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Interplay of Phosphorylation and Dephosphorylation in Vision: Protein Phosphatases of Bovine Rod Outer Segments†

Charles Fowles and Muhammad Akhtar*
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, England
Philip Cohen
Department of Biochemistry, University of Dundee, Medical Sciences Institute, Dundee, Tayside DD1 4HN, Scotland
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ABSTRACT: Two types of protein phosphatases were identified in carefully prepared bovine rod outer segments (ROS). Extraction of the ROS with a medium-salt buffer solubilized protein phosphatase activity that was mainly type 2A, since it was active toward phosphorylase *a* in the absence of divalent cations, was not retained by heparin–Sepharose, dephosphorylated the α-subunit of phosphorylase kinase faster that the β-subunit, and was unaffected by inhibitor 2. Further extraction of the resulting membranes with a high-salt buffer solubilized additional phosphatase activity which was predominantly type 1, since it was retained by heparin–Sepharose and was blocked by inhibitor 2. The molecular mass of the type 2A phosphatase estimated by gel permeation chromatography on Superose 12 was 100 kDa, suggesting it may be the $2A_2$ form. Only the ROS type 2A phosphatase dephosphorylated opsin and rhodopsin efficiently. Concordant with this finding, the purified catalytic subunit of protein phosphatase 2A from rabbit skeletal muscle dephosphorylated opsin efficiently, while the type 1 catalytic subunit isolated from this tissue was inactive. Together, the results suggest that the ROS type 2A protein phosphatase plays an important role in regenerating rhodopsin from the various phosphorylated species in vivo. The activity of the enzyme per retina (~85 pmol of P_i released/min) is comparable to that of rhodopsin kinase (100 pmol of phosphate transferred/min).

Rhodopsin is the major protein present in the outer segments of rod cells of animal retinas. The activation of rhodopsin by light leads to the formation of a series of well-defined intermediates, and one of these, metarhodopsin II (hereafter referred to as Rho*),[1] plays a pivotal role in vision. The interaction of Rho* with transducin initiates a cascade of biochemical events leading to the transmission, and eventually to the registration, of the visual signal (Stryer, 1986). Thereafter, as for other physiological stimuli, the visual signal must be terminated. It is known that Rho* acts as a substrate for a specific kinase [rhodopsin kinase; Miller et al., 1977; see Kühn (1978) and references cited therein] present in the ROS (rod outer segments) that catalyzes the introduction of up to

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[1] Abbreviations: PMSF, phenylmethanesulfonyl chloride; ROS, rod outer segment(s); Rho*, metarhodopsin II.

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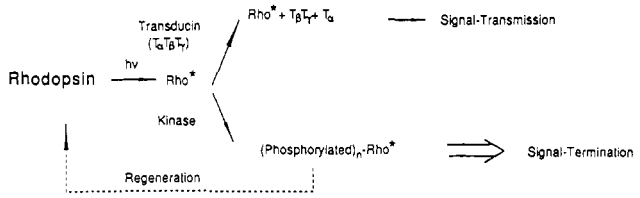

FIGURE 1: Involvement of photoactivated rhodopsin (Rho* = metarhodopsin II) in signal transmission and signal termination processes. The transformation shown by the broken arrow occurs in three steps and is elaborated in Figure 7. T = transducin.

seven phosphoryl groups in the C-terminal region of the visual protein (Sale et al., 1978; Hargrave et al., 1980; Findlay et al., 1984). This phosphorylation reaction recently studied in our laboratory (Fowles et al., 1988) is regarded to be an important part of the signal termination system (Kühn & Wilden, 1987; Bennett & Sitaramayya, 1988). The role of Rho* in the initiation and subsequent termination of visual transduction is illustrated in Figure 1.

The in vivo regeneration of rhodopsin from (phospho)$_n$-Rho* (Figure 1) should require the presence of a protein phosphatase, and an enzyme fulfilling such a role has now been identified in ROS by two groups independently (Fowles, 1988; Palczewski et al., 1989). A comprehensive account of the work summarized in this paper is described in a Ph.D. thesis (Fowles, 1988).

