly upon disruption of the photoreceptor cell. This conclusion is also supported by the data in Table 1, because if arrestin-unbound Rh1 metarhodopsin was thermally unstable in vivo, a greatly reduced amount of the residual, regenerated rhodopsin would be recovered in the *arr1*[1], *arr2*[3] mutant flies as compared to wild-type flies.

DISCUSSION

The principal findings from our experiments can be summarized as follows: (i) Rh1 metarhodopsin generated by illumination of washed, rhodopsin-containing membranes isolated from wild-type flies decayed rapidly into retinal and opsin. (ii) Illumination of these washed membranes in the presence of arrestin-2 generated Rh1 metarhodopsin which can bind arrestin; however, arrestin binding to the washed membranes did not significantly stabilize metarhodopsin. (iii) A dramatic increase in Rh1 metarhodopsin stabilization was observed when it was generated in total head homogenates, which differ from the washed membranes in that none of the cytosolic or cytoskeletal components were removed from the membrane suspension. (iv) In contrast, Rh1 metarhodopsin generated in total head homogenates from the *arr1*[1], *arr2*[3] double mutant was not stabilized. However, the addition of exogenous arrestin restores the stabilization of metarhodopsin. (v) Arrestin-unbound metarhodopsin appears to be considerably more stable in vivo than either in washed membranes or in total head homogenates. This suggests that in the unique environment of the intact photoreceptor cell, arrestin binding is not the only mechanism by which Rh1 metarhodopsin can be stabilized. (vi) Once generated in vivo, arrestin-bound Rh1 metarhodopsin can be isolated as a complex which was stabilized even in washed membrane suspensions.

The results presented above allow a more incisive analysis of the role of arrestin in Rh1 metarhodopsin stabilization. Is arrestin required for stabilizing metarhodopsin? The experiments presented in Figures 4 and 5 demonstrate unambiguously that arrestin binding greatly increases Rh1 metarhodopsin stability in vitro. However, the experiment in Figure 8 shows that in vivo, arrestin-unbound Rh1 metarhodopsin is thermally stable over a time period of at least 40 min. Thus, the environment of the intact photoreceptor cell must confer stability to metarhodopsin even in the absence of arrestin. We conclude that while arrestin binding stabilizes metarhodopsin in vitro, the interactions of Rh1 metarhodopsin with other components in the intact photoreceptor cell make a major contribution to its stabilization. Is arrestin binding sufficient for Rh1 metarhodopsin stabilization in vitro? In total homogenates, the exogenous addition of arrestin-2 is both necessary and sufficient to stabilize Rh1 metarhodopsin (Figure 5). However, in washed membranes, arrestin-2 binding is not very effective in stabilizing Rh1 metarhodopsin (Figure 6), suggesting that some additional factor or structural feature present in the total homogenates, but not in the washed membranes, is required for Rh1 metarhodopsin stabilization.

Why might the prebinding of arrestin to the membranes lead to stabilization of metarhodopsin, when exogenously added arrestin clearly binds to the washed membranes upon illumination, but does not significantly stabilize it? One possibility is that, as for bovine metarhodopsin (Arnis & Hofmann, 1993), there are two Rh1 metarhodopsin conformations, both with the ability to bind arrestin, but only one of which is stabilized by arrestin binding. If the form that can be stabilized by arrestin binding precedes the other, then rapid access of arrestin (by membrane preassociation, as opposed to diffusion from the aqueous medium) to the newly formed metarhodopsin may be critical to prevent the decay of metarhodopsin into retinal and opsin.

Our experiments do not resolve the nature of factors that stabilize metarhodopsin in vivo. One possibility is that rhodopsin and/or arrestin phosphorylation, which requires factors present only in the total homogenates, plays a key role in this process. However, this appears unlikely since no significant differences in Rh1 metarhodopsin stabilization were observed upon illumination of total homogenates from wild-type flies under a variety of conditions designed to either alter or prevent phosphorylation (A.K. and S.S., unpublished results). Another possibility is that the high local protein concentration in fly rhabdoms may result in a significant lowering of the local water concentration, leading to stabilization of metarhodopsin by slowing hydrolysis of the Schiff's base between retinal and opsin. This would not be surprising given the finding that although vertebrate Rh1 metarhodopsin I is thermally unstable when produced in isolated bovine rod outer segments, it can be stabilized under conditions of lowered humidity (Applebury et al.; 1974, Korenbrot & Pramik, 1977; Wald et al., 1950). A third, and more likely, possibility is that in intact photoreceptor cells, Rh1 metarhodopsin and arrestin interact not as a bimolecular complex, but as part of a larger multiprotein complex. Thus, this complex could act as a "scaffold" to stabilize Rh1 metarhodopsin in vivo even in the absence of arrestin. When the cells are disrupted to produce total homogenates, the scaffold could still be loosely associated so that the exogenous addition of arrestin restores stabilization, but in the absence of arrestin is no longer sufficient to stabilize Rh1 metarhodopsin (Figure 5). The procedure used to obtain washed membrane fractions could lead to a complete disruption of the scaffold, with the result that arrestin addition has only a small effect (Figure 6). An intriguing hypothesis that emerges from these results is that arrestin itself may not be freely distributed in the cytosol, but may be preassociated with the rhabdomeric membrane; immunoblot analyses (Figure 7) support this hypothesis. Thus, the absence of significant stabilization of Rh1 metarhodopsin in washed membranes (Figure 6) may reflect the loss of factors from the membrane surface that promote arrestin association. A schematic summary of rhodopsin−arrestin interactions under different conditions is presented in Figure 9.

