

LIGHT DEPENDENT PHOSPHORYLATION OF RHODOPSIN BY ATP

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1. Introduction

The photoreceptor of the retinal rod cell is located in the rod outer segment (ROS), a highly specialized membrane system containing the protein rhodopsin as a predominant component. The rod cell, a specialized neuron, is able to detect and amplify the energy contained in a single photon of light and transform it into a neural impulse. A high gain amplification process occurs within this type of cell. We are interested in studying the mechanisms by which energy is utilized in this process.

As a result of the work of G. Wald and his group [1] it is known that 11-*cis*-retinal, the prosthetic group of rhodopsin, undergoes stereochemical changes as a response to light absorption, leading eventually to all-*trans*-retinal. It is generally believed that retinal is then removed from its apoprotein opsin; the original light-sensitivity of rhodopsin is restored when, through a cycle of reactions, 11-*cis*-retinal is again linked to the protein. Little is known about other chemical reactions in this cycle.

We have found an *in vitro* reaction in which rhodopsin is phosphorylated by $AT^{32}P$. The reaction is stimulated by prior exposure of ROS membranes to light. It is possible that this reaction also occurs *in vivo* as a part of the functional cycle of photoreceptors.

2. Materials and methods

2.1. Preparation of ROS* membrane from bovine eyes

We followed essentially the procedure of McConnell et al. [2] with some modifications [3]. All manipulations were done at 4° under dim red light. The

final ROS suspension in 8% sucrose (conc. 3.0 mg/ml protein) was stored frozen in small portions at -20° in the dark.

Electron microscopy of the membrane suspension revealed that the outer envelope membrane of the ROS had been ruptured during the final purification and freezing steps. The rod discs were present in the form of flat membranes and vesicular structures (D. Papermaster, unpublished results).

Protein concentrations were determined by amino acid analysis of acid hydrolyzed samples using a Beckman Model 121 analyzer. The amino acid composition of the whole membrane was similar to the published composition of purified rhodopsin [4].

2.2. Quantitative labeling experiments

Standard labeling conditions: each sample tube contained 30 µg of membrane proteins. The volume was 50 µl. The concentrations were, unless stated otherwise in the text: 0.6 mg/ml protein; 20 mM Tris-HCl (pH = 7.3); 20 mM KCl; 6 mM NaCl (from ATP disodium salt); 2% sucrose; 3.0 mM $MgCl_2$; and 3.0 mM $AT^{32}P$. The ATP stock solution was neutralized to pH 7.3 with Tris, and the concen-

* Abbreviations used:

ROS = rod outer segment
ATP = adenosine-5'-triphosphate
AMP = adenosine-5'-monophosphate
TCA = trichloroacetic acid
SDS = sodium dodecyl sulfate
EDTA = ethylenediamine tetraacetate
P-ser = phosphoserine
P-thr = phosphothreonine

tration was checked by UV absorption at 259 nm assuming a molar extinction coefficient of 15,400 at neutral pH. Specific activities ranged between $1-5 \times 10^7$ cpm/ μ mole.

The reaction was started by adding ROS to the prewarmed (2–3 min at 37°) mixture under dim red light. The sample was shaken briefly using a Vortex mixer, and put into a water bath at 37° for the time and illumination conditions indicated in each case. The reaction was stopped by adding 1.5 ml of ice-cold 5% TCA. The mixture was filtered (after addition of 3 ml more of 5% TCA) through a millipore filter HAWP 013 00, 0.45 μ , using a slight pressure, and the filter was washed 4 times with 5 ml of 5% TCA each time. The filters were dried overnight at room temp and counted after addition of 10 ml of the scintillation fluid (toluene:liquifluor (NEN) = 960:42) in a Beckman scintillation counter. Blank controls: AT³²P was filtered together with 5% TCA and TCA-precipitated, non-labeled ROS membrane, under the same conditions as the actual samples. The blank cpm values were subtracted from the sample values. The blank values were about 3% of the sample values at maximum incorporation (10,000–30,000 cpm). At lower incorporation, therefore, the background is more significant than at higher.

