Control of Rhodopsin Multiple Phosphorylation†

Hiroshi Ohguro,‡ Richard S. Johnson,§ Lowell H. Ericsson,§ Kenneth A. Walsh,§ and Krzysztof Palczewski*,‡,§,∥

Departments of Ophthalmology, Biochemistry, and Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195-0001

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ABSTRACT: The inactivation of photolyzed rhodopsin requires phosphorylation of the receptor at multiple sites near the C-terminus by rhodopsin kinase and binding of a regulatory protein, arrestin. In the present study, the phosphorylation sites were examined in a partially reconstituted system under several experimental conditions. Initial phosphorylation sites were found to be ^{338}Ser, ^{343}Ser, and ^{334}Ser based on analysis by mass spectrometry of proteolytic peptides from the C-terminus. The extent of phosphorylation was found to be limited by two mechanisms: (1) binding of arrestin to phosphorylated rhodopsin (one to three phosphate groups) appeared to prevent further phosphorylation (arrestin has also been observed to promote the initial phosphorylation of rhodopsin at ^{338}Ser in rod outer segment homogenates); and (2) reduction of the photolyzed chromophore *all-trans*-retinal to *all-trans*-retinol prevented phosphorylation at more than three sites. We propose that previous observations of higher levels of rhodopsin phosphorylation may be the result of the removal of endogenous arrestin, or of exceeding the capacity of retinol dehydrogenase activity by intense bleaches (e.g., by exhausting endogenous NADPH).

Transduction of a light impulse into a visual sensory signal, quenching of the signal, and restoration of the dark condition are accomplished through a cycle of enzymatically controlled reactions [reviewed by Chabre and Deterre (1989), Hargrave and McDowell (1992), and Lagnado and Baylor (1992)]. During the transition of photolyzed rhodopsin to inactivated opsin, the receptor assumes three relatively stable conformations named metarhodopsin I, II, and III [reviewed by Yoshizawa and Kandori (1991)]. Metarhodopsin II binds to a G protein (transducin), and initiates the signal-amplifying cascade of reactions. Concurrently, metarhodopsin I (Paulsen & Bentrop, 1983; Pulvermüller et al., 1993), metarhodopsin II (Seckler & Rando, 1989), or both are phosphorylated by rhodopsin kinase (Bownds et al., 1972; Kühn & Dreyer, 1972; Frank et al., 1973). The dissociation of the kinase is followed by the binding of arrestin (Wilden et al., 1986; Bennett & Sitaramayya, 1988). The further decay of metarhodopsin II and III, in equilibrium with free *all-trans*-retinal and opsin, results from the reduction of the photolyzed free *all-trans*-retinal into *all-trans*-retinol by disk membrane-associated retinol dehydrogenase (Ishiguro et al., 1991). Once this reduction has occurred, arrestin dissociates from the inactive phosphorylated opsin (Hofmann et al., 1992).

Recent reports from three laboratories demonstrate that rhodopsin is multiply-phosphorylated in the C-terminal region, preferentially at ^{338}Ser, ^{343}Ser, and ^{336}Thr residues, when homogenates of rod outer segments (ROS) are examined (Ohguro et al., 1993; McDowell et al., 1993; Papac et al., 1993). Minor differences in these reports suggest that the kinetics of phosphorylation at specific sites may be influenced by unknown factors. For instance, phosphorylation of ^{343}Ser predominated in certain phosphorylated preparations (McDowell et al., 1993; Papac et al., 1993), whereas we found phosphorylation primarily at ^{338}Ser (Ohguro et al., 1993). In other *in vitro* procedures, rhodopsin was phosphorylated to a much higher extent (an average of six to eight phosphate groups per rhodopsin) (Wilden & Kühn, 1982; Miller & Dratz, 1984); however, this high stoichiometry was difficult to reproduce in any of the recent studies. Under physiological conditions, only one or two phosphates appear to be necessary for rapid inactivation of metarhodopsin II (Sitaramayya, 1986; Bennett & Sitaramayya, 1988).

We now address whether steps in quenching and regeneration of rhodopsin, namely, binding of arrestin and removal of photolyzed chromophore, influence rhodopsin phosphorylation by rhodopsin kinase. In this study, two mechanisms were identified that prevent rhodopsin phosphorylation to high stoichiometry: (1) binding of arrestin to phosphorylated rhodopsin; (2) reduction of photolyzed chromophore *all-trans*-retinal to *all-trans*-retinol. We postulate that earlier observations of high levels of phosphorylation are a result of exceeding the capacity of retinol dehydrogenase in ROS homogenates.

