Phosphorylation and Dephosphorylation of Frog Rod Outer Segment Membranes as Part of the Visual Process*

(Received for publication, September 30, 1974)

JULIE ANN MILLER AND REINHARD PAULSEN

From The Laboratory of Molecular Biology and Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

The light-activated phosphorylation of rod outer segment membranes may be an intermediary process controlling photoreceptor responses. We have measured phosphorylation of the opsin moiety of rhodopsin in isolated frog retinas and in rod outer segment suspensions and have demonstrated a phosphorylation-dephosphorylation sequence in the suspensions. The results indicate that these reactions take place in vivo and may be physiologically relevant.

Extraction of a protein kinase activity from rod outer segment membranes renders the membranes incapable of phosphorylation, but the light-activated reaction can be reconstituted by mixing the soluble extract and the "depleted" membranes. Illumination of the extract is without effect. Thus the light activation mechanism resides in the membranes. Regeneration of rhodopsin from opsin and 11-cis retinal does not influence the phosphorylation. Once activated, the reaction may use either rhodopsin or opsin as the substrate. Furthermore, 11-cis retinal regenerates rhodopsin from phosphorylated opsin without releasing bound phosphate. The isolated rod outer segment which contains regenerated rhodopsin thus differs from one that is dark adapted in that phosphate can remain bound and the phosphorylation reaction remains activated. Dark adaptation in vivo must include at least two membrane-associated reactions beyond regeneration of rhodopsin's spectral properties: dephosphorylation, and the inactivation of the phosphorylation process.

The frog rod outer segment is a cylindrical structure, composed of a plasma membrane which encircles 1000 to 2000 membranous discs. Rhodopsin, the visual pigment, accounts for over 80% of the membrane protein (1). Absorption of light by the retinal chromophore of rhodopsin causes isomerization from the 11-cis configuration to all-trans, initiating a series of conformational changes which finally produce all-trans retinal and the photoproduction opsin (2). In the visual cycle opsin finally reacts with 11-cis retinal to form a rhodopsin molecule, which is indistinguishable in its light absorbance from unbleached rhodopsin (3).

The processes which link rhodopsin photochemistry to the receptor potential and its control have not been established. A light-activated phosphorylation of photoreceptor membranes observed in frog outer segments in our laboratory (4) and in cattle outer segments (5, 6) seems likely to be one of the controlling reactions. In this reaction illumination triggers a kinase-catalyzed, Mg2+-dependent transfer of the terminal phosphate of ATP to serine residues on the protein moiety, opsin (4, 5). Maximum incorporation is observed 10 to 20 min after bleaching and ranges from 0.5 (6) to 4.0 (7) mol of phosphate/mol of rhodopsin present. The light-activated reaction is not stimulated by adenosine 3':5'-monophosphate (5, 8, 9), although rod outer segments may contain another kinase which is adenosine 3':5'-monophosphate dependent (10).

The early reports of light-activated phosphorylation in isolated rod outer segments did not establish that the reaction occurs under conditions approaching those in vivo. The rod outer segments had been extensively purified and sometimes fragmented. The possibility of this reaction being an artifact has led us to ask whether phosphorylation takes place in freshly isolated retinas, using phosphate incorporated by the retina's own metabolism. Establishing the physiological relevance of the phosphorylation reaction further requires that a phosphorylation-dephosphorylation sequence be observed. Since individual rhodopsin molecules are bleached and regenerated repeatedly in a living frog, phosphates which bind after illumination must be removed or all the available sites soon would be reacted. Moreover, if the phosphorylation influences another process, such as membrane permeability or an enzymatic activity, there must be a dephosphorylation mechanism to reverse the effect. Kühn has observed dephosphorylation subsequent to light-activated phosphorylation in living frogs injected with 32P (11). In this paper we demonstrate that such a dephosphorylation also occurs in isolated rod outer segments.