2.3. Exposure to light during incubation

A General Electric reflector flood lamp, 120 V/150 W, was installed vertically above the water bath in order to shine directly (without penetrating glass) into the test tubes. The light intensity at a 100 cm distance from this lamp was about 100 footcandles, as measured with a General Electric light meter.

2.4. Calculation of the molar concentration of rhodopsin

The molar quantitation of rhodopsin (figs. 2 and 3) was based on 2 assumptions: (a) the molecular weight of rhodopsin is 28,000 [4,5] and (b) rhodopsin makes up about 85% of the total proteins of our membrane preparations*.

Scanning of non-stained SDS-acrylamide gels [6] at 280 nm indicated about 95% of the total UV ab-

* Earlier findings indicating that a significant part of the ROS protein is low molecular weight structural protein [8] have been retracted [9, 3].

GEL ELECTROPHORESIS + RADIOAUTOGRAPHY

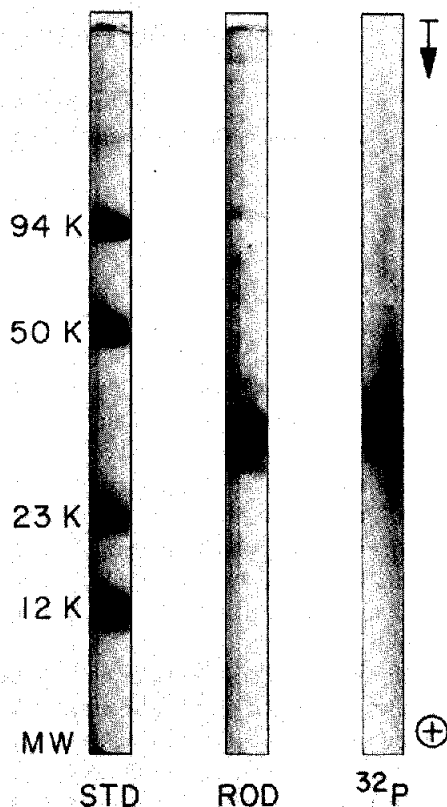


Fig. 1. Gel electrophoresis of ³²P-labeled ROS membrane. The procedure of Fairbanks et al [6] was used, the gels containing 5.8% acrylamide and 1% SDS. STD = standard gel containing phosphorylase A (M.W. 94,000), gamma-globulin (M.W. 50,000 and 23,000) and cytochrome c (M.W. 12,000). ROD = ³²P-labeled ROS membrane, stained with Coomassie Blue. ³²P = radioautograph of the non-stained gel ROD. The M.W. of the rhodopsin is about 30,000. The incubation mixture (50 μ l) contained bleached membrane. Concentrations: 0.6 mg/ml protein; 0.4 mM γ -³²P-ATP (specific activity 0.4 Ci/mM); 0.5 mM MgCl₂; 20 mM Tris-HCl (pH 7.3); 20 mM NaCl; 2% sucrose. The sample shown was incubated at 37° for 20 sec in daylight. (Longer incubation times up to 1 hr revealed similar results except the higher amount of radioactivity incorporated.) The membrane was precipitated with 5% TCA, centrifuged, and the washed pellet was solubilized using Fairbanks [6] 1% SDS cocktail, for 30 min at 37°. The gel was fixed for 5 min in 10% acetic acid and then cut into 2 halves of equal thickness using a razor blade. One half was stained with Coomassie Blue [6], the other half was wrapped with thin plastic foil and radioautographed on X-ray film for 15 hr.

