Schiff-base deprotonation is mandatory for light-dependent rhodopsin phosphorylation

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The absorption of light by rhodopsin leads to the formation of an activated intermediate (R^*) capable of catalysing the exchange of GTP for GDP in a retinal guanine-nucleotide-binding regulatory protein (transducin). The ability of R^* to function as a catalyst is terminated by the rhodopsin kinase. The 10 non-active-site lysine residues of rhodopsin can be reductively dimethylated to form permethylated rhodopsin (PMRh). This derivative is phosphorylated to the same extent as rhodopsin after photolysis. The monomethylation of the active-site lysine residue of PMRh yields active-site-methylated rhodopsin (AMRh). It had previously been shown, by using AMRh, that the formation of R^* and its spectroscopic signature metarhodopsin II requires the photochemically induced deprotonation of the active-site Schiff base [Longstaff, Calhoun & Rando (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4209–4213]. Here it is demonstrated that active-site Schiff-base deprotonation is also mandatory in the formation of the form of photolysed rhodopsin that is susceptible to phosphorylation by rhodopsin kinase. In terms of the spectroscopically defined rhodopsin intermediates, this means that only metarhodopsin II and possibly metarhodopsin III are the actual substrates for rhodopsin kinase

INTRODUCTION

The absorption of light by rhodopsin causes the *cis-to-trans* isomerization of rhodopsin's Schiff-base chromophore [1]. This photoisomerization initiates a series of spectroscopically well-characterized conformational changes in the protein. These changes are accompanied by a temporary rise in the biochemically defined 'activated rhodopsin', R^* . R^* activates a guanine-nucleotide-binding regulatory protein (G-protein, transducin) and thereby initiates the visual cascade leading to signal transduction [2]. In addition, R^* can become phosphorylated by rhodopsin kinase [3,4] in a regulatory reaction believed to be involved in signal turn-off and/or adaptation [5–9]. These pathways may constitute a general mechanism of signal transduction and regulation, since mammalian hormone reception has primary-sequence similarity with rhodopsin and exhibits activated forms, analogous to R^* , that trigger G-proteins and become phosphorylated [2,10]. Furthermore, the interacting proteins are partially interchangeable with transducin and rhodopsin kinase [2,10]. Taken together, this makes a study of the control of R^* of general interest.

The Schiff-base charge of rhodopsin appears to be central to its function. Protonation of the Schiff base is critical in energy storage and in achieving some of the high-energy intermediates of photolysed rhodopsin [11]. On the other hand, deprotonation of the Schiff base also plays a critical role in the formation of R^* [12]. The question addressed here is whether deprotonation of the Schiff base is also important in forming that conformer of rhodopsin recognized by rhodopsin kinase. We investigated this question by using active-site-methylated rhodopsin (AMRh) [12]. Monomethylation of non-active-

site permethylated rhodopsin (PMRh) at its active-site lysine residue produces a pigment (AMRh) which, when photolysed, leads to a stabilized form of metarhodopsin I which is slowly hydrolysed to opsin and all-*trans*-retinal [12]. When incorporated into lipid vesicles, AMRh is not significantly phosphorylated by rhodopsin kinase in the light. It can be concluded (1) that deprotonation of the active-site Schiff base is essential to achieving that form of rhodopsin capable of being phosphorylated by the kinase, and (2) that the phosphorylation of bleached rhodopsin both must occur at a photointermediate(s) which appears after metarhodopsin I.

EXPERIMENTAL

Materials

Dodecyl maltoside was obtained from Behring Diagnostics. Phospholipids were obtained from Avanti (Pelham, AL, U.S.A.). [γ - 32 P]ATP (sp. radioactivity 0.1–3 Ci/mmol) was a product of Amersham Corp. Dark-adapted retinas were obtained from Lawson.

Methods

Preparation of rhodopsin. Rod outer segments (ROS) and solubilized rhodopsin were prepared as previously described [13].

Preparation of the rhodopsin kinase extract. The kinase extract was prepared in the dark by ultrasonic homogenization of bovine ROS (prepared as in [16], omitting the sucrose and the gradient) in 100 mM-potassium phosphate (pH 7)/200 mM-KCl/1 mM-dithiothreitol, containing 100 μ g each of aprotinin, leupeptin, pepstatin

Abbreviations used: AMRh, active-site-methylated rhodopsin; PMRh, permethylated rhodopsin; ROS, rod outer segments; OPA, *o*-phthalaldehyde; G-protein, guanine-nucleotide-binding regulatory protein (transducin); R^* , activated rhodopsin.

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and trypsin inhibitor/ml (about 10 ml/50 retinas), followed by ultracentrifugation (150 000 *g*, 90 min). The supernatant (4–7 mg/ml of protein) contained rhodopsin kinase activity which was stable for at least 3 weeks when kept on ice.

