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Mechanistic studies on the phosphorylation of photoexcited rhodopsin

Charles Fowles, Ram Sharma and M. Akhtar

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, England

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The mechanism of the photophosphorylation of rhodopsin was studied using several synthetic peptides corresponding to the sequence of the phosphorylation domain. It was found that the decapeptide (residues 339–348) was effectively phosphorylated by rhodopsin kinase only when incubation was performed in the presence of both rhodopsin and light. These results are interpreted to suggest that in the dark-adapted state rhodopsin kinase exists in an inactive conformation and that this is converted into a catalytically competent form only after interaction with metarhodopsin II (Rho*)

Rhodopsin; Rod outer segment; Rhodopsin kinase; Synthetic peptide; Peptide phosphorylation; (Retina)

1. INTRODUCTION

Photoactivation of rhodopsin leads to the formation of bathorhodopsin as the earliest detectable intermediate, the latter then decaying to a longer lived species referred to as metarhodopsin II. There is much circumstantial evidence to suggest that the latter species (designated Rho*) is involved in signal transmission via interaction with transducin (fig.1) [1–4]. Rho* also acts as a substrate for rhodopsin kinase [5,6] and this process culminates in the phosphorylation of several amino acid residues located in the C-terminal region of rhodopsin [7–9]. The resultant phosphoprotein is proposed to bind to a 48 kDa protein (arrestin) forming a complex no longer able to interact with transducin, resulting in signal termination (fig.1) [10–12]. The mechanism of the photophosphorylation has now been studied using several synthetic peptides corresponding to the sequence of the phosphorylation domain.

Correspondence address: M. Akhtar, Dept of Biochemistry, The University of Southampton, Medical and Biological Sciences Building, Bassett Crescent East, Southampton SO9 3TU, England

2. EXPERIMENTAL

2.1. *Materials*

Labscint scintillation cocktail was obtained from Lablogica (Sheffield, England); [γ-^{32}P]ATP and NCS tissue solubiliser from Amersham; leupeptin, pepstatin A, antipain and soybean trypsin inhibitor from Sigma (Poole) and all other chemicals from BDH (Poole), Sigma or Interchem.

2.2. *Isolation of rhodopsin kinase and kinase-free rod outer segments*

All operations were performed under dim-red safety-light illumination (25 W bulb with Kodak GBX-2 safelight filter) at 4°C. Freshly isolated bovine retinae were gently shaken in solution A (100 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1% β-mercaptoethanol) containing 45% (w/v) sucrose and then carried through to the sucrose density step as described [13,14]. The rod outer segments were removed, pelleted and washed with solution B (solution A with 1 μg/ml each of antipain, leupeptin, pepstatin and soybean trypsin inhibitor added). The pellet was now suspended in solution C (distilled water containing 1 μg/ml each of antipain, leupeptin, pepstatin and soybean trypsin inhibitor) at 1.2×10^{-5} M rhodopsin (calculated from E_{500} 40000 $mol \cdot l^{-1} \cdot cm^{-1}$) and extracted by homogenisation to furnish after centrifugation a supernatant that was made up to 2 mM $MgCl_2$ and is termed kinase extract.

The membrane pellet from the above was washed with solution C and then with 5 M urea. The membrane pellet was finally washed and stored in solution B at 1.2×10^{-5} M rhodopsin and this sample was found to be free of kinase and used as a source of rhodopsin in all experiments described below.

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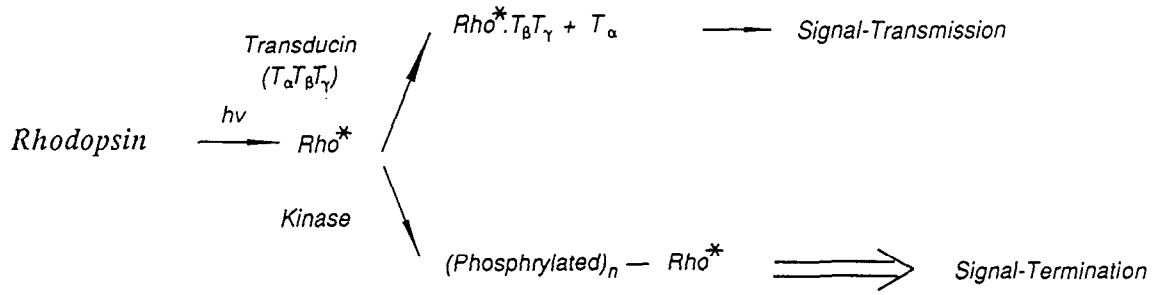

Fig.1. The involvement of photoactivated rhodopsin (Rho* = metarhodopsin II) in signal transmission and signal termination processes.