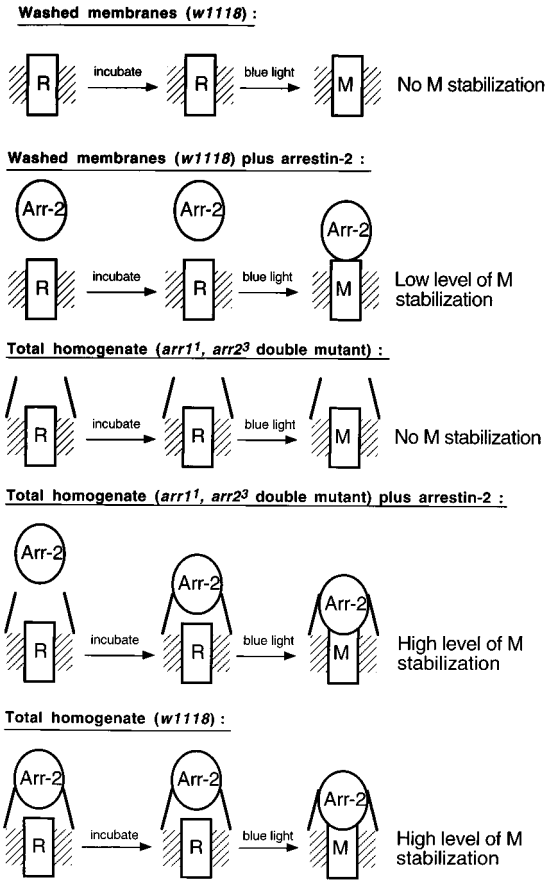

FIGURE 9: Schematic showing a hypothetical model for interactions at the membrane surface for the different experimental conditions under which Rh1 metarhodopsin stabilization was or was not observed. In washed Rh1-containing membranes obtained from wild-type flies, no stabilization of metarhodopsin was observed (panel 1; panels numbered from 1 to 5 for top to bottom, respectively). Illumination of the same washed membranes in the presence of exogenous arrestin-2 resulted in light-dependent arrestin binding, but stabilized metarhodopsin to only a small extent (panel 2). In total head homogenates from the arrestin double mutant, the absence of arrestin results in rapid decay of all the Rh1 metarhodopsin generated by illumination (panel 3). However, addition of exogenous arrestin to total homogenates from the arrestin double mutant resulted in binding of arrestin to the membranes even before illumination, and restored the stabilization of Rh1 metarhodopsin generated upon illumination (panel 4). In total homogenates from wild-type flies, arrestin was already prebound to the membranes, and produced stabilized Rh1 metarhodopsin upon illumination (panel 5).

The results in Figure 8 on the in vivo thermal stability of metarhodopsin in the *arr1*^{1}, *arr2*^{3} mutant flies are in good agreement with the prolonged depolarizing after-potential (PDA) phenomenon [reviewed by Hillman et al., (1983)], which is observed when a large fraction of the rhodopsin is converted into metarhodopsin. Studies with the arrestin mutants have led to the hypothesis that the PDA is due to the presence of arrestin-unbound Rh1 metarhodopsin (Dolph et al., 1993). It would not be possible to explain the existence of the PDA on a time scale of several minutes if Rh1 metarhodopsin decayed rapidly in vivo. Because of the long estimated lifetime (≫ 40 min) of Rh1 metarhodopsin in vivo (Figure 8), it has not been possible to directly establish whether arrestin binding also enhances the stability of Rh1 metarhodopsin in vivo. Although arrestin binding may not be required to stabilize metarhodopsin on physiological time scales in vivo, it is conceivable that it is involved in regulating the turnover of rhodopsin by protecting metarhodopsin from proteolytic degradation.

It is instructive to compare the stabilization of Rh1 metarhodopsin with studies on the stabilization of vertebrate metarhodopsin by the binding of other photoreceptor cell proteins. Experiments with intact bovine eyes show that vertebrate metarhodopsin II decays into retinal and opsin in vivo (Cone & Cobbs, 1969). However, it is possible to

stabilize bovine metarhodopsin II in vitro by the binding of either arrestin or the G-protein transducin (Emeis et al., 1982; Hofmann, 1986; Schleicher et al., 1989). In addition, bovine rhodopsin kinase can form a stable complex with activated rhodopsin, although it does not appear to stabilize metarhodopsin against eventual decay into opsin and *all-trans*-retinal (Pulvermuller et al., 1993). Although the G-proteins and/or rhodopsin kinase may also stabilize *Drosophila* Rh1 metarhodopsin, our experiments with the $arr1^{1}$, $arr2^{3}$ mutant flies provide an upper limit on the contribution of polypeptides besides arrestin in this stabilization. Thus, either the overall abundance of other proteins that can bind and stabilize Rh1 metarhodopsin is $<\sim$3% of the total rhodopsin present (Figure 4), or the binding of these other proteins does not stabilize it sufficiently under our experimental conditions.

A full understanding of the molecular mechanism of signal transduction by receptors such as rhodopsin will require knowledge of the structure in both unactivated and activated conformations. One important implication of our work is the identification of conditions to stabilize metarhodopsin on a time scale that is long enough for initiating structural studies of metarhodopsin. Another important implication is that the in vitro quantitation of stable metarhodopsin provides an effective method to investigate the formation of metarhodopsin−arrestin complexes in vivo. Thus, using site-directed mutants of rhodopsin and arrestin, it should now be possible to identify interacting regions of rhodopsin and arrestin which are important for the generation of a molecular complex. Finally, the methods developed in the course of this work provide powerful tools to critically compare protein−protein interactions important in visual transduction under both in vitro and in vivo conditions by taking advantage of the unique genetic tractability of *Drosophila*.

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