MATERIALS AND METHODS

Isolation of Bovine Rod Outer Segments (ROS). Unless otherwise stated, all procedures were performed using ice-cold solutions under dim-red light illumination. ROS were isolated from freshly dissected, dark-adapted retinas using the discontinuous sucrose gradient method (Papermaster, 1982).

Purification of Arrestin. Arrestin was purified from fresh bovine retinas as described by Buczyłko and Palczewski (1993). The arrestin concentration was determined spectrophotometrically assuming $E^{0.1\%}_{278nm} = 0.638$ (Palczewski et al., 1992).

Time Course of Rhodopsin Phosphorylation. The time course of rhodopsin phosphorylation was determined as described by Ohguro et al. (1993) with some modifications. To eliminate variable amounts of endogenous nucleotides and

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* To whom correspondence should be addressed at the Department of Ophthalmology, RJ-10, University of Washington, Seattle, WA 98195-0001. Telephone: (206) 543-9074. FAX: (206) 543-4414.

‡ Department of Ophthalmology.

§ Department of Biochemistry.

∥ Department of Pharmacology.

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soluble proteins, before initiating rhodopsin phosphorylation, ROS were suspended in 20 mM BTP (1,3-bis[tris(hydroxylmethyl)methylamino]propane) buffer, pH 7.5, containing 2 mM $MgCl_2$ and 150 mM KF, and exposed to light for 1 s using a projector lamp placed at a distance of 30 cm (approximately 10–15% of the rhodopsin was bleached). Despite the presence of rhodopsin kinase, rhodopsin remained unphosphorylated, due to the lack of ATP. This procedure allowed us to eliminate arrestin to undetectable levels as determined by SDS–PAGE. ROS membranes were then separated by centrifugation at 16000*g* for 10 s, and the pellet was immediately resuspended in the same buffer at a final rhodopsin concentration of 0.7 mg/mL. Without delay, rhodopsin phosphorylation was initiated by the addition of ATP (γ-S-ATP, [γ-^{32}P]ATP, or [γ-^{35}S]ATP) to give a final concentration of 2–3 mM, and the sample was illuminated at 30 °C from a distance of 30 cm with a projector lamp. For electrospray mass spectrometry (ES/MS), an aliquot was withdrawn at 0, 4, 7.5, 15, 30, or 45 min and added to an equal volume of 250 mM potassium phosphate buffer, pH 7.2, containing 200 mM ethylenediaminetetraacetic acid (EDTA), 5 mM adenosine, 100 mM KF, and 200 mM KCl. Phosphorylated ROS membranes were collected by centrifugation at 16000*g* for 10 min, and the pellet was subsequently washed with (1) water, (2) 200 mM KCl, and (3) 10 mM Hepes, pH 7.5. For radioactivity measurements, the phosphorylation reaction was stopped by the addition of 10% trichloroacetic acid; the precipitates were washed extensively with 10% trichloroacetic acid, solubilized with 100% formic acid, and analyzed by scintillation counting. The stoichiometry of rhodopsin phosphorylation was determined by the amount of radioactive phosphate, ^{32}P (or ^{35}S when [γ-^{35}S]ATP was used), incorporated into rhodopsin (Aton et al., 1989). Nucleotide purity and specific activity were verified by HPLC using a Nucleosil column 100-10SB (10 μm, 4 × 250 mm) employing a quaternary pump system HPLC (Hewlett Packard, Model 1050). The column was equilibrated with 100 mM potassium phosphate buffer, pH 5.4, and nucleotides were eluted with a 30-min linear gradient up to 100 mM potassium phosphate buffer, pH 2.6, containing 800 mM KCl at a flow rate of 1 mL/min. Nucleotides were detected by the absorption at 260 nm, their concentrations were measured spectrophotometrically, and their specific radioactivity was analyzed by scintillation counting. The specific radioactivity was the same, within experimental error, before and after the phosphorylation reaction.