2.3. *Assay for kinase activity*

Rhodopsin kinase activity was measured in solution B in a final volume of 100 μl containing kinase extract (24 μg protein), 2.34×10^{-5} M rhodopsin and 3 mM [γ-^{32}P]ATP. Incubation was performed at 30°C and the incorporation of ^{32}P into the protein determined as in [15]. The kinase extract had a specific activity of 15 nmol phosphate transferred/min per mg protein present in the original extract.

2.4. *Separation and analysis of phosphopeptide*

Samples of the incubation mixtures (5–10 μl) were centrifuged and the supernatant applied to a cellulose TLC plate (Merck 5716, 20 × 20 cm cellulose TLC plates) in 1 cm strips. The plate was then subjected to electrophoresis in pyridine:acetic acid:water (1:10:89) buffer (pH 3.5) for 2 h at 7°C and after drying in air was exposed to Fuji NIF RX X-ray film for 10 h. Thereafter the film was developed to view radioactive bands corresponding to the phosphopeptide. These areas of cellulose were then scraped off the plate and analysed by scintillation counting in Labscint using a Philips PW470 liquid scintillation counter programmed for automatic quench correction.

3. RESULTS

Four peptides containing residues 318–348, 329–348, 329–339 and 339–348 (peptides I–IV, respectively) of bovine rhodopsin [16] were prepared by the Merrifield solid-phase synthesis method [17]. Initially, these peptides were screened for their ability to inhibit the light-dependent phosphorylation of rhodopsin. It was found that although all four peptides inhibited the phosphorylation reaction, the decapeptide IV was by far the most effective. These results are qualitatively similar to those reported for similar (but not identical) peptides by Palczewski et al. [18].

A number of experiments were designed in which the peptides (I–IV) were incubated with [γ-^{32}P]ATP, rhodopsin kinase and rod outer segments in the presence of light and in complete darkness. Since the most significant results were obtained with the decapeptide IV, experiments pertaining to this peptide are considered in detail.

H_2N-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala-COOH
339 348

(Peptide IV)

Fig.2 shows the analysis of various incubation mixtures performed under different conditions. In

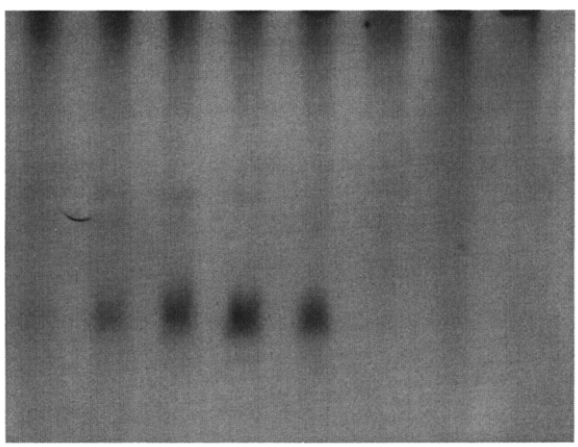

Fig.2. Phosphorylation of the synthetic peptide IV under various conditions. Incubations were performed for 1 h at 30°C under white light illumination (except lane 7) in volumes of 100 μl solution B (section 2.2). Final concentrations were: rhodopsin as indicated, 24 μg rhodopsin kinase, 1 mM peptide IV (unless stated) and 3 mM [γ-^{32}P]ATP (16400 cpm/nmol ATP). After incubation the suspensions were centrifuged and 5-μl samples of the supernatant analysed by cellulose TLC electrophoresis as in section 2.4. Rhodopsin concentrations were: (lanes) 1, 8×10^{-7} M; 2, 2.4×10^{-6} M; 3, 3.6×10^{-6} M; 4, 1.2×10^{-5} M; 5, 2.4×10^{-5} M; 6, no rhodopsin; 7, complete system as in lane 5 but incubated in darkness; 8, as in lane 5 but without the peptide.

the experiments in which incubation was performed either in the absence of rhodopsin (lane 6), or with rhodopsin but in complete darkness (lane 7), or in the absence of peptide (lane 8), no significant radioactivity was observed in a region corresponding to a putative phosphopeptide. However, in the experiments containing all components (peptide, [γ-^{32}P]ATP, kinase and rod outer segments) performed in continuous white light, there was an impressive amount of radioactivity associated with a band that migrated 10 cm from the origin towards the cathode. The intensity of the band depended on the amount of rhodopsin (lanes 1–5) and optimal phosphorylation of the decapeptide was obtained when the rhodopsin concentration was 1.2×10^{-5} M. The phosphorylation of a peptide (residues 337–348 of rhodopsin) by the kinase without rhodopsin has been reported when incubation was performed for several hours [18].

