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Rhodopsin Phosphorylation Occurs at Metarhodopsin II Level*

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Abstract. Photolyzed rhodopsin was phosphorylated in bovine rod outer segments incubated at −10° C. In the experiment in which urea-treated outer segments and rhodopsin kinase were incubated with ATP in the presence of 30% glycerol, the extent of phosphate incorporation at −10° C was about 30% of that at 37° C. Separation of phosphorylated rhodopsin by isoelectric focusing indicated that a limited number of sites were phosphorylated at −10° C. The partially phosphorylated pigment incorporated more phosphates when the temperatures was raised to 37° C. This was partly due to decreased inhibition of phosphorylation by glycerol at higher temperature. Since the maximum phosphorylation at −10° C (at which metarhodopsin II is stable) occurred at a pH value (6.0) lower than the pKa for metarhodopsin I-metarhodopsin II transition, metarhodopsin II was suggested to be the preferred substrate for rhodopsin kinase at −10° C. Limited proteolysis with thermolysin of rhodopsin phosphorylated at 37° C released peptides containing about 50% of the total phosphate incorporated. In contrast, proteolytic digestion of rhodopsin phosphorylated at −10° C released negligible amounts of phosphate-containing peptides. The results were taken to suggest that the incorporation of phosphates at metarhodopsin II level under the present condition occurred in the residues other than those removed by thermolysin digestion.

Key words: Rhodopsin phosphorylation – Rod proteins – Rhodopsin kinase – Isoelectric focusing

Introduction

Light activation of cyclic nucleotide phosphodiesterase in rod photoreceptors (Miki et al. 1973) is believed to occur as a sequence of discrete events: (*i*) photoconversion of rhodopsin to an active form of rhodopsin, (*ii*) activation of a

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GTP-binding protein by active rhodopsin, and (*iii*) activation of phosphodiesterase by the GTP binding protein (Liebman and Pugh 1980; Fung et al. 1981; Shichi 1981).

It has been suggested that active rhodopsin involved in the light activation of frog photoreceptor phosphodiesterase is probably metarhodopsin II (meta II) (Liebman 1981; Fukada et al. 1981). Furthermore, the phosphorylation of rhodopsin is suggested to be the mechanism by which the activity of active rhodopsin is turned off (Liebman and Pugh 1980). In fact, the capability of active rhodopsin to activate phosphodiesterase is reduced by 50% if the activation is carried out under the condition that rhodopsin is phosphorylated (Shichi and Somers 1980).

If rhodopsin phosphorylation plays a regulatory role and active rhodopsin is meta II, the phosphorylation reaction has to occur at meta II level or at steps preceding the formation of this intermediate. In order to test this hypothesis, we have investigated in this work the phosphorylation of rhodopsin at a temperature (−10° C) at which meta II is stable (Matthews et al. 1963). We have found that meta II is phosphorylated at this temperature but to a considerably lesser extent than that to which rhodopsin is phosphorylated at 37° C. The polypeptide region phosphorylated at −10° C is not cleaved off by limited proteolysis, whereas rhodopsin phosphorylated at 37° C loses 50% of its phospate with a peptide fragment released by the same treatment. The significance of these findings will be discussed.

Methods

Rod outer segments (ROS) were prepared from fresh bovine retinas as described (Shichi and Somers 1978). Urea-treated ROS were prepared by sonicating ROS in 50 mM *Tris* HCl (pH 8.0)-5 mM EDTA containing 5 M urea (Shichi and Somers 1978). Rhodopsin kinase was purified according to the method described previously (Shichi and Somers 1978).

For phosphorylation of ROS proteins, a mixture containing 25 µl ROS (about 3 nmole rhodopsin) in 0.5 M *Tris* HCl (pH 7.4), 25 µl 40 mM $MgCl_2$, 25 µl 15 mM γ-(^{32}P) ATP (New England Nuclear, 200–300 DPM/pmole), 50 µl deionized water, and 125 µl 60% (w/w) glycerol was incubated at −10° C or 37° C either in the dark or under constant irradiation with white light (about 100 ft candles at the side of tube containing sample). Rhodopsin in urea-treated ROS was phosphorylated under similar conditions by incubating a mixture of 25 µl urea-treated ROS (0.2 nmole rhodopsin) in 0.5 M *Tris* HCl (pH 7.4), 25 µl 40 mM $MgCl_2$, 25 µl 1 mM γ-(^{32}P) ATP (200–300 DPM/pmole), 50 µl rhodopsin kinase (2–3 µg protein), and 125 µl 60% (w/w) glycerol. The reaction was stopped by the addition of 250 µl 10% trichloroacetic acid containing 5 mM H_3PO_4 (Shichi and Somers 1978). After 30 min at 0° C, the precipitate was filtered (Millipore HA, 0.45 µm pore size), washed and counted. Phosphorylated ROS samples for isoelectric focusing were centrifuged at 20,000 *g* for 20 min at 0° C to stop the reaction. At 37° C, rod protein phosphorylation and