Isolation of ^{330}DDEASTTVSKTETSQVAPA by Endoproteinase Asp-N Digestion. The C-terminal peptide (^{330}DDEASTTVSKTETSQVAPA) of rhodopsin was isolated following endoproteinase Asp-N (Boehringer Mannheim) digestion at an enzyme to rhodopsin ratio of 1:7000 (w/w) in 10 mM Hepes buffer, pH 7.5, for 12 h at ~18 °C as described by Palczewski et al. (1991). The C-terminal peptide was separated from membranes by centrifugation at 200000*g* for 20 min, and the supernatant was loaded onto a C4 column (BU-300, 2 × 40 mm; Applied Biosystem Inc.). After the column was washed with 0.05% trifluoroacetic acid and 10 mM ammonium acetate buffer, pH 6.5, the peptides were eluted with 52% acetonitrile in 10 mM ammonium acetate buffer, pH 6.5. The peptides were detected at 220 nm.

We tested the effects of nonspecific protein phosphatase inhibitors [50 mM KF and 10 mM pyrophosphate used by Papac et al. (1993)] and okadaic acid (Palczewski et al., 1989b) on the extent of spontaneous dephosphorylation during the endoproteinase Asp-N digestion (performed at approximately 18 °C with ROS prepared from fresh retinas). Less than 25% (variable) spontaneous dephosphorylation was observed in control conditions and in the presence of phosphatase inhibitors. However, the KF/pyrophosphate mixture inhibited 50–70% the rhodopsin cleavage by endoproteinase Asp-N. This effect of KF/pyrophosphate on proteolysis may be related to changes in the physical properties of membranes at higher ionic strength, since poor cleavage of rhodopsin by endoproteinase Asp-N was also observed in the presence of 100 mM KCl, which does not inhibit endoproteinase Asp-N. There is no evidence that dephosphorylation changes the ratio of the initial phosphorylation sites as proposed by Papac et al. (1993).

Subdigestion of ^{330}DDEASTTVSKTETSQVAPA. Subdigestion of ^{330}DDEASTTVSKTETSQVAPA (approximately 5–10 nmol) was performed as described by Ohguro et al. (1993). The C-terminal peptides were treated with either 15 pmol of thermolysin (Boehringer Mannheim), 0.2 nmol of trypsin-TPCK (Worthington), or 2 nmol of *Staphylococcus aureus* protease V8 (Boehringer Mannheim) in 50 μL of 80 mM Tris-HCl buffer, pH 8.0, at 30 °C for 16, 1, or 2 h, respectively. Digestion of ^{342}TSQVAPA by leucine aminopeptidase (Sigma Chemical) was performed in 50 μL of 80 mM Tris-HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ at a 1:20 enzyme:peptide molar ratio for 2 h at 30 °C. Peptides were purified on a reverse-phase HPLC column (2.1 × 250 mm, C18, Vydac 218TP52) employing a linear gradient from 0 to 24% acetonitrile and from 0.08 to 1% trifluoroacetic acid for 60 min.

Electrospray and Tandem Mass Spectrometry. Electrospray mass spectra (ES/MS) and tandem mass spectra (MS/MS) were acquired on a Sciex API III triple-quadrupole mass spectrometer fitted with a nebulization-assisted electrospray ionization source (PE/Sciex, Thornhill, Ontario) as described by Ohguro et al. (1993).

Regeneration of Phosphorylated Opsin with 11-cis-Retinal. Phosphorylated opsin was prepared as described by Hofmann et al. (1992), washed extensively with 120 mM sodium phosphate buffer, pH 7.2, containing 2 mM $MgCl_2$, and suspended in 0.25 mL of the same buffer (~8 mg/mL). A 3-fold molar excess of 11-*cis*-retinal was added to the suspension in the dark and incubated for 3 h at room temperature and overnight at 4 °C.

Binding Assay for Arrestin and Photolyzed Phosphorylated Rhodopsin. The binding assay for arrestin and photolyzed phosphorylated rhodopsin was performed as described by Hofmann et al. (1992). Phosphorylated rhodopsin (100 μg) in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl and 1 mM $MgCl_2$, was mixed with purified arrestin (10 μg) in a total volume of 100 μL under dim red light. The sample was illuminated by a 150-W lamp for 10 min from a distance of 10 cm at 30 °C. The sample was centrifuged at 200000*g* for 5 min. The supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The pellet was washed with 1 mL of 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl, centrifuged again under the same conditions, and dissolved in 55 μL of 1% SDS containing 0.1% 2-mercaptoethanol for SDS–PAGE.