Active-site methylation of rhodopsin. Permethylation, active-site methylation and *o*-phthalaldehyde (OPA)/ β -mercaptoethanol treatment were performed by the previously published procedure [13]. As a control, permethylated opsin was treated identically.

Vesicle preparation. Vesicles were prepared by sonicating the pigment in 10 mM-Pipes/HCl/100 mM-NaCl (pH 6.5)/6 mM-dodecyl maltoside with a dried mixture of 90% (w/w) phosphatidylcholine and 10% (w/w) phosphatidylserine, resulting in a final lipid:protein ratio of 100:1 (mol/mol). The detergent/lipid ratio was $\geq 3:1$ (w/w) to facilitate the formation of mixed micelles [14]. The detergent was subsequently removed by batch chromatography on Bio-Beads SM2 [2 g of wet beads pretreated with 10 mM-Pipes (pH 7)/10 mM- Na_2EDTA /100 mM-NaCl/1 mM-dithiothreitol/glycerol (120 mg/ml) per 10 mg of detergent to be removed] for 1–3 h [15].

Rhodopsin kinase assay. Pigment-containing vesicles (4–10 mM) were incubated in 100 mM-potassium phosphate, pH 7, with 1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 0.1–3 Ci/mmol), 1 mM- MgCl_2 and rhodopsin kinase extract (0.8–1.4 mg of protein/ml as determined with bovine serum albumin as standard in a dye-binding assay [17]) in a total volume of 100–250 μl , depending on the number of time points to be considered. The amount of vesicles

applied depended on their chromoprotein concentration (judged by the absorption at their absorption maximum, namely 500 and 520 nm respectively for the two pigments). In the case of the control vesicles, where this was not possible, protein concentration was determined as described in [18], with rhodopsin as standard. Incubations were performed in the dark (dim red light) or with bleached pigments (where the phosphorylation reaction was allowed to proceed in the dark after an orange-light bleach for 1 min or under continuous illumination by 60 W white light) at 30 °C for 0–15 min, at 22 °C for 0–20 min or on ice (0 °C) for 0–150 min. The reactions were stopped after various time intervals by pipetting a 30–50 μl aliquot on to a 1.2 cm $\times$ 2 cm Whatman 3 MM filter-paper strip and immediately dropping it into a solution of 20% (w/v) trichloroacetic acid/5 mM- H_3PO_4 /10 mM-ATP (5 ml/filter) with further processing as described in [19].

RESULTS

Phosphorylation of photolysed PMRh and AMRh

The preparation of AMRh requires first that all ten non-active-site lysine residues of rhodopsin be dimethylated to form permethylated rhodopsin (PMRh) [13]. PMRh has been shown to behave identically with rhodopsin with respect to both its bleaching behaviour at 4 °C and its ability to activate transducin [12]. When PMRh is incorporated into phospholipid-based vesicles and photolysed in the presence of rhodopsin kinase, it is phosphorylated, as is the rhodopsin itself under the same conditions (Table 1). PMRh contains 20 methyl groups adducted to its peripheral lysine residues [13]. The

Table 1. Light-dependent phosphate incorporation into active-site methylated rhodopsin

Phosphate incorporation into rhodopsin and its modified analogues was determined by incubating the bleached reconstituted pigments with a rhodopsin kinase-containing ROS supernatant and ATP. Incorporation was calculated as follows:

$$\text{Average relative phosphate incorporation} = \frac{\text{Radioactivity}_{\text{sample}} - \text{Radioactivity}_{\text{kinase alone}}}{\text{Radioactivity}_{\text{PMRh}} - \text{Radioactivity}_{\text{kinase alone}}} \times 100$$

The assays were performed in triplicate with two to four time points per assay. The Table compares the following compounds with respect to their ability to become light-dependently phosphorylated: concanavalin A-Sepharose-purified reconstituted rhodopsin and its permethylated form, PMRh; the active-site-modified pigment active-site methylated rhodopsin (AMRh), which was purified by hydroxyapatite chromatography [12] and reconstituted into vesicles; and active-site OPA/ β -mercaptoethanol permethylated opsin control. Data from incubating the different vesicle preparations with the rhodopsin kinase extract in the dark are also given. The data represent averages of values derived from incubations at 30 °C (0–15 min duration), 22 °C (0–20 min) and 0 °C (0–150 min) for all dark values and light-dependent phosphorylations of PMRh and AMRh, with no measurable differences in the relative phosphate incorporation at these different temperatures. Control vesicles were only investigated at 30 and 22 °C, whereas only data derived from incubations at these two temperatures were used for the calculation of the relative phosphorylation of rhodopsin (cf. Fig. 2).

