# PHOSPHORYLATION OF RHODOPSIN: MOST RHODOPSIN MOLECULES ARE NOT PHOSPHORYLATED

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Summary: Phosphorylation of rod membrane proteins is a light-dependent reaction. Most rhodopsin molecules, however, are not phosphorylated. The protein that is highly phosphorylated(>3 moles phosphate per mole phosphorylated protein) appears to be a rhodopsin species that is different from the rest or is located in different parts of the rod membrane system.

Introduction: The visual pigment rhodopsin is the major protein component of the rod photoreceptor membranes(1,2). Several investigators have recently reported the enzymatic phosphorylation of rhodopsin by ATP under *in vitro* conditions(3-6). Kühn(7) has shown that phosphorylation also takes place in the living eye of a light-adapted frog. Since rhodopsin is dephosphorylated during a subsequent dark period, he has suggested that the phosphorylation-dephosphorylation reaction may play a role in the mechanism of light-dark adaptation of the rods.

In this communication we present evidence that when isolated rod membranes are incubated *in vitro* with ATP only a minor fraction of rhodopsin is phosphorylated and that most of the rhodopsin molecules in rod membranes are not phosphorylated at all either in the light or in the dark.

Materials and Methods: Bovine rod outer segments retaining high kinase activity were prepared from fresh eyes essentially following the method of Kühn *et al*(6). Rhodopsin was labelled with [$^{3}$H]leucine and purified on Agarose by the method described by O'Brien *et al*(8). Labelled outer segments were extracted with 1% Emulphogene BC 720 in 0.05 M Tris, pH 8.5 and chromatographed with 0.3% Ammonyx LO in 0.05 M Tris (pH 7.8). Rod outer segments(from 4 retinas) were suspended in the reaction medium (2 ml, pH 7.3) containing 2 mM ATP(0.2 mC $\gamma$-[$^{32}$P]ATP), 50 mM Tris, 2 mM $Mg^{+2}$ and 4 mM $Na^{+}$, and phosphorylated by incubation at 37°C for 5 minutes in the dark and 10 minutes in the light. After phosphorylation, bleached outer segments were incubated

with 2.5-fold rhodopsin equivalents of 9-cis retinal(Sigma) to regenerate the pigment as described elsewhere(9). For inhibitor studies, samples were incubated in the presence of $10^{-4}$M oubain(Sigma) or 30 mM KCl. For studies on the lability of the phosphate linkage, phosphorylated outer segments were exposed to 50 mM Tris(pH 9.0) or 500 mM $NH_2OH$(pH 7.4) for 10 minutes, centrifuged and washed by recentrifugation. Radioactivity was determined with a Searle Analytic Mark II Liquid Scintillation Spectrometer using a standard scintillation fluid.

Results:When the phosphorylated protein of rod membranes was extracted in detergent and chromatographed on calcium phosphate-Celite(10), the phosphorylated peak could not be separated from the visual pigment. On an Agarose column, however, the {$^{32}$P}-labelled fraction was separated, although not completely, from the visual pigment fraction(Fig. 1). Reproducibility of the chromatographic separation of the {$^{32}$P}-peak from the visual pigment peak was assured by six consecutive experiments. The visual pigment fraction did not show a shoulder corresponding to {$^{32}$P}-labelled eluates. On the other hand, when {$^{3}$H}-leucine-labelled rod membranes were phosphorylated and chromatographed, the {$^{3}$H}-radioactivity peak showed a symmetrical shape and coincided with the visual pigment fraction. Phosphorylation of the component was light-dependent and increased almost proportionally with the extent of bleaching of rhodopsin. Assuming the same $\varepsilon_M$ value(43,000) for the phosphorylated component and

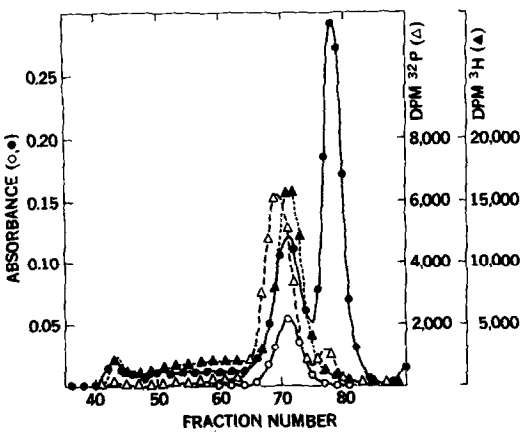


Fig. 1. Chromatographic separation on Agarose of {$^{32}$P}-labelled component from the visual pigment. ○——○, $A_{485}$; ●——●, $A_{278}$; △--△, DPM{$^{32}$P}; ▲--▲, DPM{$^{3}$H}.