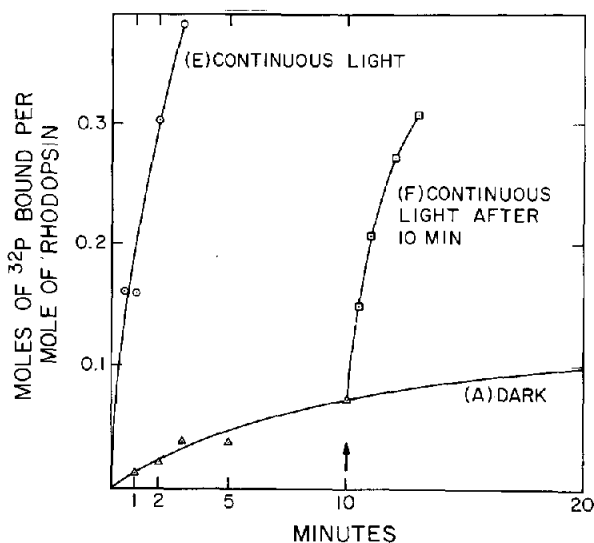


Fig. 2. Phosphorylation rate of rhodopsin, light dependence. Curve A: Samples were mixed under dim red light and incubated at 37° in the dark for the times indicated. Curve E: samples were mixed under dim red light; immediately after starting the incubation at 37° they were exposed to white light (intensity 400 footcandles) for the whole incubation time. Curve F: Samples were mixed under dim red light, incubated in the dark for 10 min, and then (arrow) exposed to white light (400 footcandles) for the rest of the incubation time. ATP concentration in all samples was 3mM. For experimental details see Materials and methods.

sorbing proteins to be rhodopsin; and scanning of Coomassie Blue stained gels at 550 nm, considering concentration dependence of color in a series of different loadings, indicated at least 85% to be rhodopsin [3]. Although we know that Coomassie Blue stain tends to overemphasize minor bands, we assumed the lower, and more conservative value of 85% as a basis for molar calculations. It should be mentioned that other workers [7] have found a significantly lower relative amount of rhodopsin in ROS proteins. When we applied their denaturation method, using low SDS concentrations, to our membrane preparation, we also found larger amounts of material with a molecular weight higher than rhodopsin. This was probably due to incomplete dissociation. Treatment with 1% SDS [6] at 37° , however, dissociated these aggregates down to a molecular weight of 30,000.

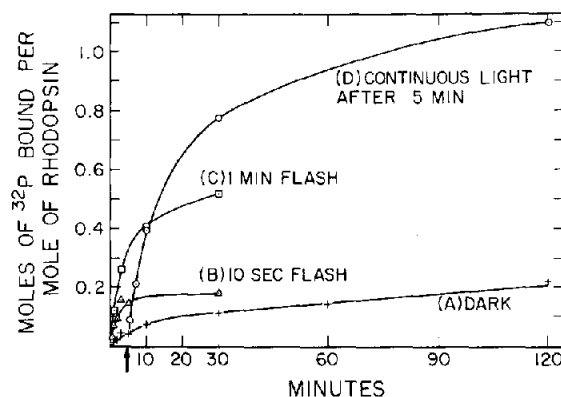


Fig. 3. Extent of the phosphorylation, light dependence. Curve A: Samples were mixed under dim red light and incubated at 37° in the dark. Curve B: Samples were mixed under red light. Immediately after starting the incubation at 37° , they were flashed for 10 sec with white light (100 footcandles). Rest of the incubation was in the dark. Curve C: The same as B, except that the light flash was 1 min at 200 footcandles. Curve D: Samples were mixed under red light, incubated for 5 min in the dark, and then (arrow) exposed to white light (100 footcandles) for the rest of the incubation period. The concentration of ATP and $MgCl_2$ was 3 mM in both A and D, and 2 mM in both B and C. If the dark experiment (A) was done with 2 mM ATP/ $MgCl_2$, the same values were obtained as with 3 mM ATP. For further experimental details see Materials and methods, standard labeling procedure.