Sample	No. of different methylations	No of different vesicle preparations	No. of assays performed	No. of triplicates	Average relative phosphate incorporation (mean $\pm$ S.D.)
Rhodopsin	—	2	9	20	102.13 $\pm$ 19.01
PMRh	3	3	19	62	100
AMRh	3	6	10	39	3.56 $\pm$ 1.21
OPA-treated permethylated opsin control	2	2	2	4	1.02 $\pm$ 0.28
Dark reaction (kinase extract + vesicles)	7	10	7	76	0.63 $\pm$ 0.60

Fig. 1. Phosphorylation of bleached modified rhodopsin at different temperatures

Results are means for triplicate determinations ($\pm$ S.D.) of phosphate incorporation observed in 100 μ l standard assay (see the legend to Table 1) when incubating the kinase with vesicles containing 10 μ M previously bleached (1.5 min orange light) rhodopsin (●), PMRh (Δ) or AMRh ($\blacktriangledown$) or without added vesicles (○) at 30 °C (a), 22 °C (b) and 0 °C (c) respectively. The left-hand scale applies to (a) and (b), the right-hand scale to (c).

addition of one more methyl group at its active-site lysine residue produces AMRh [13], which, when bleached as described above in the presence of rhodopsin kinase, shows less than 5% of the phosphate incorporation of either PMRh or rhodopsin itself (Table 1). Up to six phosphate groups were incorporated per available rhodopsin molecule.

The inability of photolysed AMRh to be significantly phosphorylated by rhodopsin kinase suggests that the metarhodopsin I-like intermediate that is formed cannot be phosphorylated. The hydrolysis of the metarhodopsin I-like intermediate AMRh to its corresponding opsin derivative and all-*trans*-retinal is somewhat more rapid than the decay of the metarhodopsin II-like intermediate of PMRh. After a 10 s bleach with orange light, 75% of the metarhodopsin I-like intermediate of AMRh is hydrolysed after 3, 7, 15, 55 and 75 min at 30, 25, 20, 10 and 4 °C respectively. However, lowering the incubation temperature for 30 °C to either 22 °C or 0 °C, ensuring that substantial quantities of the metarhodopsin I-like intermediate would be present, did not increase the relative light-dependent phosphorylation of AMRh when compared with PMRh at the same temperatures (Fig. 1). Thus the stabilized metarhodopsin I-like intermediate of AMRh is not susceptible to significant rhodopsin kinase-mediated phosphorylation.

The small amount of phosphorylation of AMRh always observed (Table 1 and Fig. 1) could be caused by minor contamination with PMRh, estimated to be 2–4% by referring to the standard curve (Fig. 2). The procedure used to prepare AMRh involves the incomplete methylation of the active-site lysine residue of bleached PMRh (Lys-296) [13]. The unmethylated, free-lysine-containing material was removed by treatment with OPA/ β -mercaptoethanol, a reagent specific for primary amines [20]. This treatment, when applied to unbleached PMRh, produced no effect on this pigment's ability to serve as a rhodopsin kinase substrate (results not shown). If the reaction does not go to completion, then a small amount of PMRh will result. This is clear from transducin-activation studies, where transducin activation by these samples was found (B. Seckler & R. R. Rando, unpublished work), as well as from a control experiment.

Fig. 2. Phosphorylation of partially bleached PMRh

Average relative light-dependent phosphate incorporation into 10 μ M partially bleached PMRh vesicles is shown. Standard 100 μ l incubations were done at 30 °C for 5 or 10 min (for experimental details, see the legend to Table 1). This led to the incorporation of about 0.3 mol of phosphate/mol of PMRh when the fully bleached pigment was incubated. Partially bleached PMRh was prepared by mixing defined amounts of unbleached and freshly bleached (1 min orange-light bleach on ice) vesicles at the same concentration as described in [12].

The blocking of the active-site lysine residue without its prior methylation resulted in a modified permethylated opsin, which, after regeneration with 11-*cis*-retinal and reconstitution into lipid vesicles, still exhibited some light-dependent phosphate incorporation (Table 1). Further evidence for the possibility of minor contamination by PMRh comes from the experiment the results of which are shown in Fig. 3. AMRh, but not PMRh, is susceptible to chemical bleaching by high concentrations of hydroxylamine in the dark (Fig. 3a). Under conditions where 80% of the AMRh was chemically bleached by hydroxylamine, the phosphorylation of the sample was only slightly affected (Fig. 3b). Thus it is unlikely that the small amount of phosphorylation observed is related to the presence of AMRh.