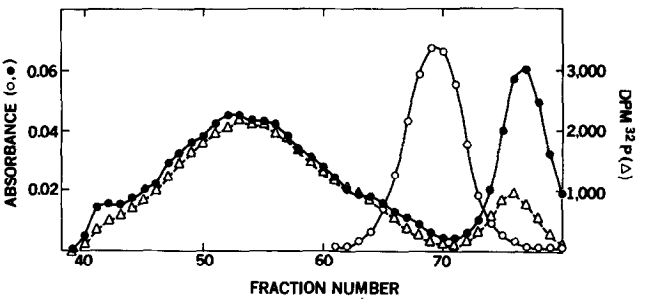


Fig. 2. Agarose chromatography of bleached [$^{32}$P]-labelled extract. Unlabelled rhodopsin was cochromatographed as marker. o——o, $A_{485}$; ●——●, $A_{278}$; Δ– –Δ, DPM[$^{32}$P].

isorhodopsin, it was estimated that no less than 3 moles of phosphate were incorporated per mole of the component.

When the phosphorylated protein was re-bleached by light and chromatographed, the radioactivity of the bleached product was eluted exactly in the same fractions as the bleached aggregates of unphosphorylated opsin(Fig. 2). If the [$^{32}$P]-labelled component was extracted from bleached rods that had not been regenerated with 9-cis retinal and chromatographed on Agarose, the [$^{32}$P]-labelled fractions were found also in those fractions containing opsin aggregates. In order to study the effect of chromophore regeneration on phosphorylation, bleached rods were incubated with 9-cis retinal and phosphorylated. Phosphorylation of the regenerated rods was found to be light-dependent. This indicates that the phosphorylation sites in membranes that have been exposed upon bleaching of the chromophore are again masked by regeneration of the chromophore.

Table I summarizes the effect of Na K ATPase inhibitors on the phosphorylation reaction. Both light- and dark-phosphorylation of rod membrane proteins were not affected by oubain. High concentrations of $K^+$ ion which are known to stimulate dephosphorylation of phosphorylated ATPase(11) had no effect on the phosphorylation reaction in rod membranes. Treatment of phosphorylated rod outer segments with hydroxylamine or at pH 9 did not result in dephosphorylation. The results indicate that the phosphate group is not attached to the acyl group of an acidic amino acid residue as is the case with Na K ATPase. In phosphorylated rod membranes, serine

Table I. Effect of Na K ATPase inhibitors on light-dependent phosphorylation of rod membrane proteins.

| | Phosphate incorporated* ( nmoles ) |
|---|---|
| Control(pH 7.4) | 32.3 |
| Treatment at pH 9 after phosphorylation | 31.2 |
| Treatment with $NH_2OH$ after phosphorylation | 32.8 |
| $10^{-4}$M oubain | 33.7 |
| $3 \times 10^{-2}$ M $K^+$ | 34.8 |

*The total rhodopsin concentration was 46.2 nmoles. Therefore, moles of phosphate incorporated per mole of total rhodopsin was approximately 0.7. A comparable value for dark phosphorylation was less than 0.1.

and threonine residues have been previously shown to be phosphorylated(3,4).

Discussion:We have confirmed previous observations(3-6) that when isolated rod membranes are incubated with ATP, i.e. under in vitro conditions, a light-dependent phosphorylation of membrane proteins takes place. However, the main $[^{32}P]$-labelled component was not found in the visual pigment fractions collected from a chromatographic column, although the radioactivity of $[^{3}H]$-leucine incorporated into rod membranes was closely associated with the pigment fractions. From these results, we conclude that most rhodopsin molecules are not phosphorylated and the phosphorylated protein constitutes only a very minor fraction of rod membrane proteins.

Since even the least contaminated rod preparations demonstrate some Na K ATPase activity(5), it was possible that a part or all of the incorporated $[^{32}P]$ radioactivity might be accounted for by the phosphorylated enzyme. However, this possibility was excluded because oubain and high $K^+$ concentrations which are known to inhibit phosphorylation of ATPase(11) did not affect phosphorylation of rod membrane proteins and on the ground that, unlike the phosphorylated ATPase, the phosphate group was not cleaved by hydroxylamine or at alkaline pH.

The phosphorylated protein is not likely to be a rhodopsin precursor reported to be present in disc membranes(12) since the precursor is supposedly devoid of the retinal chromophore and its phosphorylation would not be light-dependent. The stability of the protein in such detergents as Emulphogene BC 720 and Ammonyx LO argues against the possibility of the component being a cone pigment, as cone pigments are readily decomposed(bleached) in these detergents(Shichi, H., unpublished).

The following lines of evidence support the conclusion that the phosphorylated component is a rhodopsin. (a)The phosphorylated component is light-sensitive and, upon bleaching by light, the component behaves on an Agarose column like bleached rhodopsin. (b)The phosphorylation sites exposed upon the bleaching of rhodopsin become inaccessible to kinase by regeneration of the chromophore. (c)The action spectrum of the phosphorylation reaction reported by Bownds et al(4) coincides with the absorption spectrum of rhodopsin.

It has yet to be investigated whether the phosphorylated component is indeed a species of rhodopsin different from the majority of rhodopsin or whether the component is phosphorylated because it is localized in specific parts(e.g. plasma membrane) of the rod membrane system.

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