The Role of Arrestin and Retinoids in the Regeneration Pathway of Rhodopsin*

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Phototransduction results from a cascade of reactions that culminate in a neuronal signal. Photosomestications of rhodopsin (II/III), the chromophore, 11-cis-retinal to all-trans-retinal, leads to the formation of the activated photoprodut metarhodopsin II (Meta II). Subsequently, Meta II initiates the excitation events by activating many copies of the rod cell-specific G-proteins (Gt, or transducin). To terminate the signal, the long-lived Meta II must be quenched. Deactivation of Meta II involves phosphorylation by rhodopsin kinase followed by the binding of arrestin. In order to recycle rhodopsin for phototransduction, arrestin must dissociate, and the chromophore must be replaced. In this study, we show that the reduction of the photolyzed chromophore all-trans-retinal to all-trans-retinol is essential for recycling photoactivated rhodopsin. Once this reduction has occurred, the arrestin blockade of the receptor is removed, the chromophore site becomes accessible for regeneration, and the phosphates can be hydrolyzed. If the reduction does not occur, we demonstrate that free all-trans-retinal can react with the apoprotein to form pseudo-photoproducts that are spectrally identical to the photoinduced metarhodopsin species (Meta I/II/III). The Meta II-like product, M3s0, interacts tightly with arrestin and kinase, however, it does not measurably interact with Gt. The persistent blockade by arrestin and the low affinity for Gt together prevent activation of the visual cascade. Therefore, any insufficiency in the reduction of all-trans-retinal to all-trans-retinol may lead to an accumulation of M3s0-arrestin in situ, which may effect adaptational processes.

Visual transduction begins with the fast 11-cis to all-trans photosomerization of the protonated Schiff base retinyl chromophore of rhodopsin. Through a series of intermediates, the photoproduct Meta II, absorbing maximally at 380 nm, is formed. Meta II is a long-lived, activated receptor that is distinguished from all other rhodopsin intermediates by the deprotonation of the retinal Schiff base between the chromophore and apoprotein (reviewed in Ref. 1). This chromophore-protein interaction is necessary for rhodopsin to bind to Gt (1-3). Meta II catalyzes the GDP/GTP exchange of several thousand molecules of Gt (4); then, after seconds, the binding of rhodopsin kinase leads to phosphorylation of Meta II at several sites on its COOH-terminal extension (reviewed in Ref. 5). Phosphorylation of Meta II is required to switch from a state in which it activates Gt to a state in which it binds the inhibitory protein arrestin (6). Bound arrestin blocks activation of transducin and the excitation cascade of phototransduction (6-8). Thus, rhodopsin-mediated activation of the visual transduction cascade is shut off after 2-3 s (9), long before the spontaneous decay of Meta II. During its lifetime, Meta II is in equilibrium with its predecessor Meta I and decays via Meta III and/or directly to the apoprotein opsin and free all-trans-retinal (1, 10, 11).

We have reinvestigated the post-Meta II decay and regeneration of photolyzed rhodopsin and the influence of arrestin and retinoids on the regeneration pathway of rhodopsin. We present evidence that a key step for the decay reaction of photoactivated rhodopsin is the reduction of the photolyzed chromophore all-trans-retinal into all-trans-retinol. In the presence of all-trans-retinal, opsin reacts with the chromophore to form pseudo-photoproducts that bind arrestin and rhodopsin kinase. Upon reduction of all-trans-retinal to all-trans-retinol, arrestin is released from phosphorylated opsin.

MATERIALS AND METHODS

All reagents were purchased from Sigma. 11-cis-Retinal was a gift from P. Brown (Hoffmann-La Roche, Nutley, NY) and from the National Eye Institute of the National Institutes of Health. 

Protein Preparation—Rod outer segment (ROS) membranes were isolated from fresh, dark-adapted bovine retinas (12). Rhodopsin, in the native disc membranes, was prepared by removing the soluble and membrane-associated proteins at low ionic strength (13). Opsin was prepared from rhodopsin by thorough bleaching with white light for 30 min at 30 °C in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl in the presence of 25 mM NH4OH to facilitate conversion of retinals to their retinal oxime (14). Membranes were collected by centrifugation (45,000 × g for 15 min), and the supernatant containing excess NH4OH was discarded. Trace amounts of NH4OH were neutralized with 10 mM formaldehyde for 5 min at 0 °C (15). Finally, opsin was washed twice with 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl, and suspended in the same buffer.