Understanding the mechanism of the phosphorylation reaction requires that its components be separated and identified. Kühn et al. (12) have separated kinase activity from the membranes of bovine outer segments and have demonstrated restoration of light-activated phosphorylation in the reconstituted sys-
tem. They did not demonstrate the site of the light effect. We have extended these experiments to determine whether activation of the reaction resides in the membrane or in the soluble fraction. We have further tested one model for the mechanism of light activation: that the kinase is specific for the photoprotein opsin, with the stoichiometry of the reaction regulated simply by the amount of opsin substrate present. We have regenerated rhodopsin from opsin with 11-cis retinal, before, during, and after phosphorylation of the opsin moiety. The results suggest the existence of several distinct enzymatic reactions during dark adaptation in rod outer segments.

MATERIALS AND METHODS

Chemicals—11-cis retinal was a gift from Paul K. Brown, Biological Laboratories, Harvard University. Hepes was obtained from Calbiochem; hexadecyltrimethyl ammonium chloride from Eastman Kodak. Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) and ATP were purchased from Sigma; [γ-32P]ATP, [γ-32P]-GTP, and Aquasol from New England Nuclear.

Rod Outer Segment Preparations—Retinas were dissected from the bushytail tenrec, under dim red light. Preparations of crude rod outer segments were obtained by swirling two retinas in 0.5 ml of an isotonic Ringer solution containing 115 mM NaCl, 2 mM MgCl₂, 2 mM KCl, and 10 mM Hepes, pH 7.5 (Hepes-Ringer). This procedure released most of the rod outer segments. The retinas were rinsed out of the suspension. Washed rod outer segment preparations were obtained by centrifuging the crude preparation at 1200 × g for 2 min and resuspending the pellet in Hepes-Ringer. Purified rod outer segment preparations contained outer segments isolated from crude preparations by centrifugation on a Ficoll gradient (7).

Phosphorylation of Rod Outer Segment Membranes—Outer segment suspensions containing about 10⁻⁴ M rhodopsin were divided into portions for light and dark conditions. The light portions were illuminated by orange light (Corning sharp cut filter CS-3-67 and heat filters) using either a continuous light source or flash illumination, bleaching 70 to 80% of the rhodopsin present. Appropriate amounts of [γ-32P]ATP or [γ-32P]Pi were added to incubation mixtures either before or after bleaching of light portions. Incubation temperature was 22°C. Samples of 0.01 to 0.05 ml were taken during the incubation period and pipetted into 2 ml of 10% trichloroacetic acid containing 50 mM sodium phosphate. The precipitate was collected and washed on Millipore filters which then were dissolved in 5 ml of Aquasol and counted for radioactivity.

The concentration of rhodopsin in incubation mixtures was determined by difference spectroscopy after dissolving the rod outer segments in 0.004 M sodium phosphate buffer, pH 7.2, containing 4% sodium dodecyl sulfate and 2% 2-mercaptoethanol. Storing this extract less than 4 hours at room temperature avoided the formation of polymers described previously (7). The solution then was made 1% sodium dodecyl sulfate and 5% sucrose. Samples containing 20 to 50 μg of opsin were applied to each gel. Electrophoresis followed the method described by Weber and Osborn (13) using gels (6 × 70 mm) containing 12.5% acrylamide, 0.08% methylenebisacrylamide, and 0.4% sodium dodecyl sulfate in 0.05 M sodium phosphate buffer, pH 7.2. The electrode buffer was 0.04 M sodium phosphate buffer, pH 7.2, with 0.2% sodium dodecyl sulfate. Gels were run for 4 hours at 7.5 mA per gel. Unstained duplicates of Coomassie stained gels were sliced in 2-mm sections which were dissolved by 0.2 ml of 30% hydrogen peroxide. Aquasol, 5 ml, was added to each sample and counted for radioactivity in a scintillation counter.