Experimental Procedures

Materials. Cattle eyes were collected from a local slaughterhouse (FMC Meat, Salisbury, Wilts, U.K.); [γ-^{32}P]ATP and NCS tissue solubilizer were obtained from Amersham International (Amersham, Bucks, U.K.); Labscint scintillation cocktail was obtained from Lablogica (Sheffield, Yorkshire, U.K.) and *all-trans*-retinal from Fluka (Glossop, Derbyshire, U.K.). All other chemicals were from Sigma or BDH (Poole, Dorset, U.K.) or Interchem (Ipswich, Suffolk, U.K.).

Phosphorylase *a* (Antoniw et al., 1977), phosphorylase kinase (Stewart et al., 1981), inhibitor 2 (Cohen et al., 1988a), and the catalytic subunits of type 1 and type 2A protein phosphatases (Cohen et al., 1988b) were purified from rabbit skeletal muscle.

Solutions. The following buffers used for the various preparations described in this paper contained 0.1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Except for buffers A and B, they also contain 1 mM *p*-aminobenzamidine dihydrochloride and 0.1 mM PMSF. Buffer A was 100 mM potassium phosphate, pH 7.0, and 2 mM $MgCl_2$; buffer B, 100 mM Tris-HCl, pH 7.0; buffer C, 50 mM Tris-HCl, pH 7.0; buffer D, 20 mM Tris-HCl, pH 7.0, and 500 mM NaCl; buffer E (equilibration/low-salt running buffer), 20 mM Tris-HCl, pH 7.0, and 5% glycerol.

Preparation of Rod Outer Segments (ROS). Retinas were excised from freshly killed cattle (less than 15 min after slaughter) and immediately stored in a light-proof container at 0 °C for transportation to the laboratory. The retinas were next either processed immediately to rod outer segments or stored in the dark overnight at 0 °C for processing the next day, using a modification of the procedure described by Sale et al. (1977).

Procedures for isolation of rod outer segments were performed in a light-sealed room at 4 °C under red safelight illumination (25-W bulb; Kodac GBX-2 red safelight filter). The buffer used throughout the preparative procedure depended on the final destination of the ROS isolated. Buffer A was used to prepare ROS for phosphorylation and buffer B when ROS were used to isolate protein phosphatases.

A total of 60–120 retinas were shaken vigorously in buffer containing 45% (w/v) sucrose for 1 min at a concentration of 1 retina/mL to release the ROS from the retinas, and the resultant suspension was centrifuged for 30 min at 10000*g*. The supernatant was collected; the pellet was again resuspended in 45% sucrose-containing buffer (0.5 mL/retina), shaken vigorously for 1 min, and centrifuged for 30 min at 10000*g*. The walls of the centrifuge tubes were scraped with a spatula to release the attached red ROS material, taking care to ensure that the pellet was not disturbed, and the two sets of supernatants were combined. The mixture was next diluted with 0.5 volume of buffer and centrifuged for 45 min at 40000*g*. The sedimented ROS pellet was resuspended carefully in buffer containing 34% (w/v) sucrose (10 retinas/mL) by suction through a Pasteur pipet and layered onto discontinuous sucrose density gradients (40 retinas/gradient) consisting of 5 mL each of 36% (w/v) sucrose and 34% (w/v) sucrose. Gradients were centrifuged for 1.5 h at 110000*g* using a swing-out centrifuge rotor. Two red ROS-containing bands were observed at the loading solution–34% (w/v) sucrose interface and 34–36% (w/v) sucrose interface, termed bands 1 and 2, respectively. For the experiments described in this paper, these bands were removed from the gradients with a syringe, combined, diluted with 0.3 volume of buffer, and centrifuged for 25 min at 40000*g* to collect the ROS pellet. ROS pellets were either used immediately or stored at −70 °C until required.

The total rhodopsin yield was typically 0.75 mg of rhodopsin per retina as estimated by the difference in absorption at 500 nm before and after bleaching (ΔA_{500} using a rhodopsin molar extinction coefficient of 40 000 mol L^{-1} cm^{-1}). The purity of ROS, assessed by comparing the 280:500-nm ratio, was typically 3 ± 0.2.