rhodopsin phosphorylation in the presence of 30% glycerol were 30% and 60% less than corresponding controls without glycerol, respectively.

Isoelectric focusing of phosphorylated rhodopsin was performed by a modification of the method described by Kühn and McDowell (1977). Ten mM H_3PO_4 was used for the anode and 20 mM NaOH for the cathode. Cylindrical gels (11 × 0.6 cm) containing 5% (w/v) polyacrylamide, 0.5% (v/v) Triton X-100, 1% Pharmalyte (pH 3–10) and 4% (v/v) Pharmalyte (pH 4–6.5) were pre-electrofocused for 1 h at 1 mA/tube. Pharmalyte was purchased from Pharmacia. Samples were applied and electrofocused for 16 h at 0.25 mA/tube at 3° C in the dark. Each gel was cut into disks 5 mm in thickness and extracted with 2% Emulphogene BC 720 (GAF Corp.). The radioactivity and absorbance at 498 nm of extracts were determined. Rhodopsin was regenerated by incubating bleached ROS (or bleached urea-treated ROS) with three times as much 11-*cis* retinal as opsin at 3° C overnight in the dark.

Phosphorylated rhodopsin was digested by thermolysin according to Pober and Stryer (1975). A reaction mixture (0.2 ml) containing the membrane suspension in 10 mM *Tris* acetate (pH 7.4), 2 mM $CaCl_2$ and thermolysin (0.02 mg/mg rhodopsin) was incubated at 23° C for a given time. After stopping the reaction by the addition of 0.2 ml 10 mM EDTA, the mixture was filtered through a Millipore HA (0.45 μm pore size) and washed with 5 mM EDTA. The radioactivity of both the precipitate and filtrate was determined.

Results

Phosphorylation of Rod Proteins

Rod proteins were phosphorylated at −10° C both in the dark and in the light (Fig. 1B). The reaction was essentially complete in 72 h. The extent of phosphorylation in the light was about four times as high as that in the dark. After correction for dark phosphorylation, the maximum light-dependent phosphorylation was calculated to be about 0.6 mole of phosphate per mole rhodopsin (Fig. 1A). The maximum level of phosphorylation attained in 60 min at 37° C is shown in Fig. 1A. If the temperature of the reaction mixture was raised to 37° C at different intervals during the low-temperature incubation, phosphorylation was markedly increased to a level of about 1.2 moles phosphate per mole rhodopsin (Fig. 1A). The constant level of phosphorylation indicated that the enzyme involved in the reaction was not inactivated during the prolonged incubation at −10° C.

Phosphorylation of Rhodopsin

The phosphorylation of proteins in irradiated rod outer segments does not necessarily represent rhodopsin phosphorylation. In order to demonstrate the phosphorylation of rhodopsin, it is necessary to prepare rod membranes which