Phosphorylated opsin was prepared from fresh bovine retinas (16). A stoichiometry of 3 to 4 P/rhodopsin was determined using radioactive [γ-32P]ATP as a tracer. After phosphorylation, membranes were collected by centrifugation (45,000 × g for 10 min). The pellet

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*The abbreviations used are: Gt, rod cell-specific G-protein (transducin); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Meta II, metarhodopsin II; PAGE, polyacrylamide gel electrophoresis; ROS, rod outer segments; SDS, sodium dodecyl sulfate.
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**A.** The centrifugation assay of the light-dependent binding of arrestin to phosphorylated Meta II. Under red light, phosphorylated rhodopsin (30 μM) was mixed with purified arrestin (5 μM) in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl (total volume of 60 μL). The sample was either incubated in the dark (labeled −) or illuminated for 5 min under white light (labeled +) at 30 °C. Illumination of rhodopsin with a 180-watt lamp at 10-cm distance yielded 45–50% bleached rhodopsin. The arrestin/phosphorylated Meta II mixture was centrifuged using an air-fuge (Beckman, 180,000 × g) for 2 min. Supernatant was stored for SDS-PAGE (labeled S). Pellet was washed with 180 μL of the Hepes buffer, centrifuged for 2 min at 180,000 × g, and dissolved in 55 μL of 1% SDS, containing 0.1% 2-mercaptoethanol, for SDS-PAGE analysis (labeled P).

**B.** The instability of phosphorylated Meta II-arrestin complex in the presence of NH$_2$OH. The experiment was performed as described in A, and the supernatants were analyzed using SDS-PAGE. After illumination, the sample was incubated in the dark at 30 °C for 5 or 10 min with either 10 mM Hepes buffer, pH 7.5, and 100 mM NaCl or with the same buffer supplemented with 1 mM NH$_2$OH. Supernatants were analyzed as indicated. In the control experiment, the sample was neither illuminated nor exposed to NH$_2$OH. C, influence of NH$_2$OH on flash-induced light scattering signals at 820 nm from a suspension of phosphorylated rhodopsin and arrestin. Phosphorylated rhodopsin (5 μM) in the native disc membrane was incubated with arrestin (0.3 μM) in 100 mM Hepes buffer, pH 7.0, in the presence or absence of 2 mM NH$_2$OH, in a 10-mm cuvette at 20 °C. As indicated, the sample was illuminated by a 500 ± 20 nm flash of light (f), which photolyzed 8% of the rhodopsin suspension. Light-scattering signals were recorded at 820 nm. In the control experiment, the sample was prepared without arrestin or NH$_2$OH.

**D.** Influence of arrestin on NH$_2$OH-induced decay of phosphorylated Meta II. Phosphorylated rhodopsin (15 μM) in the native disc membrane was incubated with or without arrestin (3 μM) in 100 mM Hepes buffer, pH 7.0, in the presence of 1 mM or 2 mM NH$_2$OH (marked on the figure) at 20 °C. As indicated, the sample was illuminated by a flash of green light (g), which photolyzed 20% of the rhodopsin suspension, and the absorption differences between 450 and 417 nm were recorded. The flash-induced transition from P-rhodopsin (500 nm) to a photoproduct (450-470 nm) is observed as the subsequent slow positive absorption change (21). The rate of decay depends on NH$_2$OH and arrestin. The level of absorption that is reached after the decay of Meta II is higher in the control than in the sample containing arrestin. This reflects the formation of decay photoproducts absorbing in the 450–470 nm range which are inhibited by arrestin (analogous to observations with added G, (21)).

was washed with 100 μL of 67 mM sodium phosphate buffer, pH 7.5, and 100 μL of water. Incubation with and removal of NH$_2$OH, and two washing steps were performed as described for the preparation of opsin (above).