Phosphorylation of Rhodopsin. All procedures were performed under red safelight illumination at 4 °C in a light-proof room unless stated otherwise. ROS prepared in buffer A and containing 5–35 mg of rhodopsin were resuspended in the same buffer at a rhodopsin concentration of 1 mg/mL. The ROS suspension was hand-homogenized and transferred to a stoppered glass round-bottom flask, and [γ-^{32}P]ATP (1000–180 000 dpm/nmol) was added to a final concentration of 0.1, 2, or 3 mM ATP, depending on the extent of phosphorylation required. Samples were sonicated for 1 min and then incubated for 5 min at 30 °C, and the light-dependent phosphorylation process was initiated by illumination with continuous white light (150-W bulb in a photoflood lamp at a distance of 30 cm). After the desired incubation period, samples were centrifuged for 20 min at 40000*g*, and the pellet was washed 3 times with phosphorylation buffer and 3 times with water. Each washing entailed resuspension of the pellet by vortex mixing, mild sonication for 30 s, and the recovery of the pellet by centrifugation as above. The phosphoopsin pellet was stored at −20 °C. Prior to use, two further washes were performed as above, using the buffer required in subsequent experimentation.

The [^{32}P]phosphate content of rhodopsin was assessed by precipitating aliquots in trichloroacetic acid essentially as described by Guy et al. (1981), except that the final washed pellet was dissolved in 0.1 mL of NCS tissue solubilizer and 0.9 mL of Labscint scinitillation cocktail. The concentration of rhodopsin in phosphorylation incubations was assessed spectrophotometrically at A_{500} prior to the onset of illumination. Results are presented as moles of phosphate incorporated

per mole of opsin. Typically, incubations performed for 1 h at 0.1 mM ATP contained 0.1 ± 0.05 mol of phosphate/mol of opsin; samples incubated with 2 mM ATP gave phosphoopsin with 1 ± 0.5 mol of phosphate/mol of protein and samples incubated with 3 mM ATP 3 ± 1.5 mol of phosphate/mol of opsin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was also performed on samples, and the resultant gels were analyzed for radioactivity to ensure that greater than 95% of the protein-bound phosphate copurified with phosphoopsin (results not shown). When phosphorhodopsin was required, samples of phosphoopsin were regenerated (90–97%) with 11-*cis*-retinal (Sale et al., 1978).

Extraction of Protein Phosphatases from Dark-Adapted ROS. All extraction procedures described were performed under red safelight illumination at 4 °C in a light-proof room. Dark-adapted ROS were suspended at 15 mg of rhodopsin/mL in buffer C, hand-homogenized, mildly sonicated for 30 s, and left for 1 h at 0 °C before a second mild sonication was performed. The suspension was centrifuged for 60 min at 110000*g* and the resultant supernatant termed "medium salt extract". Typically, this contained 2–3 mg of protein/mL. The pellet obtained was resuspended in buffer D at 15 mg of rhodopsin/mL, and the extraction procedure described above was repeated to give a "high salt extract", which typically contained 1–2 mg of protein/mL.

Protein Phosphatase Assay. Well-washed phosphoopsin or phosphorhodopsin was resuspended in buffer C (20–200 μL), mildly sonicated for 30 s, and preincubated for 5 min at 30 °C. ROS extracts were then added to give a final volume of 30–300 μL. Aliquots removed at various times were precipitated with 20% (w/v) trichloroacetic acid, and released ^{32}P radioactivity was measured essentially as described by Cohen et al. (1988b) in the presence of Labscint scintillation cocktail. Controls were performed in which extract was omitted and these values subtracted. In some experiments, aliquots of the trichloroacetic supernatant were extracted with 0.6 mL of 1.25% (w/v) ammonium molybdate in 1.2 M HCl containing 20 μM potassium phosphate. After mixing, 0.9 mL of 2-methyl-2-propanol/benzene (1:1) was added and the tube agitated for 20 s using a vortex mixer. Once the two layers had separated, the organic phase was removed, extraction was repeated once more, and the combined organic phases were pooled and counted after addition of scintillation cocktail. Results obtained by direct analysis of trichloroacetic acid supernatants and phosphomolybdate complexes were identical within experimental error, demonstrating that protein phosphatase activity and not proteinase activity is measured by the first method.