Results

Phosphorylation by Endogenous and Exogenous Nucleotides—We have observed light-activated phosphorylation in rod outer segments in the presence of ATP concentrations ranging from 0.01 to 100 mM. The optimum ATP concentration is about 5 mM. ADP and adenosine inhibit the reaction when present in concentrations similar to that of ATP, with adenosine inhibiting more strongly than ADP (Table I). Phosphate from GTP is incorporated to a lesser extent than from ATP, suggesting either that GTP is also a substrate or that the γ-phosphate of GTP and ATP exchange during the incubation. There is no indication that GTP is the preferred substrate for the reaction.

We find that the light-activated phosphorylation reaction in crude and purified outer segment suspensions, incubated with ATP, is similar in rate and extent of phosphate incorporation. In crude preparations, however, as many as 50% of the outer segments remain attached to the mitochondria-rich inner segments. Using this preparation we have been able to demonstrate that Pᵢ can be incorporated into opsin, presumably via endogenous ATP synthesis. The initial time courses for light-activated incorporation in incubations of rod outer segment with Pᵢ and a Maximum phosphate incorporation of bleached control is 0.6 mol of phosphate per mol of rhodopsin present.

Inhibition of light-activated phosphorylation

Crude rod outer segments were suspended in 0.5 ml Hepes-Ringer, pH 7.5, which contained 10 mM of the indicated compound and either 1 mM ATP (15 mCi of [³²P]ATP/mmol) or 0.1 mM ADP and 0.5 mM carrier-free [³²P]Pi. The results are presented as percent of maximum phosphate incorporation by the relevant bleached control. The 100% value represents incorporation of 300 cpm for the ATP control and 30,000 cpm for the Pi control. Standard deviations are given for the means of three or more separate experiments. Single values are the means of three to five samples within an experiment.

Table I

<table>
<thead>
<tr>
<th>Inhibitors of light-activated phosphorylation</th>
<th>Source of Pᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>[γ-³²P]ATP</td>
</tr>
<tr>
<td>Source</td>
<td>Bleached</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>86.0</td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>116.8 + 10.3</td>
</tr>
<tr>
<td>ADP</td>
<td>42.3</td>
</tr>
<tr>
<td>Adenosine</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* Maximum phosphate incorporation of bleached control is 0.6 mol of phosphate per mol of rhodopsin present.

1. Maximum phosphate incorporation of bleached control is 0.6 mol of phosphate per mol of rhodopsin present.

2. Incubation with Hepes-Ringer, pH 7.0, containing 0.5 mM MgCl₂, 3 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid, 10% of calf serum, 10⁻¹ M papaverine, and 10⁻³ M dithiothreitol.
Fig. 1. Initial time course of light-activated phosphorylation in rod outer segments. Carrier-free $^{32}$P$_i$, 0.15 mCi (●), or 0.75 μmol of ATP (30 mCi of $^{32}$P/mmol; ○) was added to 0.15 ml of crude rod outer segment suspensions in Hepes-Ringer. Orange light bleached 75% of the rhodopsin present. Unbleached controls, not shown, incorporated less than 7 and 30% of the maximum $^{32}$P light incorporation for ATP and $P_i$, respectively. The maxima represent 220 cpm for ATP and 25,000 cpm for $P_i$.

with ATP are identical (Fig. 1), although the amount of background incorporation in dark samples is higher with $P_i$ than with ATP (Table I). Sodium cyanide and sodium azide, inhibitors of mitochondrial ATP synthesis, dramatically decrease incorporation of added $P_i$ but not of phosphate from ATP (Table I).

Isolated retinas also undergo a light-activated phosphorylation. When retinas are incubated with $^{32}$P$_i$; for 8 min, phosphate incorporation into rod outer segment membranes in the light is higher than in the dark. The product of the light-activated incorporation is located in the peak protein band on sodium dodecyl sulfate polyacrylamide gels (Fig. 2), as it is in incubations of isolated rod outer segments with [$\gamma$-$^{32}$P]ATP (see Fig. 6). This band has been identified as the opsin moiety of rhodopsin and its photoproducts (1, 6). Thus in retinas, as in isolated rod outer segments, the protein moiety, opsin, is phosphorylated only after exposure to light.