3. Results

3.1. Gel electrophoresis

When a suspension of ROS membrane was incubated with γ - ^{32}P -ATP and $MgCl_2$ in the presence of light, radioactivity was incorporated into the membrane. SDS gel electrophoresis of the acid-precipitated material, solubilized by 1% SDS, showed that the radioactivity is bound to rhodopsin (fig. 1), the main protein component of this membrane.

With much higher specific activities and longer radioautography exposure times, a second radioactive band appears at molecular weight 60,000; it is faint compared to the main band, and is thought to be due to a residual dimer of rhodopsin. None of the minor bands that appear on the Coomassie Blue stained gels was ever found to be radioactively labeled. Quantitative scintillation counting of the whole acid-precipitated material could therefore be carried out

without further purification of rhodopsin.

No radioactivity was bound to rhodopsin when α - ^{32}P -ATP instead of γ - ^{32}P -ATP was used, as checked by gel electrophoresis-radioautography as well as by scintillation counting on filters. This indicates that rhodopsin is phosphorylated and that the nucleoside is not bound to the protein.

3.2. Kinetics, light dependence

The rate of phosphorylation was found to be stimulated after exposure of the membranes to light, as shown in fig. 2. Some phosphorylation occurred in the dark under our reaction conditions (curve A), but illumination with white light increased the reaction rate dramatically (curves E and F).

The reaction approached a constant level at about (or slightly above) 1 mole of $^{32}\text{PO}_4^{3-}$ bound per mole of rhodopsin (curve D, fig. 3) when we used long incubation times, continuous illumination during the incubation, and an initial ATP concentration as high as 3 mM. When only a single light flash was applied at the beginning of the incubation period, the phosphorylation was also stimulated to an extent depending on the intensity and the time length of the flash (curves B and C). It should be noted that most of the ^{32}P was incorporated *after* the actual flash, when the samples were kept again in the dark; this indicates that the phosphorylation is a dark reaction rather than an immediate response to the light flash. The final level of the curves (B and C) depends on the amount of light flashed, that is, presumably, on the amount of rhodopsin available in the bleached form. In the experiments shown in fig. 2 and 3, the membrane was kept dark-adapted before ATP was added and the incubation was started. However, if a membrane sample was bleached in bright light for several min or even hr before addition of AT^{32}P , the phosphorylation occurred at almost the same initial rate as that shown in curve D though to a somewhat lower extent.

Under the reaction conditions described, the phosphorylation was essentially irreversible. After the radioactive phosphate was bound to rhodopsin, it was not possible to displace it with an excess of 20 mM cold ATP (incubated for 30 min at 37° after the labeling with γ - ^{32}P -ATP).

3.3. Reaction conditions

The phosphorylation also occurs at ATP concentrations lower than 3 mM. Preliminary experiments showed that the initial reaction rate is not significantly influenced by the ATP concentration in a range from 1–9 mM. However, the final extent of phosphorylation is lower at lower ATP concentrations. This is probably due to other, competing ATP consuming processes [10–12]. Accordingly, 1 mM Ouabain which is known to inhibit the Na^+/K^+ -dependent ATPase in ROS [11] increased the extent of the phosphorylation of rhodopsin at low ATP concentrations. The increase is about 50% at 1 mM ATP.

Mg^{2+} ions are necessary for the phosphorylation; EDTA inhibits. Na^+ and K^+ seem to have little influence. No phosphorylation occurs when the structure of the membrane has been destroyed before addition of ATP. This was determined after heat denaturation at 100° for 5 min, and after separate treatment with different detergents: 1% SDS, 1% Emulphogene, and 0.04 M cetyl-trimethylammonium bromide. The latter 2 detergents are reported to conserve the spectral properties of rhodopsin; apparently they do not conserve the phosphorylating system.