RESULTS

Rhodopsin Phosphorylation under Different Conditions. Rhodopsin phosphorylation was studied employing conditions recently described (Ohguro et al., 1993) with some modifications. To eliminate variable amounts of small molecules (such as nucleotides) and soluble proteins, fresh bovine ROS were homogenized and bleached. Membranes containing

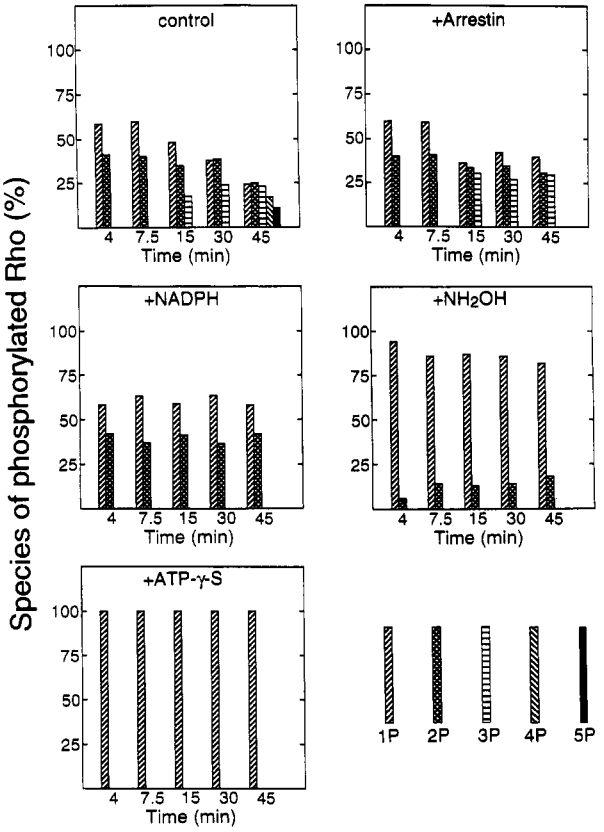

FIGURE 1: Distribution of phosphorylated forms of rhodopsin at multiple sites under different conditions. The time course of rhodopsin phosphorylation (Figure 2) was carried out in the presence of 0.7 mg/mL rhodopsin, 0.7 mg/mL arrestin, 0.1 mM NADPH, or 10 mM NH_2OH. Thiophosphorylation employed 2 mM γ-S-ATP in place of ATP. After phosphorylation for the indicated times, the C-terminal peptide was released by endoproteinase Asp-N, purified by HPLC, and directly analyzed by ES/MS. Ratios of phosphorylated species of rhodopsin (1P–5P) were estimated from the relative ion abundances.

photolyzed rhodopsin, bound rhodopsin kinase, transducin, and phosphodiesterase were collected by centrifugation and resuspended in a buffer containing exogenous nucleotides. This approach allowed us to eliminate most of the endogenous arrestin, ATP, and NADP/NADPH, and to avoid detergent that would otherwise be needed for efficient rhodopsin kinase purification. The light in our experiments bleached 50% of rhodopsin in 3 min and was chosen to ensure maximal phosphorylation of rhodopsin at the four Thr and three Ser residues near the C-terminus (Palczewski et al., 1991; Ohguro et al., 1993; Papac et al., 1993; McDowell et al., 1993).

The C-terminal 19-residue peptide of rhodopsin, containing all sites phosphorylated by rhodopsin kinase, was analyzed by mass spectrometry (Figure 1). In combination with the analysis of total ^{32}P or ^{35}S incorporation into rhodopsin (Figure 2), the effect of different experimental conditions on the distribution of phosphates among the phosphorylation sites could be examined [see Methods in Ohguro et al. (1993) and Palczewski et al. (1991)]. In the control experiment, no phosphate was incorporated at the initial condition (Figure 1; Ohguro et al., 1993), and four phosphates were incorporated per rhodopsin molecule, resulting in formation of tetra- and pentaphosphorylated species during 45-min phosphorylation (Figures 1 and 2). Under these conditions, addition of GTP, which promotes transducin activation and dissociation (data not shown), had an insignificant effect on rhodopsin phosphorylation when compared to the control conditions (Figure 2A). In contrast, arrestin restricted rhodopsin phosphorylation

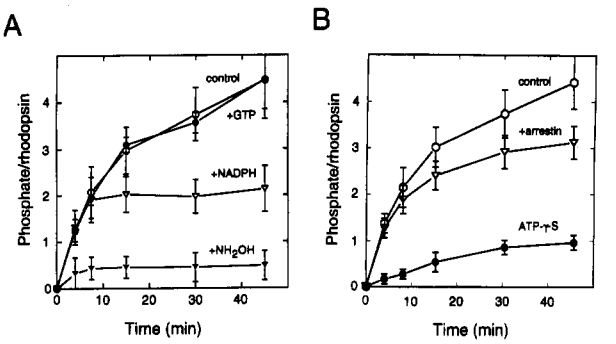

FIGURE 2: Time course of rhodopsin phosphorylation or thiophosphorylation under different conditions as described in Figure 1. After phosphorylation for the indicated times, the reaction was terminated by adding 10% trichloroacetic acid (TCA), the ROS membranes were washed extensively with freshly-prepared 10% TCA, and the phosphate content was measured by scintillation counting.