Phosphorylated rhodopsin was prepared by regeneration of phosphorylated opsin with 11-cis-retinal. Phosphorylated opsin was suspended in 10 μL of 67 mM sodium phosphate buffer, pH 7.5, 1 mM MgCl$_2$, and 1 mM dithiothreitol. A 5-fold molar excess of 11-cis-retinal was added to the sample in the dark, followed by incubation for 3 h at room temperature, and then overnight at 5 °C. After regeneration (105–110%), phosphorylated membranes were collected by centrifugation (45,000 × g for 10 min). The pellet was washed with 100 μL of 67 mM sodium phosphate buffer, pH 7.5, and the excess of 11-cis-retinal was converted to the retinal oxime by incubation with NH$_2$OH. The washing procedure was identical to that described for opsin (above).

Arrestin was purified as described (17), and G, was isolated from thoroughly washed ROS by elution with GTP (13). SDS-PAGE was performed according to Laemmli (18).

**Assay of Retinol Dehydrogenase**—The activity of retinol dehydrogenase was assayed in 10 mM Hepes buffer, pH 7.5, containing 20 μM phosphorylated rhodopsin and 20 μM arrestin. The reaction mixture was illuminated for 5 min under white light (a 180-watt lamp at 10-cm distance) at 30 °C. The sample was transferred to dark, and 80 μM NADP or NADPH was added. The sample was incubated for 1, 3, 5, 10, or 20 min at 20 °C. All-trans-retinol was extracted (19), and the retinol formation was monitored by following the increase in fluorescence intensity at $\lambda_{em} = 470$ nm ($\lambda_{ex} = 327$ nm) (20) using a Perkin-Elmer LS 50 Luminescence Spectrometer. The calibration was performed using commercial all-trans-retinol.

**Spectrophotometry**—A two-wavelength spectrophotometer (Shimadzu UV360) was used to measure the differences of absorption at 450 and 417 nm. The absorption at 417 nm is the isobestic point between Meta I and II, and normalizing this wavelength subtracts light-scattering artifacts from the absorption signal. Samples containing phosphorylated rhodopsin (15 μM) and arrestin (3 μM) were prepared in 100 mM Hepes buffer, pH 7.0, at 20 °C. The formation of Meta II was induced by flash photolysis of 20% of the rhodopsin in the sample. The principle of the decay of Meta II in the presence of G, has been described previously (21).

**Light Scattering**—Light scattering changes at an angular range of 16 ± 2° were monitored using 820 nm of light (22) in a 10-mm cuvette.
Centrifugation. The concentration of nucleotides and retinoids was 2 mM and 120 μM, respectively. The mixture was illuminated for 5 min, and 2 μl of either 95% ethanol (final concentration 0.2%) or 11-cis-retinal (final concentration 230 μM) was added. After 1, 10, 20, 30, and 40 min of incubation in the dark at 30 °C, the supernatant after centrifugation, in the presence and absence of 11-cis-retinal, was added. The remainder was subjected to protein analysis using the Bradford method (26). The time course of rhodopsin regeneration was analyzed by the binding signal of arrestin, and accordingly, the negative initial deflection is the Meta I1-related N-signal which causes irreversible decay of Meta I1 by forming all-trans-retinal oxime, a second flash evokes a full signal (when normalized to the lower photoexcitation, Fig. 1C). This shows that the reaction of rhodopsin leads to the translocation of soluble arrestin from the supernatant to the membrane fraction. After the fast binding, the release of arrestin from phosphorylated Meta II is slow (Fig. 1B) and requires removal of all-trans-retinal from rhodopsin’s binding pocket, e.g. by NH₂OH (Fig. 1B). Binding and release of arrestin was also monitored in real time by kinetic light scattering-binding signals. The data in Fig. 1C are from two flash experiments. The negative initial deflection is the Meta II-related N-signal or rhodopsin signal (22, 23), and the subsequent slow arrestin-binding signal arises from the gain of mass of the phosphorylated membranes by the binding of arrestin (analogous to the G-binding signal (22–23)). The upper record shows that, after completion of the signal from a first flash, a second flash does not elicit a second binding signal. Thus, arrestin was quantitatively bound to the equimolar phosphorylated Meta II formed by the first flash. In the presence of 2 mM NH₂OH, which causes irreversible decay of Meta II by forming all-trans-retinal oxime, a second flash evokes a full signal (when normalized to the lower photoexcitation, Fig. 1C). This shows that the phosphorylated Meta II-arrestin complex from the first flash has been completely dissociated by adding NH₂OH. More NH₂OH proportionally accelerates the decay of the scattering signal (data not shown) and the reappearance of the particle mass with a correction factor α, which corrects the differences primary in the refractive index and density between arrestin and membranes, accordingly $dL/I_0 = 2dM/M$. Thus, $dL/I_0$ is proportional to $dM/M$.