Dephosphorylation of Phosphorylated Membranes—After phosphorylation activated by large bleaches, we have observed dephosphorylation in frog rod outer segments under three conditions: (a) during incubations with low concentrations of ATP; (b) after addition of 1 mg/ml of apyrase, an enzyme that hydrolyzes ATP; and (c) during incubations with $P_i$.

In suspensions containing less than 50 μM ATP, dephosphorylation varies in extent and appears to be more rapid in crude than in purified rod outer segments (Fig. 3). In the presence of 10 μM ATP, maximum incorporation is reached within 5 min and then decreases 20 to 60%. The incomplete dephosphorylation observed and the more rapid dephosphorylation measured in crude preparations may be explained as a loss of activity of the phosphatase or, alternatively, as lack of some essential factor from the rod inner segment. There may also be phosphorylation occurring concurrently with dephosphorylation. During the observed dephosphorylation, outer segments retain their ability to undergo phosphorylation, for addition of more ATP causes a second increase in phosphate incorporation (Fig. 4). The difference between crude and purified preparations also might result from a higher concentration of ATP hydrodases in the crude preparation. Decreasing ATP concentrations would cause less phosphorylation to compete with dephosphorylation. If the initial ATP concentration is increased to 1 mM or higher, total phosphate incorporation dramatically increases, the dephosphorylation reaction can no longer be detected, and additional ATP does not produce a second peak of incorporation.

Dephosphorylation, however, can be observed if the enzyme apyrase is added after a period of light-activated phosphorylation with 1 mM ATP. In the presence of apyrase, 50 to 80% of the bound phosphate is released. Under these conditions removal of ATP probably prevents further phosphorylation from obscuring the dephosphorylation reaction. However we cannot rule out the possibility that apyrase or a contaminating enzyme catalyzes hydrolysis of opsin-phosphate bonds.
Separation of kinase activity from rod outer segment membranes

Crude rod outer segments were suspended in 10 mM Tris-Cl, pH 8.0, and 5 mM EDTA for 10 min and the membranes were pelleted by centrifugation, 90,000 × g for 30 min. The supernatant was stored on ice. The pellet was sonicated in 2.7 M urea, centrifuged, and washed free of the urea. The unfractionated control also was sonicated. The indicated fractions were illuminated and combined, then assayed for phosphorylation after incubation for 20 min in 20 mM Tris-Cl containing 2.2 mM ATP (1.1 mCi of $^{32}$P/mmol), 5 mM MgCl$_2$, and 20 mM KCl. The maximum incorporation represents 700 cpm. The results are typical of four individual experiments.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Moles phosphate bound/mol rhodopsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Illuminated</td>
</tr>
<tr>
<td>Unextracted rod outer segments</td>
<td>0.340</td>
</tr>
<tr>
<td>Isolated fractions</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.014</td>
</tr>
<tr>
<td>Pellet (membrane)</td>
<td>0.025</td>
</tr>
<tr>
<td>Recombined supernatant and pellet</td>
<td>0.517</td>
</tr>
<tr>
<td>Supersnatant only illuminated</td>
<td></td>
</tr>
<tr>
<td>Pellet only illuminated</td>
<td>0.490</td>
</tr>
</tbody>
</table>

* The supernatant activity is expressed as moles of phosphate bound per mol of rhodopsin in the original suspension.

We also have observed dephosphorylation in 8 out of 10 experiments with crude preparations and inorganic phosphate. The total amount of phosphate bound decreased 20 to 80%, from the maximum within 30 min. These results demonstrate that dephosphorylation occurs in the presence of the ATP concentrations endogenous to the isolated rod outer segments.