to three or less phosphates per rhodopsin without changing the initial extent of phosphorylation in the first 4 min (Figures 1 and 2B). Increased amounts of the triply-phosphorylated species of rhodopsin were repeatedly observed (in five independent experiments) after 15–30 min, but not the more highly phosphorylated species (Figure 1). Bennett and Sitaramayya (1988) reported also that, in the presence of arrestin, the initial rate of phosphorylation was unchanged, although no analysis of phosphorylated species of rhodopsin was performed. In other relevant studies, Buczyłko et al. (1991) have shown that highly phosphorylated species of rhodopsin inhibit rhodopsin kinase, whereas arrestin by formation of a complex with these species acts as an apparent activator. Our data extend these observations and demonstrate that the extent of phosphorylation is lower in the presence of arrestin, implying that the binding sites for arrestin and rhodopsin kinase may overlap.

NADPH or NH_2OH also inhibited the incorporation of phosphate, resulting in predominantly mono- and diphosphorylated species (Figures 1 and 2A). Both compounds shorten the lifetime of photolyzed rhodopsin, although by two different mechanisms. As a cofactor of the reaction catalyzed by retinol dehydrogenase, NADPH promotes reduction of *all-trans*-retinal to *all-trans*-retinol, whereas NH_2OH forms an oxime with photolyzed *all-trans*-retinal [Wald & Brown, 1953; reviewed by Rando (1991)]. These observations are consistent with results obtained by Pepperberg and Okajima (1992), who found that NH_2OH inhibits rhodopsin phosphorylation in the isolated retina, and with previous observations that rhodopsin kinase is activated only by photoactivated form(s) of rhodopsin (Bownds et al., 1973; Kühn, 1978; Fowles et al., 1988; Palczewski et al., 1991). Interestingly, replacement of ATP by γ-S-ATP (Palczewski et al., 1990) led to incorporation of a single thiophosphate per rhodopsin (Figures 1 and 2B).

It is important to note that the data depicted in Figure 1 are derived from comparisons of ES/MS ion abundances and, as such, should not be considered quantitative with regard to concentration ratios. A comparison of the UV absorbance with the mass spectral response of unphosphorylated and monophosphorylated C-terminal peptides (data not shown) suggests that in this case the addition of one phosphate group reduces the ES/MS signal by approximately half. This apparent reduction in the mass spectral response upon the addition of phosphate groups may account for discrepancies between the data presented in Figures 1 and 2. Nonetheless, these results suggest that the extent of rhodopsin phosphorylation is strongly decreased by shortening the lifetime

Table 1: Subdigestion of C-Terminal Phosphopeptides and Thiophosphopeptides of Rhodopsin[a]

peak designation phosphopeptides	thiophos-phopeptides	sequence
	Thermolytic Peptides	
Ta		DDEAS$\underline{T}$TVSKTETSQ
Tb	Ty	DDEA$\underline{S}$TTVSKTETSQ
Tc	Tz	VSKTETS$\underline{Q}$VAPA
T(b+c)		DDEAST$\underline{T}$VSKTETSQVAPA
T(b+d)		DDEASTT$\underline{V}$S$\underline{K}$TETS$\underline{Q}$
	Thermolytic/Tryptic Peptides	
Tr-a		DDEASTTVSK
Tr-b	Tr-y	DDEA$\underline{S}$TTVSKTETSQ
Tr-c	Tr-z	TETS$\underline{Q}$VAPA
	Thermolytic/*S. aureus* Proteolytic Peptide	
V8-b		ASTTVS$\underline{K}$TE
V8-c		TS$\underline{Q}$VAPA

[a] Sequence and phosphorylation sites (underlined) determined by ES/MS, MS/MS, and aminopeptidase digestion.

of photolyzed rhodopsin, and by competition of arrestin with rhodopsin kinase for binding to partially phosphorylated receptor.