**RESULTS**

**Does NH₂OH Induce the Release of Arrestin from Phosphorylated ROS Membranes?**—To investigate the role of arrestin on the quenching pathway of photolyzed rhodopsin, the binding of arrestin to ROS membranes and its subsequent release were investigated. Binding and release of arrestin from phosphorylated Meta II was assayed using a centrifugation method and SDS-PAGE/colorimetric method to determine the amount of protein either bound to the membrane pellet or present in the supernatant (Fig. 1A and B). Fig. 1A shows that the photoactivation of rhodopsin leads to the translocation of soluble arrestin from the supernatant to the membrane fraction. After the fast binding, the release of arrestin from phosphorylated Meta II is slow (Fig. 1B). Binding and release of arrestin was also monitored in real time by kinetic light scattering-binding signals. The data in Fig. 1C are from two flash experiments. The negative initial deflection is the Meta II-related N-signal or rhodopsin signal (22, 23), and the subsequent slow arrestin-binding signal arises from the gain of mass of the phosphorylated membranes by the binding of arrestin (analogous to the G-binding signal (22–23)). The upper record shows that, after completion of the signal from a first flash, a second flash does not elicit a second binding signal. Thus, arrestin was quantitatively bound to the equimolar phosphorylated Meta II formed by the first flash. In the presence of 2 mM NH₂OH, which causes irreversible decay of Meta II by forming all-trans-retinal oxime, a second flash evokes a full signal (when normalized to the lower photoexcitation, Fig. 1C). This shows that the phosphorylated Meta II-arrestin complex from the first flash has been completely dissociated by adding NH₂OH. More NH₂OH proportionally accelerates the decay of the scattering signal (data not shown) and the reappearance of the particle mass with a correction factor α, which corrects the differences primary in the refractive index and density between arrestin and membranes, accordingly $dL/I_0 = 2dM/M$. Thus, $dL/I_0$ is proportional to $dM/M$.

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of arrestin in the supernatant</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 7.7%</td>
<td>9</td>
</tr>
<tr>
<td>In the light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (without retinoids and nucleotides)</td>
<td>22 ± 4.3</td>
<td>8</td>
</tr>
<tr>
<td>+ NAD</td>
<td>21 ± 2.2</td>
<td>4</td>
</tr>
<tr>
<td>+ NADP</td>
<td>22 ± 1.2</td>
<td>4</td>
</tr>
<tr>
<td>+ NADH</td>
<td>26 ± 2.8</td>
<td>4</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>52 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>+ 11-cis-Retinal</td>
<td>56 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>+ 9-cis-Retinal</td>
<td>43 ± 2.2</td>
<td>4</td>
</tr>
<tr>
<td>+ All-trans-retinal</td>
<td>4 ± 2.2</td>
<td>4</td>
</tr>
<tr>
<td>+ All-trans-retinox</td>
<td>24 ± 3.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Release of arrestin analyzed as in Figs. 1 and 2; the samples after bleaching have been incubated in the dark for 20 min at 20 °C before centrifugation. The concentration of nucleotides and retinoids was 2 mM and 120 μM, respectively.
arrestin in the supernatant. Thus, arrestin is released when phosphorylated Meta II decays, and the dissociated arrestin can immediately rebind to phosphorylated Meta II formed by the second flash. Fig. 1D shows that the NH₂OH-induced spectroscopic decay of phosphorylated Meta II is influenced by bound arrestin. The 450/417-nm absorbance difference drops when phosphorylated Meta II is formed and recovers with the decay of Meta II into photoproduct(s) in the 450–480-nm range that precede the decay to opsin and retinal oxime (Aₓmax = 365 nm). The time scale in Fig. 1D was chosen to show the decay of Meta II to photoproducts absorbing in the 450–480 nm. Phosphorylated Meta II, like unphosphorylated Meta II (21) decays faster at higher concentrations of NH₂OH. Similar observations were made for the phosphorylated Meta II-arrestin complex, except that the decay of Meta II to retinal oxime is slower in proportion to the increase of bound arrestin. The recovery of the absorbance difference signal (after full occupation of phosphorylated Meta II by arrestin) has a time course that is comparable to the decay of the scattering signal (Fig. 1C). Fig. 1 demonstrates that bound arrestin does not prevent reaction of NH₂OH with the photocytolyzed chromophore, but rather slows the decay reaction.