Separation of Kinase Activity from Rod Outer Segment Membranes—When outer segments are extracted to yield a rodopsin-free supernatant and a washed membrane pellet, neither fraction assayed alone displays more than 10% of the original light activated phosphate incorporation (Table II). The reaction is completely restored if the fractions are recombined. (The difference between the values of phosphorylation of the recombined fractions and unextracted rod outer segments in Table II was not observed in three other experiments.) Supernatants which are heated to 98° for 10 min before recombining with the pellets do not restore activity. These experiments indicate that the supernatant contains an essential element of the kinase activity, either the kinase or a heat-labile cofactor.

Table II also demonstrates the consequences of illuminating the membrane and the soluble fractions separately. Exposing the supernatant to light before adding an unbleached pellet suspension does not activate the reaction. Bleaching the membrane before adding an unilluminated supernatant does activate phosphorylation. Therefore, the rod outer segment membrane is the site of the initial light activation.

Phosphorylation during Rhodopsin Regeneration—Regeneration of rhodopsin and light-activated phosphorylation seem to be separate reactions of the opsin molecule. In flash-photolysed rod outer segments the time course of phosphorylation is not influenced by the presence of simultaneous rhodopsin regeneration (Fig. 5). Furthermore, rod outer segment membranes, after their rhodopsin has been bleached and regenerated 80 to 95% by addition of 11-cis retinal, can be phosphorylated by ATP without additional bleaching (Fig. 5). (The decrease in maximum phosphorylation which is observed when the reaction is measured after a 30-min time interval is typical of several experiments.) We find also that 100% of the phosphorylated opsin from isolated frog rod outer segments can be regenerated to rhodopsin in the presence of 11-cis retinal without decreasing the amount of phosphate bound. This result is in agreement with data obtained for cattle outer segment fragments (12). Gel electrophoresis patterns (Fig. 6) show that phosphate is still bound to the opsin moiety both after phosphorylation of regenerated rhodopsin and after regeneration of rhodopsin from phosphorylated opsin.
in the presence of the long-lived photoproducts, metarhodopsin II and metarhodopsin III (14). Frank et al. have suggested that metarhodopsin III is the preferred substrate (6). Since rhodopsin regenerated from opsin by 11-cis retinal can be a substrate, the conformational changes that result from the binding of the 11-cis chromophore to opsin do not influence its substrate activity. Hence, there must be less obvious changes in the opsin moiety or in its position in the membrane which are recognized by the kinase.

Support for the second mechanism includes the observation of Bownds et al. (9) that in frog rod outer segments bleaches of less than 5% of the rhodopsin present cause incorporation of about 20 mol phosphate per mole of rhodopsin bleached. The simplest explanation of this result is that a second substrate or unbleached rhodopsin molecules have been phosphorylated. The activation of a kinase, which is caused by the bleaching of rhodopsin and which catalyzes the phosphorylation of unbleached rhodopsin molecules, is one mechanism that would permit such an amplification. The recombination experiments do not rule out such a mechanism because kinase activation might require interaction with an intermediary site on the membrane which has been altered after illumination.

Whatever the mechanism of light activation, our results indicate that the chromophore rhodopsin and the activation of phosphorylation occupy distinct sites on the rod outer segment membrane, although both may be on the rhodopsin molecule. The sites are related in their response to light but separate in the reversal of the light effects. Outer segment membranes first illuminated and then regenerated to their full rhodopsin complement can be phosphorylated. Unilluminated membranes containing rhodopsin cannot be phosphorylated. The complete "visual cycle," then, must involve not only the regeneration of rhodopsin but also the return of the second membrane site to its dark adapted state.

The physiological role of the phosphorylation reaction has still not been established. We now think it most likely that this reaction serves as part of the sensitivity-controlling mechanism which operates during dark adaptation in photoreceptors.

Acknowledgments—We thank Dr. Deric Bownds for advice and support and for providing the context for these experiments. We are grateful to Paul K. Brown for the generous gift of 11-cis retinal.