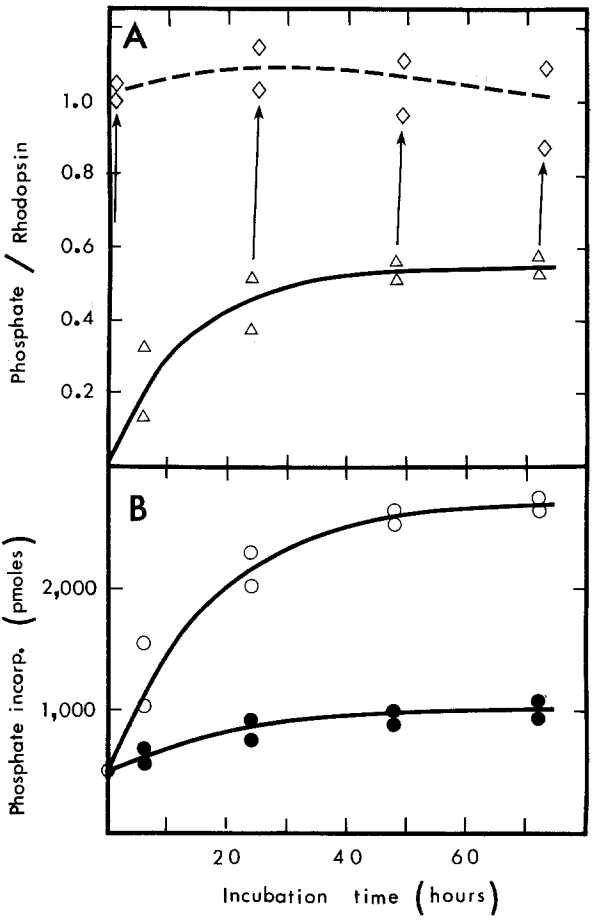

Fig. 1A and B. Phosphate incorporation into bovine rod outer segments at −10° C. Phosphate incorporation in the dark (●) and in the light (○) is shown in **B**. Open triangles (△) in **A** represent light-dependent phosphate incorporation calculated as moles of phosphate per mole rhodopsin. At given time points (indicated by arrow), the temperature of the reaction mixture was raised to 37° C and incubated for 60 min to determine the maximum level of phosphate incorporation (◇)

are free of associated proteins and enzymes. We prepared such membranes by treatment of intact rods with urea and incubated them with purified rhodopsin kinase. Rodopsin in urea-treated membranes was phosphorylated at −10° C and the reaction was virtually complete within 48 h after the onset of incubation (Fig. 2B). The pH at which the maximum phosphorylation occurred in 7 h at −10° C was about 6 (data not shown). Phosphorylation in the dark was negligible. In the light, phosphate incorporation was about 0.25 mole per mole rhodopsin (Fig. 2A). Exposure of the reation mixture to 37° C at different intervals during the incubation resulted in a three-fold increase in the extent of phosphorylation (Fig. 2A). The difference in the extent of phosphorylation between at 37° C and −10° C is partly attributed to higher inhibition of

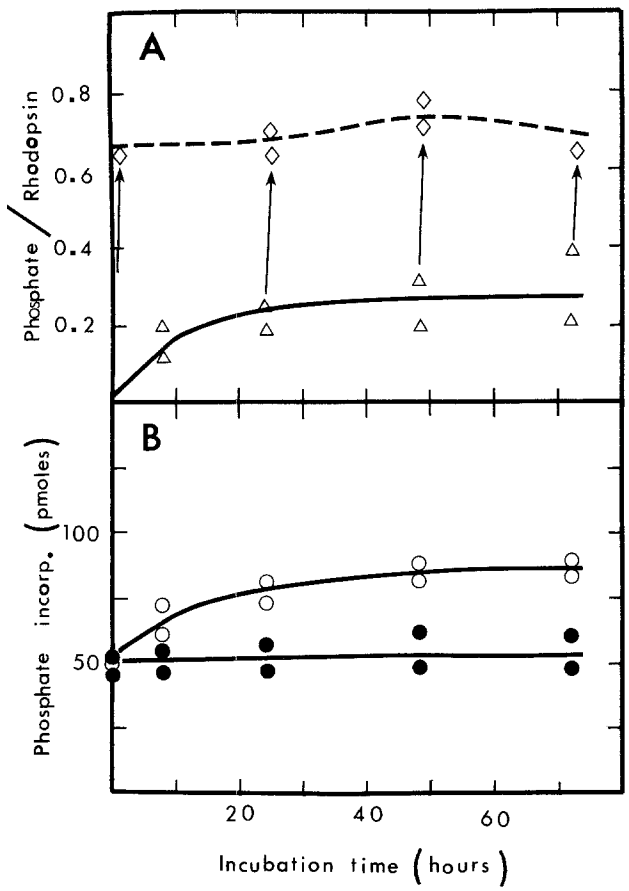

Fig. 2A and B. Phosphorylation of rhodopsin at −10° C. The reaction mixture contained urea-treated outer segments, rhodopsin kinase and ATP (see text for detail). Dark (●) and light (○) phosphorylations are presented in **B**. Light-dependent phosphorylation is shown by open triangles (△) in **A**. The maximum phosphorylation (◇) was determined at 37° C by raising the temperature of sample at the time points indicated by arrow and incubating for 60 min