Identification of Phosphorylation Sites in Rhodopsin. Sites of phosphorylation in rhodopsin in the presence of NADPH were determined essentially as described by Ohguro et al. (1993). The C-terminal rhodopsin peptide was obtained by proteolysis of phosphorylated rhodopsin (phosphorylated for 45 min) with endoproteinase Asp-N; it was then subdigested with thermolysin and trypsin or *S. aureus* protease V8, and the proteolytic products were analyzed by mass spectrometry. Initial subdigestion by thermolysin yielded a mixture of three monophosphopeptides (Ta, Tb, and Tc; see Table 1) and two diphosphopeptides [T(b+d) and T(b+c); see Table 1]. The monophosphorylated peptides were more readily separated from each other by HPLC after tryptic digestion [see Ohguro et al. (1993)]. Analysis by ES/MS and MS/MS demonstrated that peptide Tr-a was phosphorylated at ^{334}Ser, peptide Tr-c was phosphorylated at ^{343}Ser, and Tr-b was phosphorylated at ^{338}Ser. The MS/MS spectrum of T(b+d) delineated sites of phosphorylation at ^{338}Ser and ^{336}Thr [identical MS/MS spectrum; shown by Ohguro et al. (1993)]. Two *S. aureus* preoteolytic peptides from T(b+c) were identified as ^{333}ASTTV(phospho-S)KTE (V8-b) and ^{342}T(phospho-S)-QVAPA (V8-c), the phosphorylation sites of which were determined using leucine aminopeptidase digestion in conjunction with ES/MS. However, diphosphopeptides, including ^{334}Ser, were not detected (Ohguro et al., 1993).

The sites of phosphorylation in the presence of arrestin were identical to those occurring in the presence of NADPH. As under the control conditions, the first three phosphates were incorporated predominantly into ^{338}Ser, ^{343}Ser, and ^{336}Thr. The phosphorylation sites of rhodopsin monophosphorylated in the presence of NH_2OH were identical to the initial sites of phosphorylation found in the presence of NADPH.

Sites of thiophosphorylation in rhodopsin were determined as described above. A mixture of monothiophosphopeptides (Ty and Tz; see Table 1) was obtained by proteolysis of rhodopsin C-terminal peptides by thermolysin and subdigestion by trypsin and then analyzed by mass spectrometry. ES/MS and MS/MS identified Tr-y and Tr-z as ^{330}DDEASTTV-(thiophospho-S)KTETSQ and ^{340}TET(thiophospho-S)QVA-PA, respectively.

When the initial sites of phosphorylation were analyzed as described above, phosphorylation was observed only at

Table 2: Initial Phosphorylation Sites in Rhodopsin under Several Conditions[a]

condition	rhodopsin	percent ^{334}Ser	percent ^{338}Ser	percent ^{343}Ser
(A) pH 7.5, 30 °C	meta: I < II	8	85	7
(B) pH 8.0, 10 °C	meta: I > II	9	77	14
(C) pH 6.0, 10 °C	meta: I < II	22	78	trace
(D) pH 8.0, 37 °C	meta: I < II	21	72	7
(E) pH 7.5, 30 °C, +*all-trans*-retinal (opsin was used instead of rhodopsin)	meta: I, II < III	15	77	8
(F) pH 7.5, 30 °C, +arrestin	meta: I < II	4	93	3
(G) pH 7.5, 30 °C, +NADPH	meta: I < II	12	78	10
(H) pH 7.5, 30 °C, +NH_2OH	meta: I < II	7	66	27
(I) pH 7.5, 30 °C, +γ-S-ATP	meta: I < II	trace	49	51

[a] Rhodopsin was phosphorylated for 7.5 min using fresh ROS under the following conditions: (A) 20 mM BTP buffer (pH 7.5)/2 mM $MgCl_2$ (buffer 1) at 30 °C (control conditions); (B) 120 mM potassium phosphate buffer (pH 8.0)/2 mM $MgCl_2$ (buffer 2) at 10 °C; (C) 120 mM potassium phosphate buffer (pH 6.0)/2 mM $MgCl_2$ (buffer 3) at 10 °C; (D) buffer 2 at 37 °C. (E) Before phosphorylation, rhodopsin was completely bleached to opsin by exposure to light in the presence of 10 mM NH_2OH for 10 min on ice. Opsin was collected by centrifugation (16000*g* for 3 min), incubated with 10 mM formaldehyde for 5 min on ice to neutralize trace amounts of NH_2OH, and incubated with a 10 molar excess of *all-trans*-retinal for 15 min at room temperature (Hofmann et al., 1992). Then, phosphorylation was started by adding ATP and exposing to light. (F) Control + 0.7 mg/mL arrestin; (G) control + 0.1 mM NADPH; (H) control + 10 mM NH_2OH; (I) control + 2 mM γ-S-ATP instead of ATP. After phosphorylation, the C-terminal peptides were obtained by proteolysis with endoproteinase Asp-N and subdigested with thermolysin. Mixtures of thermolytic monophosphopeptides were separated after proteolysis by trypsin and analyzed by mass spectrometry. Proteolytic and chromatographic conditions are described under Materials and Methods. The relative amounts of peptides were estimated from their UV absorbance at 220 nm.