Note Added in Proof—Since the submission of this paper, two papers about light-activated phosphorylation in bovine rod outer segments have been published (15, 16). The paper by Weller et al. (15) includes data in agreement with ours on the extraction of kinase activity from outer segment membranes and on the dephosphorylation of rhodopsin. However, on the basis of an observed linear relationship between rhodopsin bleaching and phosphorylation, they concluded that the kinase specifically catalyzes phosphorylation of opsin. They did not attempt to phosphorylate regenerated rhodopsin. Our data indicate that rhodopsin regenerated by addition of 11-cis retinal can also be substrate for the kinase. Recently we have repeated this experiment four times and have found that regeneration causes no significant decrease in the extent of phosphorylation. In contrast to these results, Shichi et al. (16) have reported that regeneration of visual pigment with 9-cis retinal prevents phosphorylation. They did not include their complete experimental procedure nor any quantitative data on this point. Therefore we cannot suggest reasons for the differences between these results.

**DISCUSSION**

Our experiments, along with those of Kühn in living frogs (11), demonstrate that light-activated phosphorylation takes place in rod outer segments under physiological conditions. Since the reaction can be measured in intact retinas, it is not an artifact of trauma caused by isolation procedures. The observation that inorganic phosphate can be incorporated into the opsin moiety, by either retinas or crude outer segments, suggests that ATP produced in the inner segments can be used for phosphorylation of outer segment membranes.

Our data are consistent with two alternative mechanisms for activation of the phosphorylation reaction. One possibility is that light activates the substrate, rhodopsin. The other is that activation involves the kinase or other intermediaries which have not been identified.

If the effect of light is to activate the substrate, the kinase must differentiate between a rhodopsin photoproduct and unilluminated rhodopsin. The preferred substrate might be opsin or earlier photoproducts. We find that phosphorylation occurs if ATP is added to bleached rod outer segments at a time when only opsin is present. Further, the time course of phosphorylation in flash-photolyzed outer segments shows that phosphorylation starts about 60 phosphate groups then would be bound to a single opsin moiety, requiring the phosphorylation of all the serine and threonine residues present. We feel that this possibility is unlikely in a highly hydrophobic molecule like rhodopsin.

Even though we have measured complete regeneration of rhodopsin during phosphorylation, the results could be explained by assuming that there is a small fraction of the rhodopsin which does not regenerate and which incorporates a high number of phosphate groups. About 60 phosphate groups then would be bound to a single opsin moiety, requiring the phosphorylation of all the serine and threonine residues present. We feel that this possibility is unlikely in a highly hydrophobic molecule like rhodopsin.

**FIG. 6. Electrophoresis on sodium dodecyl sulfate polyacrylamide gels of rod outer segment membranes before and after regeneration of rhodopsin by 11-cis retinal.** A washed preparation was divided into aliquots a, b, c, and d. The aliquots b, c, and d were bleached (80%). 11-cis retinal was added to a and b. After 25 min 95% of the rhodopsin bleached in aliquot b was regenerated. Thirty minutes after bleach, ATP (1.0 mCi of 32P/mmol) was added to each aliquot (final ATP concentration, 10 mM). Sixty minutes after bleach, 11-cis retinal was added to c, regenerating 100% of the rhodopsin bleached within 10 min. Sodium dodecyl sulfate extracts of a, b, c, and d containing 30 μg of opsin were applied per gel. •—• a, unbleached control; Δ—Δ, b, membranes regenerated before phosphorylation; U—U, c, membranes regenerated after phosphorylation; O—O, d, phosphorylated membranes not regenerated. — , protein pattern after staining gels with Coomassie Blue.
REFERENCES

Phosphorylation and dephosphorylation of frog rod outer segment membranes as part of the visual process.

J A Miller and R Paulsen


Access the most updated version of this article at http://www.jbc.org/content/250/12/4427

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/12/4427.full.html#ref-list-1