Do Retinoids Alter the Release of Arrestin from Phosphorylated ROS Membranes?—The release of bound arrestin is influenced specifically by the presence of retinoids. For example, 11-cis-retinal enhances the release with respect to the control (Fig. 2, A and B) which parallels the regeneration of phosphorylated opsin (Fig. 2C). Similarly, the enzymatic reduction of endogenous all-trans-retinal to retinol, after the decay of Meta II, should favor the dissociation of the arrestin-bound Meta II-arrestin complex, except that the decay of Meta II to retinal oxime is slower in proportion to the increase of bound arrestin. The recovery of the absorbance difference signal (after full occupation of phosphorylated Meta II by arrestin) has a time course that is comparable to the decay of the scattering signal (Fig. 1C). Fig. 1 demonstrates that bound arrestin does not prevent reaction of NH₂OH with the photocytolyzed chromophore, but rather slows the decay reaction.

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<table>
<thead>
<tr>
<th>Sample</th>
<th>Retinol dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the dark</td>
<td></td>
</tr>
<tr>
<td>Arrestin + NADP</td>
<td>0</td>
</tr>
<tr>
<td>Arrestin + NADPH</td>
<td>0</td>
</tr>
<tr>
<td>In the light</td>
<td></td>
</tr>
<tr>
<td>+ NADP</td>
<td>0</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>7 ± 1.25</td>
</tr>
<tr>
<td>Arrestin + NADP</td>
<td>0</td>
</tr>
<tr>
<td>Arrestin + NADPH</td>
<td>4.25 ± 0.75</td>
</tr>
</tbody>
</table>

* The activity is expressed in nmole of retinol formed/minute/μmole of rhodopsin, and the results are the average of five independent experiments.