From the aforementioned experiment it appears that an effective phosphorylation of the exogenously added peptide was intimately linked to the photoexcitation of rhodopsin. This aspect was further studied by performing experiments in which the phosphorylation of rhodopsin and the decapeptide was monitored simultaneously under different conditions. Fig.3 shows the time course of phosphorylation of rhodopsin and the decapeptide and emphasises that both these processes were dependent on photoactivation. No significant phosphorylation was observed when all components of the reaction mixture were present but

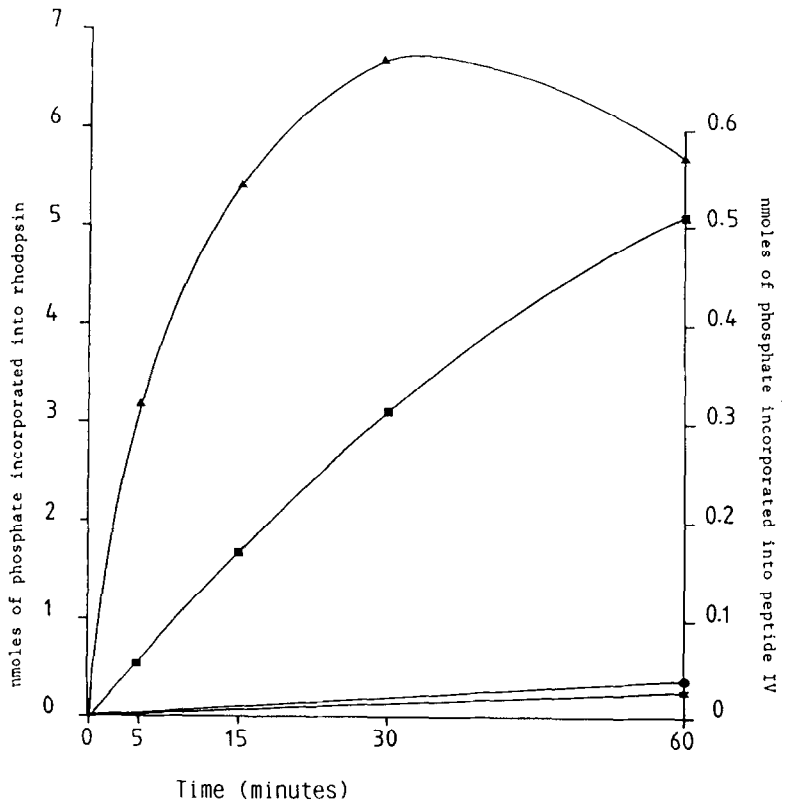

Fig.3. Time course for the phosphorylation of rhodopsin and the synthetic peptide IV. Phosphorylation of (▲) rhodopsin in light; (●) rhodopsin in darkness; (■) peptide IV in light; (★) peptide IV (complete system) in darkness or in the absence of rhodopsin. Incubations were performed at 30°C in volumes of 100 μl in the presence of white light or in complete darkness as indicated. Final concentrations were 2.4×10^{-5} M rhodopsin, 60 μg rhodopsin kinase, 1 mM peptide IV and 3 mM [γ-^{32}P]ATP (80000 cpm/nmol ^{32}P) in solution B (section 2.2). At various time intervals, aliquots of the incubation mixtures were centrifuged and the pellet processed for the determination of protein-bound radioactivity (section 2.3) and the supernatant subjected to cellulose TLC electrophoresis as in section 2.4. For all curves the radioactivity incorporated is shown as that calculated for the total incubation mixture (100 μl).

incubation was performed in the dark. The comparison of curve (■) with (★) (fig.3) provides the most dramatic illustration of the requirement of light as well as rod outer segments for the phosphorylation of the peptide. The phosphorylation of rhodopsin was maximum at 30 min when 6.5 nmol phosphate groups were incorporated into protein whereas the incorporation into the peptide was 0.3 nmol phosphate at this time.

The remarkable fact that dark-adapted rhodopsin is resistant to the action of the kinase, but becomes an effective substrate following photoexcitation, may be rationalised by at least two alternative hypotheses. First, the 'site-accessibility' hypothesis assumes that the C-terminal of rhodopsin, which is the site for the action of the kinase [7–9], is inaccessible to the enzyme when rhodopsin is in the dark-adapted state and it is only after photoexcitation that a new conformation of the visual protein is obtained in which the C-terminal region becomes available for modification by the kinase (pathway A, fig.4). The second possibility is that in the dark state the kinase exists in an inactive form and is activated after binding to photoexcited rhodopsin (pathway B, fig.4). The domain in rhodopsin responsible for the activation may either be the C-terminal region and/or another part of the rhodopsin molecule that undergoes profound conformational change following photoexcitation.