^{338}Ser, ^{343}Ser, and ^{334}Ser (Table 2). Conditions favoring metarhodopsin II (conditions A, C, and D; Parkes & Liebman, 1984) resulted in preferential phosphorylation at ^{338}Ser. This was also observed under conditions favoring metarhodopsin I (condition B), although a slight increase in phosphorylation was detected at ^{343}Ser. At low temperature and low pH (condition C) or at high pH and high temperature (condition D), phosphorylation of ^{334}Ser was enhanced (up to 22%). When phosphorylation was performed in the dark using opsin and *all-trans*-retinal (condition E; Hofmann et al., 1992), the ratios of the initial sites of phosphorylation were not substantially changed. The addition of arrestin to the phosphorylation mixture favored initial phosphorylation at ^{338}Ser (condition F; Ohguro et al., 1993). The presence of NADPH (condition G) led to similar ratios observed in the control, whereas addition of NH_2OH (condition H) increased the phosphorylation of ^{343}Ser. Thiophosphorylation occurred in nearly equal concentrations at ^{338}Ser or ^{343}Ser (condition I).

To determine the minimum number of C-terminal rhodopsin phosphorylation sites required for binding of arrestin, rhodopsin was phosphorylated to varying extents and incubated with arrestin, and ROS membranes were collected by centrifugation. Both the pellets and supernatants were analyzed by SDS–PAGE (Figure 3), where it was found that arrestin binds in a light-dependent manner regardless of the degree of phosphorylation. These data suggest that arrestin binding is secured as soon as one phosphate is present per photolyzed rhodopsin, consistent with the data of Bennett and Sitaramayya (1988) and Schleicher et al. (1991), and suggest that the initial site of phosphorylation, whether ^{338}Ser or ^{343}Ser, will support the binding. However, we cannot exclude the possibility that different degrees of phosphorylation will

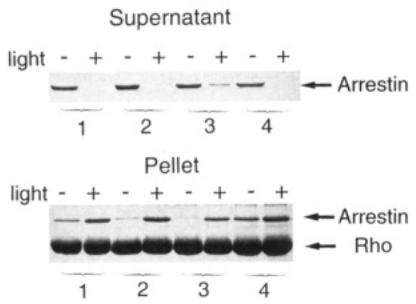

FIGURE 3: Binding of arrestin to rhodopsin phosphorylated to different extents. Rhodopsin was phosphorylated by illuminating ROS for 45 min in the presence of either 0.1 mM NADPH, 10 mM NH_2OH, or 2 mM γ-S-ATP, and each was regenerated with 11-*cis*-retinal. The phosphate incorporation per rhodopsin molecule is as follows: (1) control, 4.5; (2) NADPH, 1.5; (3) NH_2OH, 0.3; (4) γ-S-ATP, 0.8. Under dim red light, phosphorylated rhodopsin (2.5 nmol) was mixed with purified arrestin (0.21 nmol) in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl. The reaction preceded with or without illumination for 10 min, the arrestin/metarhodopsin II complex was centrifuged at 200000*g* for 5 min, and the supernatant was subjected to SDS–PAGE (upper panel). The pellet was washed with 1 mL of the Hepes buffer, centrifuged for 5 min at 200000*g*, dissolved in 55 μL of 1% SDS containing 0.1% 2-mercaptoethanol, and subjected to SDS–PAGE (lower panel).

influence the affinity of phosphorylated and photolyzed rhodopsin toward arrestin. Interestingly, thiophosphorylated rhodopsin, which contains a high proportion of thiophosphorylated ^{343}Ser, was also capable of binding arrestin in the dark. In summary, these results suggest that phosphorylation is significantly modulated by the presence of factors and conditions used for rhodopsin phosphorylation (in particular, NADPH) and that the accessibility of sites of phosphorylation may be influenced by conformational changes of photolyzed rhodopsin and the presence of arrestin.