3.4. Phosphorylated peptides and amino acids from rhodopsin

^{32}P -labeled ROS membrane was digested with subtilisin (ratio enzyme to substrate = 1:10) at pH 8.5 and 37° for 2 days, a treatment which solubilized about half of the membrane protein. The insoluble residue was then solubilized almost completely by partial acid hydrolysis using 2 N HCl at 100° for 5 hr. The phosphorylated peptides and amino acids (which are acidic) were separated from the bulk of neutral and basic material by chromatography on a small Dowex 50 X2 column equilibrated and eluted with 1% HCOOH (pH 2). The Dowex fractions were subjected to paper electrophoresis at pH 1.7 [13] followed by radioautography (fig. 4). A series of ^{32}P -phosphorylated peptides (migrating slowly towards the cathode) appear in both the subtilisin digest and the partial acid hydrolysate. In the latter, 3 additional spots appear which were identified as phosphoserine, phosphothreonine, and inorganic phosphate. When the peptides from the subtilisin digest were eluted from the paper and subjected to partial acid hydrolysis, they also revealed these 3

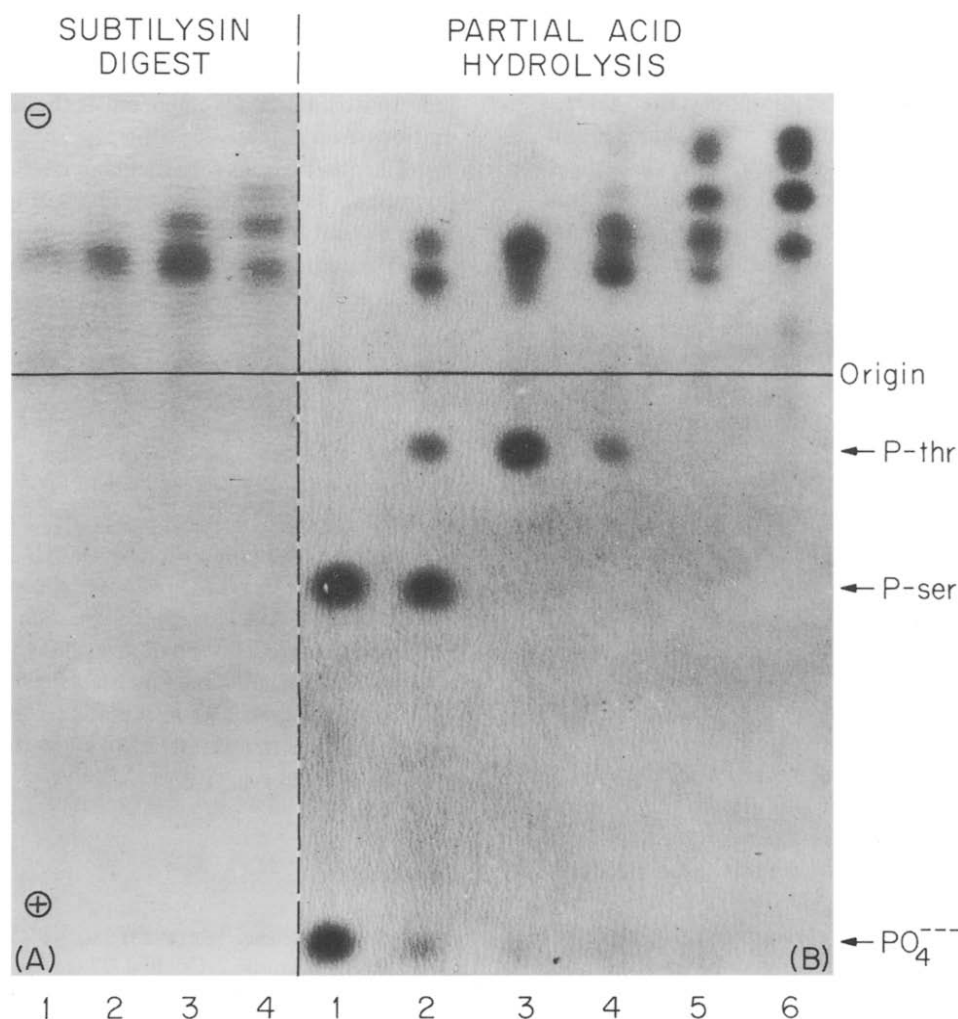


Fig. 4. Radioautograph of a paper electropherogram (6.7% HCOOH, pH 1.7, 65 min at 7,800 V, Gilson Model DW Electrophorator) containing cleavage products of 10 mg of ^{32}P -labeled rhodopsin. Samples (A) 1–4 were obtained by subtilisin digestion, samples (B) 1–6 by partial acid hydrolysis. The peptide mixtures were prepurified by ion exchange chromatography on Dowex 50 and the fractions containing acidic peptides were spotted onto the paper in the sequence in which they emerged from the column. For further details see text.

anionic compounds in subsequent paper electrophoresis.

The ^{32}P -ser and ^{32}P -thr bands were eluted from the paper and identified in 3 different systems by comparison with authentic P-ser and P-thr standards: electrophoresis at pH 1.7 (6.7% HCOOH), at pH 3.5 (1% pyridine–10% acidic acid–water), and detection of the amino acids serine and threonine (together with radioactive inorganic phosphate) after complete

hydrolysis in 6 N HCl.

In different experiments, P-thr was found in different amounts relative to P-ser, ranging from only 10% of P-thr to almost equimolar amounts of both. We are not yet certain of the explanation for this finding. It might be due to a slower phosphorylation rate of the threonine by ATP or the result of transphosphorylation from phosphoserine to an adjacent threonine during the partial acid hydrolysis,

a process favored by the fact that phosphothreonine is more stable against acid than phosphoserine.