phosphorylation by glycerol at lower temperature. The maximum level of phosphorylation at 37° C was about 0.7 mole phosphate per mole rhodopsin and remained virtually constant throughout the incubation period. Thus, rhodopsin kinase was not denatured during the incubation period at −10° C. The kinase to rhodopsin ratio was 27 for the experiment of Fig. 1 (intact ROS) and 300 for the experiment of Fig. 2 (urea-treated ROS). Therefore, the lower degree of phosphate incorporation into urea-treated membranes cannot be explained by a lack of the availability of sufficient amounts of enzyme to urea-treated rod membranes. The higher phosphate incorporation into untreated membranes may be attributed to an increase in the phosphorylation of each rhodopsin molecule or, alternatively, to the phosphorylation of membrane components other than rhodopsin.

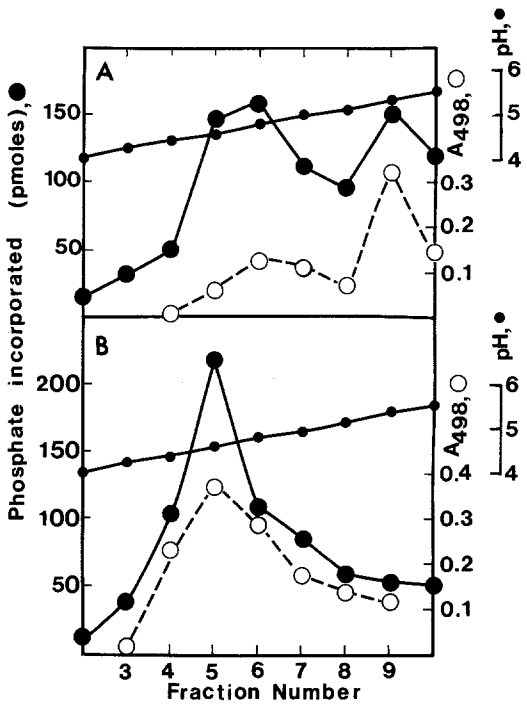

Fig. 3A and B. Separation of phosphorylated rhodopsin by isoelectric focusing. After phosphorylation at −10° C (**A**) or 37° C (**B**), outer segments were solubilized with Tween-80 and electrofocused. Large filled circles (●), phosphate incorporated in pmoles; open circles (○), A_{498}; small filled circles (•), pH values of gel slices

Isoelectric Focusing of Phosphorylated Rhodopsin

Whether the sample be ROS or urea-treated ROS, there was a difference in the final level of phosphorylation between the sample incubated at −10° C and that incubated at 37° C. This is explained either as increased phosphate incorporation into a fixed population of rhodopsin molecules at 37° C as compared to phosphate incorporation at −10° C or as an increase in the population of phosphorylated rhodopsin at higher temperature. In order to determine which is the case, phosphorylated rhodopsin at either temperature was solubilized with detergent and examined by isoelectric focusing. Rhodopsin phosphorylated at −10° C was separated into two major peaks with isoelectric points at 5.3 and 4.8 (Fig. 3A). On the other hand, rhodopsin phosphorylated at 37° C migrated as one major peak with an isoelectric point of 4.6 (Fig. 3B). Essentially the same electrophoretic pattern was obtained both with phosphorylated intact membranes and phosphorylated urea-treated membranes. The phosphorylated proteins were undoubtedly rhodopsin because (^{32}P)-radioactivity and absorbance at A_{498} approximately paralleled with each other. Since the isoelectric point of rhodopsin would shift toward the acid with increased incorporation of phosphate groups (Kühn and McDowell 1977), the results of Fig. 3 indicate that rhodopsin phosphorylated at −10° C (Fr. 9) contained less phosphate than that phosphorylated at 37° C. Since we did not attempt to resolve phosphorylated rhodopsin species any further, it is possible that each peak of Fig. 3 may not represent a homogeneous population with respect to phosphate content.