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**Fig. 3.** Reversible binding of arrestin to phosphorylated opsin and the presence of retinoids and formation of pseudophotoproducts. A, binding of arrestin to phosphorylated opsin and phosphorylation of opsin by rhodopsin kinase; effect of retinoids. B, formation of pseudo-photoproducts: a, formation of 380-nm product (M₃₈₀) and 470-nm product (M₄₇₀) from phosphorylated opsin and all-trans retinal; b, stabilization of M₃₈₀ by arrestin at the expense of M₄₇₀; and c, absence of destabilizing effect of GTP in a mixture of opsin, all-trans-retinal, and transducin. Arrestin binding was assayed as in Fig. 1. Rhodopsin kinase was prepared and assays were performed as described (28). Briefly, purified rhodopsin kinase was added to washed and bleached disc membranes in the presence of [γ-³²P]ATP and retinoids in the dark, and the incorporation of ³²P into opsin was measured. The absorption measurements (B) employed a Shimadzu UV 3000 spectrophotometer. Quartz cuvettes (2-mm path length) were placed in the measuring and reference beam of the spectrometer, close to the end-on photomultiplier. The sample and reference were filled with the phosphorylated opsin or opsin membrane suspension (400 μL, 5 μM phosphorylated opsin or opsin) and the starting agents. Reactions were started by adding all-trans-retinal (4 μL, final concentration 10 μM) to the phosphorylated opsin sample and alcohol to the reference (a), by adding arrestin (final concentration 5 μM) to the phosphorylated opsin sample and arrestin buffer to the reference, both after equilibration with all-trans-retinal (b), or by adding guanosine 5'-O-(3-thiotriphosphate) (final concentration 25 μM) to the opsin sample and buffer to the reference, both after equilibration with all-trans-retinal and G (c). All spectra were plotted against a base line taken after equilibration. In each set (a–c), the solid line represents the final spectrum; in Bb, the dotted line represents calculated absorption due to the turbidity change from arrestin in the sample. The turbidity causes a curved base line. The dotted line fits the equation: E = a · λ² + b, where E is the absorption (turbidity) of the sample at λ (wavelength, nm), and a and b are parameters. All records were made at 2.5-min intervals, in 100 mM Hepes buffer, pH 7.6, at 8 °C.
photoproducts; Fig. 4. **Flux diagram of rhodopsin deactivation and regeneration.** Phototransduction begins by the photoisomerization of rhodopsin's chromophore, 11-cis-retinal, to all-trans-retinal, resulting in conformational changes of rhodopsin \((R)\). The active conformation \((R^*P)\), identified with the Meta II photoprodct, initiates the excitation cascade of reactions by the activation of several thousands of \(G\)-proteins \((4, 25, 27)\). To terminate the light signal, Meta II is deactivated by a second messenger-independent protein kinase, rhodopsin kinase \((5)\). Several Ser and Thr residues are phosphorylated by this kinase at the COOH-terminal of Meta II \((28)\), \((R^*P)\). Autophosphorylation of rhodopsin kinase is also taking place at this time, which may lower the affinity of the kinase to Meta II \((29)\). The dissociation of the kinase is followed by the binding of arrestin \((6)\). The further decay reaction of Meta II \((in\ equilibrium with free all-trans-retinal and pseudophotoproducts; Fig. 3, omitted in Fig. 4) results from the reduction of the photolyzed free all-trans-retinal into all-trans-retinol by disc membrane-associated retinol dehydrogenase \((30)\). Once this reduction has occurred, arrestin dissociates from now inactivated phosphorylated opsin \((O)\). The chromophore is regenerated, and phosphates are removed by protein phosphatase 2A \((PrP 2A (31))\).

**Does Opsin React with All-trans-retinal to Form Products That Can React with Arrestin, Rhodopsin Kinase, or \(G\)?**—Results presented in Table I suggest that the free trans isomer of the chromophore can either be reduced to noninteractive retinol or associated with opsin to produce an arrestin-interactive species of rhodopsin. This can be tested more directly by a protocol in which one starts from purified phosphorylated opsin and studies the binding of arrestin in the presence of retinoids. The data in Fig. 3A demonstrate that all-trans-retinal leads to the binding of arrestin from the supernatant and also to the increased phosphorylation of opsin by rhodopsin kinase. Neither 11-cis-retinal or all-trans-retinal is effective in inducing the binding of arrestin. These results indicate the formation of an arrestin-interactive species between opsin and all-trans-retinal. It was important to establish whether this interactive species formed between phosphorylated opsin and all-trans-retinal has properties similar to those of Meta II obtained from photolyzed rhodopsin. Meta II can be spectrally assayed by its equilibrium with the different tautomeric forms of Meta I \((A_{max} = 478 \text{ nm})\) and/or Meta III \((A_{max} = 470 \text{ nm})\). Under conditions that favor Meta I/III \((8 \celsius, \text{pH 7.5})\), a species with an absorption maximum in the 470-nm range \((M_{470})\) is indeed formed from phosphorylated opsin and all-trans-retinal \((Fig. 3B, a)\). When arrestin is added to an equilibrated all-trans-retinal/phosphorylated opsin mixture, the 470-nm absorption is shifted to 380 nm \((M_{380})\) \((Fig. 3B, b)\) with the same kinetics as in Fig. 3B, a. The formation of \(M_{470}\) is independent of phosphorylation \((data not shown)\); however, the binding of arrestin and formation of \(M_{380}\) requires phosphorylated opsin. These results indicate the formation of \(M_{470}\), which is in equilibrium with the arrestin-interactive species \(M_{380}\). The accurate absorption maximum has not been determined, since many different forms of all-trans-retinal contribute to this spectrum, for which most of the absorption coefficients are unknown.