We have found that of the four peptides used by us, the decapeptide IV was the best substrate for the kinase and that the phosphorylation of the peptide was greatly enhanced (15–30-fold) by the presence of light as well as rhodopsin. We believe that the simplest explanation of these results is that photoexcitation leads to the exposure of a region of rhodopsin that interacts with the kinase, converting the latter into an active form that participates in the phosphorylation of the C-terminal and that the peptide manages to 'smuggle' into the Rho*-kinase complex acting as a 'surrogate' substrate.

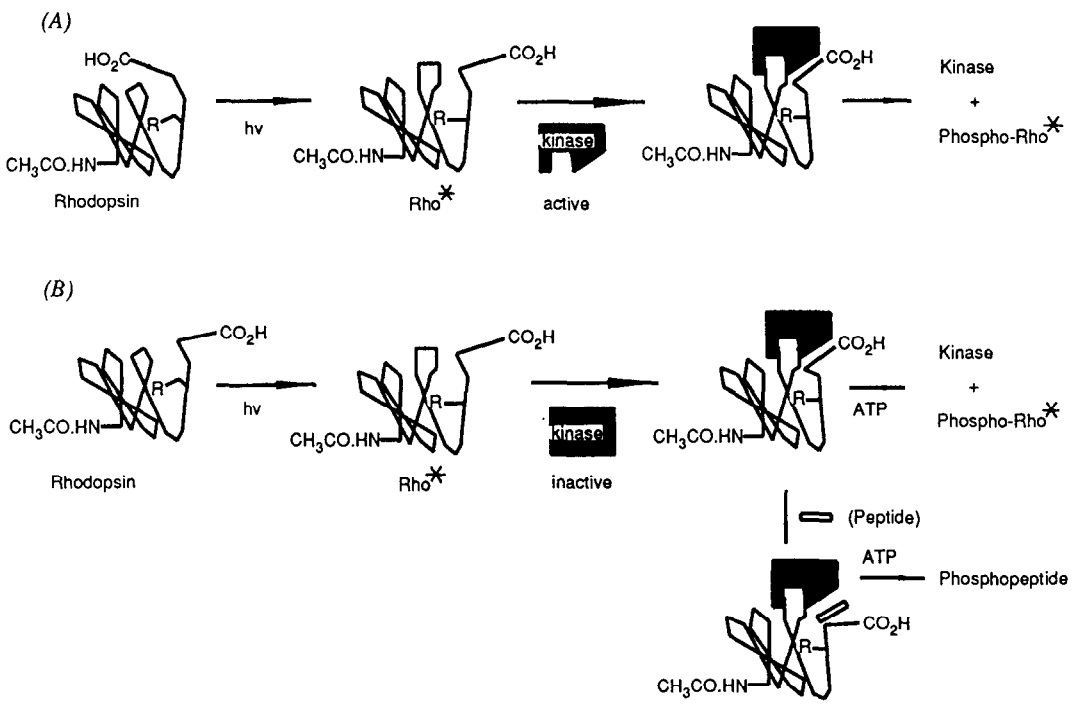

Fig.4. Two hypotheses regarding the light activation of rhodopsin phosphorylation. In (A) light excitation exposes the C-terminal of rhodopsin to the kinase, while in (B) kinase exists in an inactive form and is rendered catalytically competent only after binding to a domain in Rho*. In both the alternatives the major conformational change is shown to occur only in one of the cytoplasmic loops. This choice is completely arbitrary and is made for convenience. Peptide refers to peptide IV, comprising residues 339–348 of rhodopsin.

The model proposed in fig.4 is broadly consistent with the earlier findings of Kühn [1] who has convincingly shown that rhodopsin kinase binds more tightly to Rho* than to native rhodopsin. However, it had hitherto been assumed, albeit tacitly, that the kinase acts on Rho* only because the conformation of the latter species contains the phosphorylation sites suitably exposed to the enzyme. Though this still remains a distinct possibility, the present work has unravelled a new facet of the phosphorylation system showing that the kinase only becomes catalytically active after interacting with Rho*. In the illustration of fig.4, a ternary complex (consisting of Rho*, activated kinase and peptide) has been invoked to participate in reaction with ATP to produce the phosphopeptide. A closely related possibility in which, following interaction with Rho*, the activated form of the kinase dissociates prior to participating in the phosphorylation of the synthetic peptide has not yet been eliminated.

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