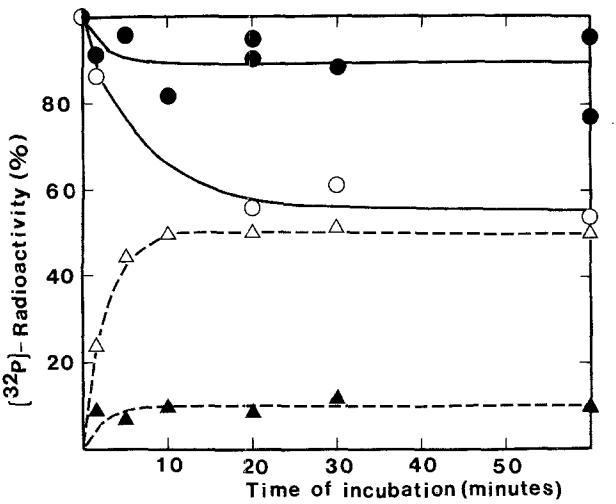

Fig. 4. Proteolytic digestion of phosphorylated rhodopsin. Filled symbols are for outer segments phosphorylated at −10° C and open symbols for those phosphorylated at 37° C. Filled circles (●) and open circles (○) represent the (^{32}P)-radioactivity of digested membranes. Filled triangles (▲) and open triangles (△) show the radioactivity of phosphorylated peptides collected in the filtrate

Proteolytic Digestion of Phosphorylated Rhodopsin

Proteolytic digestion by thermolysin of urea-treated ROS which had been phosphorylated at −10° C released no more than 10% of the phosphate with cleaved peptides (Fig. 4). On the other hand, proteolysis of membranes phosphorylated at 37° C resulted in the release of about 50% of the total phosphates into the medium (Fig. 4). With both membranes, the fraction of phosphate lost from the membrane was approximately proportional to the fraction of phosphate appeared in the medium.

Discussion

After rhodopsin photolysis, the intermediates stabilized at −10° C are meta I and meta II (Matthews et al. 1963). The present study does not determine unequivocally which intermediate is phosphorylated because the intermediates exist in equilibrium. However, in view of the fact that the equlibrium shifts from meta I toward meta II at temperatures above −15° C (Matthews et al. 1963) and that the maximum phosphorylation occurs at a pH value (6.0) lower than the pKa (6.4) of meta I-meta II transition (Matthews et al. 1963), meta II is considered to be the preferred substrate for rhodopsin kinase. Phosphorylation at −10° C is about 30% of that at 37° C. Hence, phosphorylation at meta II level is limited and additional phosphorylation occurs during the decay of this intermediate. Although the experiment with urea-treated membranes and purified kinase indicates that a single enzyme can catalyze phosphorylation both

at meta II and at later stages, the presence of a group of kinases specific for differentially phosphorylated substrates cannot be ruled out. The higher extent of phosphorylation attained with intact membranes than with urea-treated membranes may be explained by the action of such kinases associated with intact membranes.

Rhodopsin has been reported to have five phosphorylation sites (Kühn and McDowell 1977; Shichi and Sommers 1978). According to a recent paper (Wilden and Kühn 1982) as many as nine phosphorylation sites are present per rhodopsin. 30% glycerol inhibits rhodopsin phosphorylation by 30% at 37° C and 60% at −10° C. Therefore, in the presence of glycerol, only six out of nine sites, at maximum, would be phosphorylated at 37° C. The finding that phosphorylation at −10° C is about 30% of that at 37° C indicates phosphorylation of no more than two sites at −10° C under the condition. Although the level of phosphorylation in this work by no means reflects phosphorylation in vivo, these sites phosphorylated before the decay of metarhodopsin II may be of particular importance for the regulation of active rhodopsin.

In the limited proteolysis of rhodopsin, thermolysin releases the peptides $Val^{12'}$–Ser–$Lys^{10'}$–Thr–Glu–Thr–Ser–$Gln^{5'}$ and $Val^{4'}$–Ala–Pro–$Ala^{1'}$ from the carboxyl terminus of opsin (Hargrave and Fong 1977). The present result suggests that phosphates are probably not incorporated into these peptides at −10° C. To demonstrate this conclusively, thermolysin-cleaved peptides have to be isolated and analyzed for identification. Aton and Litman (1982) separated each population of differentially phosphorylated rhodopsin species by isoelectric focusing and prepared tryptic digests from each species. Their results suggest that three out of the four sites phosphorylated first are located on the residues closer to the amino terminus than $Lys^{10'}$.

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