DISCUSSION

Phosphorylation of rhodopsin is a complex reaction wherein several phosphates are incorporated into the C-terminal region of rhodopsin (Palczewski & Benovic, 1991). No systematic studies have been performed to explain whether steps in quenching and regeneration of rhodopsin, such as the binding of arrestin and the removal of photolyzed chromophore, influence rhodopsin phosphorylation by rhodopsin kinase. Recently, we reported that the phosphorylation reaction is sequential with the first phosphate transferred almost exclusively to ^{338}Ser, and subsequent phosphorylations at ^{343}Ser and ^{336}Thr (Ohguro et al., 1993). Independently, McDowell et al. (1993) and Papac et al. (1993) reported that monophosphorylated rhodopsin is a mixture of rhodopsins phosphorylated at residues ^{338}Ser or ^{343}Ser. Further, Adamus et al. (1993) detected two monophosphorylated species of rhodopsin using isoelectrofocusing.

In the present study, the initial sites of phosphorylation were examined under several conditions, such as those that favor metarhodopsin I or II, or the addition of arrestin, NADPH, or NH_2OH. In nearly all cases, phosphorylation of ^{338}Ser is the dominant initial reaction. The only exception is that thiophosphorylation occurs equally well at ^{338}Ser or ^{343}Ser (but not both), suggesting that the nucleotide binding by rhodopsin kinase is somewhat altered for γ-S-ATP compared to ATP.

Two mechanisms limit more extensive phosphorylation of rhodopsin—the binding of arrestin and the reduction of the photolyzed chromophore. The high binding affinity of arrestin for rhodopsin phosphorylated at one to three sites may prevent the formation of highly phosphorylated species of rhodopsin. These results correlate with functional assays, which show that one or two phosphates per rhodopsin are required for arrestin to efficiently block G-protein activation (Sitaramayya, 1986; Bennett & Sitaramayya, 1988). Perhaps arrestin promotes phosphorylation of rhodopsin at ^{338}Ser as observed in homogenates of fresh ROS (Ohguro et al., 1993), by preferentially binding that species.

The binding state of photolyzed rhodopsin for rhodopsin kinase is likely to be first revealed in the metarhodopsin I photoproduct and remains at a stable equilibrium with $K_D = 0.5\ \mu M$, $k_{on} = 0.5\ \mu M^{-1}\ s^{-1}$, and $k_{off} = 0.25\ s^{-1}$. Phosphorylation of rhodopsin or autophosphorylation of rhodopsin kinase leads to rapid destabilization of the complex by increasing the k_{off} rate (Pulvermüller et al., 1994). The relative simplicity of the interaction between rhodopsin kinase and photolyzed rhodopsin is exhibited in the bimolecular rate constant, which is much higher than that of the complex between arrestin and photolyzed, phosphorylated rhodopsin (Hofmann et al., 1992). The consequences of the initial strong binding of rhodopsin kinase to photolyzed rhodopsin and destabilization of the rhodopsin kinase-photolyzed rhodopsin complex by phosphorylation are in agreement with the lack of initial inhibition of rhodopsin phosphorylation by arrestin, and subsequent prevention of further phosphorylation when the k_{off} for the rhodopsin kinase-photolyzed rhodopsin complex increases. From this study, it is apparent that approximately three phosphate residues per rhodopsin are needed for arrestin to completely stop further phosphorylation.

Reduction of the photolyzed chromophore *all-trans*-retinal to *all-trans*-retinol further hinders phosphorylation of rhodopsin. NH_2OH is more effective than NADPH at limiting the degree of phosphorylation (≤1 phosphate per rhodopsin molecule). It can be proposed that *in vivo*, at high levels of bleaching, retinol dehydrogenase may effectively suppress multiple phosphorylation. This finding is particularly significant in light of recent observations that free *all-trans*-retinal interacts reversibly with opsin and forms a substrate for rhodopsin kinase (Hofmann et al., 1992). However, in the absence of information about the physiological regulation and kinetic parameters of the dehydrogenase, this point remains speculative. Higher levels of rhodopsin phosphorylation may only occur when the reducing capacity of retinol dehydrogenase is exceeded by intense bleaches in ROS or by the lack of NADPH *in vitro*. The physiological significance of rhodopsin phosphorylation at *multiple sites* will only become apparent when our attention focuses on the integrated system *in vivo*.

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