The relatively high number of radioactive peptides (fig. 4) might suggest that phosphorylation takes place at many serine and threonine residues in rhodopsin. On the other hand, neither subtilisin digestion nor partial acid hydrolysis is very specific and thus each is expected to yield a series of peptides deriving from a single "active center" sequence. Another experiment (P. A. Hargrave et al., unpublished results) showed indeed that only a limited range of the total peptide chain of rhodopsin is phosphorylated: ^{32}P -labeled rhodopsin was cleaved with CNBr, and the fragments were separated by gel permeation chromatography. Essentially all the radioactivity was eluted in a single sharp peak. The molecular weight of the fragment is about 2,000. Studies are in progress to determine the amino acid sequence of this phosphorylated peptide.

4. Discussion

The data presented indicate that rhodopsin is phosphorylated when ROS membrane is incubated with ATP, and that this reaction is greatly stimulated by light. The reaction occurs essentially as a dark reaction, after the 11-*cis* retinal pigment has been bleached by light (curves B and C, fig 3). This observation, together with the relatively slow reaction rate, suggests that the phosphorylation process may be involved in the cycle of regeneration after the bleaching of 11-*cis* to all-*trans* retinal, rather than in the immediate response to a light signal.

Adenyl cyclase activity has been reported to be high in ROS [10]. However, in preliminary experiments we could not find that the phosphorylation of rhodopsin is stimulated significantly by cyclic AMP. The reverse light dependence of the cyclase activity (inactivation by light) compared to the phosphorylation also suggests that both reactions may occur independently, or at least that the relationship between them is more complex than just activation of a rhodopsin kinase by cyclic AMP.

It is not possible at this time to assess the relevance of the phosphorylation reaction to the *in vivo* process of receptor function. In any case, the reaction is triggered by light and it appears that a specific re-

gion of the rhodopsin molecule is phosphorylated. The maximum extent of phosphorylation is about 1 mole of ^{32}P per mole of protein. Once formed, the phosphoester bond is stable under the reaction conditions used in these experiments.

The observations reported here raise a number of questions: Is bleached rhodopsin capable of catalyzing its own reaction with ATP or is a separate protein kinase involved? Does this reaction supply part of the energy for receptor function? Are other types of membrane receptors phosphorylated in an analogous reaction? A number of experiments are suggested by these observations.

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