While spectroscopically similar to Meta I and II, \(M_{470}\) and \(M_{380}\) are strikingly different from true metarhodopsin photoproducts. First, the formation of \(M_{470}\) from all-trans-retinal and phosphorylated opsin, even at low temperature, is faster than the decay of the metarhodopsin species into subsequent photoproducts \((data not shown)\). More importantly, we could not find an interaction of the reversibly formed pseudophotoproducts with \(G\). When the protocol used for arrestin \((Fig. 3B, b)\) was repeated with \(G\) and opsin \((or phosphorylated opsin)\), no significant absorption changes were observed \((data not shown)\). Moreover, Fig. 3B, c demonstrates that GTP does not produce the rise of \(M_{470}\) absorption in a retinal-opsin-G mixture, which is expected if \(G\) was activated and released. These data demonstrate that all-trans-retinal can reversibly reactivate opsin only to a limited degree, which is in agreement with the results obtained from GTPase assays \((24)\).
**DISCUSSION**

**Dissociation of the Rhodopsin-Arrestin Complex Requires Removal of the Chromophore**—We have shown that the release of arrestin from phosphorylated ROS membranes depends on the presence of photoisomerized chromophore, all-trans-retinal. Arrestin is released from phosphorylated ROS membranes when the chromophore is removed by the action of N,NH$_2$OH or reduced to inert all-trans-retinol by retinol dehydrogenase. In vivo, only the reduction reaction is physiologically significant.

**All-trans-retinal Reacts with Opsin**—We have shown that the all-trans-retinal, after its release, can rebind to the apo-protein and form pseudo-photoproducts, M$_{470}$ and M$_{380}$. Although M$_{470}$ and M$_{380}$ have similar spectroscopic properties to Meta III and II, M$_{380}$ does not measurably interact with G; however, M$_{380}$ is a substrate for rhodopsin kinase, and phosphorylated M$_{380}$ binds arrestin. Thus, for opsin-all-trans-retinal complex, only part of the interactive domain (the one for arrestin and rhodopsin kinase, denoted as A) is restored:

\[ R \rightarrow \text{Meta I} \leftrightarrow \text{Meta II}(G_t,A) \leftrightarrow \text{Meta III} \rightarrow \]

Opsin + all-trans-retinal

M$_{380}(A) \leftrightarrow M_{470}$

The binding and activation of G is more complex than the binding of arrestin. Glutamic residue E113, in the third transmembrane helix in the opsins, appears to restrict the activation of G$_A$ by opsin and all-trans-retinal, since a mutant of opsin E113Q can activate G, in the presence of all-trans-retinal (24). Conversely, the wild type of opsin cannot activate G$_A$ (24).

**Sequence of Events in the Quenching of Phototransduction**—Independent of the molecular mechanism, physiological conclusions can be suggested from this work. An irreversible key step for deactivation of phototransduction is the enzymatic reduction of all-trans-retinal, which removes this photoprodut from competition with 11-cis-retinal for binding to phosphorylated opsin (Fig. 4). Thus, the reduction of the photolyzed retinal in all-trans-retinal is essential for recycling photoactivated rhodopsin and implies a specific succession of phototransduction steps: dissociation of arrestin, and parallel dephosphorylation of opsin by a protein phosphatase(s) with regeneration of opsin by 11-cis-retinal (Fig. 4). Our results represent the first biochemical evidence for these steps in the quenching of phototransduction.

**Arrestin Could Be Involved in Bleaching Adaptation**—In the transient situation, when the irreversible reduction has not yet occurred, activation of the visual cascade is prevented by two factors: 1) arrestin does not dissociate from the receptor, and 2) the product formed is virtually inactive toward G. The mechanism is effective under scotopic conditions when only a few rhodopsin molecules/second are activated in the rod cell. However, for high levels of bleaching, the M$_{380}$-arrestin complex may accumulate and represent the form of "bleached rhodopsin" that is thought to be the origin of the bleaching adaptation pathway (17, 25). Additionally, any insufficiency in the reduction of all-trans-retinal to all-trans-retinol may lead to the accumulation of the M$_{380}$-arrestin complex in vivo, even at a low level of bleaching.

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