^{32}P-Labeled phosphorylase *a* containing 1.0 mol of phosphate/mol of subunit (Cohen et al., 1988b) and ^{32}P-labeled phosphorylase kinase containing 1.8 mol of phosphate/$\alpha\beta\gamma\delta$ unit (Stewart et al., 1981), both containing 200000 dpm/nmol, were prepared by published procedures, and their dephosphorylation was measured as described by Cohen et al. (1988b) at 10 μM phosphorylase *a* and 1 μM phosphorylase kinase. When inhibitor 2 was present, this protein was added to a final concentration of 100 nM, and the phosphatase and inhibitor were preincubated for 10–15 min prior to initiation of the reactions with substrate (Cohen et al., 1988a).

One unit of activity refers to the nanomoles of phosphate released from the particular substrate per minute per milliliter of original phosphatase extract at 30 °C unless stated.

Results

Extraction of Phosphatases from Rod Outer Segments. Preliminary experiments indicated that phosphatase activity was easily lost during the various operations involved in the isolation and purification of ROS from bovine retinas. A protocol was therefore devised that involved neither homogenization nor vortex mixing at any step of the preparation. ROS from the discontinuous sucrose density gradient were sedimented and various classes of protein solubilized employing the selective salt extraction protocol originally introduced by Kühn (1978). Polyacrylamide gel electrophoresis showed that the proteins extracted with "medium salt buffer" corresponded to those proteins located in the ROS interdiscal cytoplasmic space while the "high salt extract" contained the "peripheral membrane proteins", such as transducin, arrestin, and cGMP phosphodiesterase. Both the medium- and high-salt extracts catalyzed the dephosphorylation of opsin, although the majority of the activity was present in the "medium salt extract" (Table I). Opsin phosphatase activity was stimulated by protamine, activation being most pronounced with the medium-salt extract (Table I). It may be estimated from the data of Table I that when phosphoopsin containing 4.3 mol of phosphate/mol was used, the two extracts together catalyzed the release of 1.7 nmol of P_i/min. Since the activity was obtained from 15 mg of rhodopsin corresponding to 20 retinas, the phosphatase activity was 0.085 nmol of P_i released min^{-1} $retina^{-1}$.

Table I: Phosphoopsin Phosphatase Activity of ROS Extracts in the Presence or Absence of Protamine[a]

	^{32}P released/ 30 μL (dpm)	phosphatase act. (units)
medium-salt extract	1801	1.0
+protamine	5043	2.8
high-salt extract	1256	0.7
+protamine	1407	0.8

[a] The medium- and high-salt extracts were prepared in appropriate buffers as described under Experimental Procedures. Samples of phosphoopsin (1 nmol containing 4.3 mol of phosphate/mol; 9×10^3 dpm/nmol of ^{32}P) in 20 μL of buffer C were incubated with either medium-salt extract (10 μL containing 20 ng of protein) or high-salt extract (10 μL containing 18 μg of protein dialyzed against incubation buffer) in the presence or absence of 30 μg of protamine. Incubations were for 20 min at 30 °C. Thereafter, the mixture was analyzed for the release of [^{32}P]P_i and activity expressed as milliunits per milliliter of the original extract, each 1 mL of extract being obtained from ROS containing 15 mg of rhodopsin. Each value is the mean of duplicate determinations with control values (in the absence of extract) deduced. An identical distribution of activity was found in at least six independent experiments.

Dephosphorylation of Phosphorhodopsin and Phosphoopsin Phosphorylated at Various Levels. Samples of rhodopsin were phosphorylated at 3, 1, or 0.15 mol of phosphates per mole of polypeptide by incubation in the presence of various concentrations of ATP. Half of each sample was regenerated with 11-*cis*-retinal, and dephosphorylation of samples was performed by using the medium-salt extract. The results of these experiments are presented in Figure 2 and show that the dephosphorylation of phosphoopsin and phosphorhodopsin occurred at identical rates. As expected, the initial rate of dephosphorylation was dependent on the degree of phosphorylation of the protein; thus, with samples phosphorylated at higher levels (3 or 1 phosphates/mol), the release of P_i was significantly greater (15–30-fold) than those phosphorylated at lower levels (0.15 phosphate/mol).