Fig. 3. (a) Stability of modified rhodopsin in the presence of hydroxylamine, and (b) influence of hydroxylamine treatment on the ability of AMRh to be phosphorylated

(a) Absorption changes at the $\lambda_{\max}$ of the modified rhodopsin (—, PMRh, 500 nm; —, AMRh, 520 nm) as observed when incubating 3.75 μM -pigment in 10 mM-sodium Pipes/HCl/100 mM-NaCl/6 mM-dodecyl maltoside, pH 6.5, at 37 °C in the presence of 5 mM-hydroxylamine as shown. (b) Relative changes in pigment concentration (a and b) and in the ability to be phosphorylated (c and d) of PMRh (1) and AMRh (2) as a consequence of treatment of the vesicles with hydroxylamine (b and d) compared with untreated samples (a and c) are shown. Portions (1 ml) of PMRh vesicles and AMRh vesicles at a 3.75 mM chromoprotein concentration in 10 mM-sodium Pipes/HCl, pH 6.5, were either kept on ice or were incubated in the presence of 5 mM-hydroxylamine for 1 h at 37 °C with both vesicle populations at each temperature and in duplicate. Further manipulations were performed at 4 °C. All samples were diluted 8-fold and the vesicles sedimented by centrifugation at 150000 g for 1 h and washed twice. The pellets were resuspended to a final volume of 200 μl , corresponding to 18.75 mM initial chromoprotein concentration. Final pigment concentrations were determined by solubilizing an aliquot in detergent buffer (the buffer was as in a) and determining $A_{\lambda_{\max}}$ and $A_{370} \pm$ bleach in the presence of 20 mM-hydroxylamine. All vesicles were further investigated at 1:2.5 dilution in 100 μl standard incubation volume at 22 °C for 4 and 8 min to determine their abilities to serve as substrates for the kinase; 100 % relative phosphorylation corresponds to 494 and 24.8 pmol of ^{32}P incorporated into PMRh and AMRh respectively.

DISCUSSION

The studies described here were aimed at determining whether or not deprotonation of the Schiff base of rhodopsin is required in order to become a substrate for rhodopsin kinase. Our experimental approach relied on the fact that AMRh cannot be deprotonated on light absorption [13]. Furthermore, PMRh is phosphorylated to the same extent as rhodopsin itself after absorbing light and, hence, dimethylation of the non-active-site lysine residues is without effect. This has also been found to be true for the interaction of PMRh with transducin [12]. It is then possible to focus on the perturbation caused by active-site monomethylation. When AMRh was incorporated into phospholipid vesicles and irradiated in the presence of rhodopsin kinase and ATP, no significant phosphorylation of the protein was observed. Thus deprotonation of the Schiff base of rhodopsin must be necessary for producing the conformational transition that exposes phosphorylatable sites on the protein or rhodopsin kinase. Furthermore, when AMRh was incorporated into phospholipid vesicles and irradiated in the presence of rhodopsin kinase and ATP, no significant phosphorylation of the protein was observed. Thus deprotonation of the Schiff base of rhodopsin must be necessary for producing the conformational transition that exposes phosphorylatable sites on the protein to rhodopsin kinase. Furthermore, when AMRh was incorporated into phospholipid vesicles and irradiated in the presence of transducin, neither specific binding (M. A. Pajares & R. R. Rando, unpublished work) nor

activation [12] was observed. This means that rhodopsin conformers prior to metarhodopsin II are incapable of binding to transducin and, hence, binding is correlated with activation. That metarhodopsin II binds to and activates transducin is already established [21].

Efforts to correlate spectroscopically characterized photointermediates of rhodopsin with the biochemically defined R^* have shown that transducin activation is mediated by metarhodopsin II [12,22,23]. On the other hand, the phosphorylation of R^* has been reported to be triggered by either the lumirhodopsin-metarhodopsin I transition [24] or the metarhodopsin I-metarhodopsin II transition [25]. Our results with AMRh, which cannot form metarhodopsin II, argue that the conformational transitions of rhodopsin required to form a substrate for the kinase are not complete at metarhodopsin I. The results described here and previously are in complete accord with the view that the deprotonation of the active-site lysine residue of rhodopsin is obligatory in the formation of that specific conformer (R^*) recognized both by transducin and by rhodopsin kinase. The deprotonation of the active-site-lysine-residue Schiff base, which occurs during the metarhodopsin I-to-metarhodopsin II transition, must initiate large structural changes in the protein [21]. These structural changes must be translated both into the formation of the catalytically active R^* and into the unshielding of the serine and threonine residues located at the C-terminal end of rhodopsin, thus permitting their phosphorylation. This light-induced structural change is also critical for the binding to, and activation of, transducin. Whether

the similar active-site deprotonation of ligands or amino acid side chains in structurally similar signal-transducing receptors is also mechanistically important remains to be determined. In this regard, however, it is noteworthy that there appear not to be any quaternary-amine-containing catacholamine agonists of the β -receptor [26]. If the ethylamine side chain of a putative catacholamine agonist simulated the protonated Schiff base of rhodopsin, then it too would need to deprotonate prior to forming an R*-like intermediate. Thus only primary, secondary and tertiary catacholamines could possibly be agonists, and indeed these are the only ones that have been found [26].

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