Identification of the Major Opsin Phosphatase in ROS as a Type 2A Phosphatase. Four major types of protein phosphatase catalytic subunits have been identified in mammalian tissues. Two of these, type 1 and type 2A, are active in the absence of divalent cations and are the only enzymes with significant phosphorylase phosphatase activity. Type 1

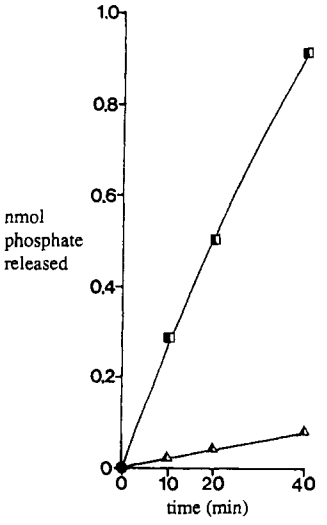

FIGURE 2: Dephosphorylation of phosphoopsin and phosphorhodopsin phosphorylated to different extents. Samples of phosphoopsin (□) or phosphorhodopsin (■) (2.3 nmol, 1.0 mol of phosphate/mol, 6 × 10^3 dpm/nmol of ^{32}P) and phosphoopsin (△) or phosphorhodopsin (▲) (2.3 nmol, 0.15 mol of phosphate/mol, 1.9 × 10^5 dpm/nmol of ^{32}P) in 0.1 mL of buffer C containing 0.15 mg of protamine were incubated with medium-salt extract (0.5 mL containing 0.1 mg of protein). Aliquots (0.3 mL) were removed at various time intervals and analyzed for released $[^{32}P]P_i$. Results are presented as nanomoles of $[^{32}P]P_i$ released per 0.15 mL of incubation mixture.

phosphatases are potently inhibited by two proteins, inhibitor 1 and inhibitor 2, that do not affect type 2A phosphatases, and type 1 enzymes dephosphorylate the β-subunit of phosphorylase kinase, whereas the α-subunit is dephosphorylated preferentially by type 2A enzymes [reviewed in Cohen (1989)]. The type 2A enzymes are not retained by heparin–Sepharose at 100 mM NaCl, whereas the type 1 enzymes bind and can be eluted with 500 mM NaCl (Erdodi et al., 1985).

The heparin–Sepharose column elution profile of the medium-salt extract of ROS in Figure 3A shows two well-separated peaks of phosphatase activity. As observed in other mammalian tissues, phosphorylase phosphatase activity not retained at 100 mM NaCl and eluting in the first and larger peak shows little inhibition on inclusion of inhibitor 2 in the assay and is presumably predominantly composed of type 2 protein phosphatases. The second smaller peak shows marked inhibition by inhibitor 2 and is assumed to comprise predominantly type 1 protein phosphatases. The elution profile of the high-salt extract is shown in Figure 3B and exhibits the same peaks of phosphatase activities; however, in this case, the second peak predominants. Again, on inclusion of inhibitor 2, it is the second peak that is strongly inhibited, suggesting it comprises predominantly type 1 phosphatase activity.

When phosphoopsin was used as substrate, and the fractions were assayed in the presence of protamine to stimulate activity, 94% of the opsin phosphatase was found in the flow-through fractions (first peak in Figure 3C); only 6% was retained. Opsin phosphatase in the flow-through fractions was essentially unaffected by 100 nM inhibitor 2 and stimulated 8-fold by 1 mg/mL protamine (not shown). When protamine was omitted, inclusion of Ca^{2+} (2 mM) did not affect the opsin phosphatase activity of the flow-through fractions, while inclusion of Mg^{2+} (2 mM) only increased activity slightly (~15%). These experiments suggested that type 2B and type 2C phosphatases, which are also excluded from heparin–Sepharose (unpublished experiments) and dependent on Ca^{2+} and Mg^{2+}, respectively, do not make a significant contribution to the opsin phosphatase activity.

The flow-through fractions from heparin–Sepharose (Figure 3C) were pooled, concentrated to 1 mL by vacuum dialysis, and subjected to gel permeation chromatography on Superose 12. The major peak of phosphorylase phosphatase activity was eluted with an apparent molecular mass of ~100 kDa and coeluted with a peak of opsin phosphatase activity (Figure 4). The opsin phosphatase activity was stimulated ~10-fold by 1 mg/mL protamine. The fractions exhibiting opsin phos-

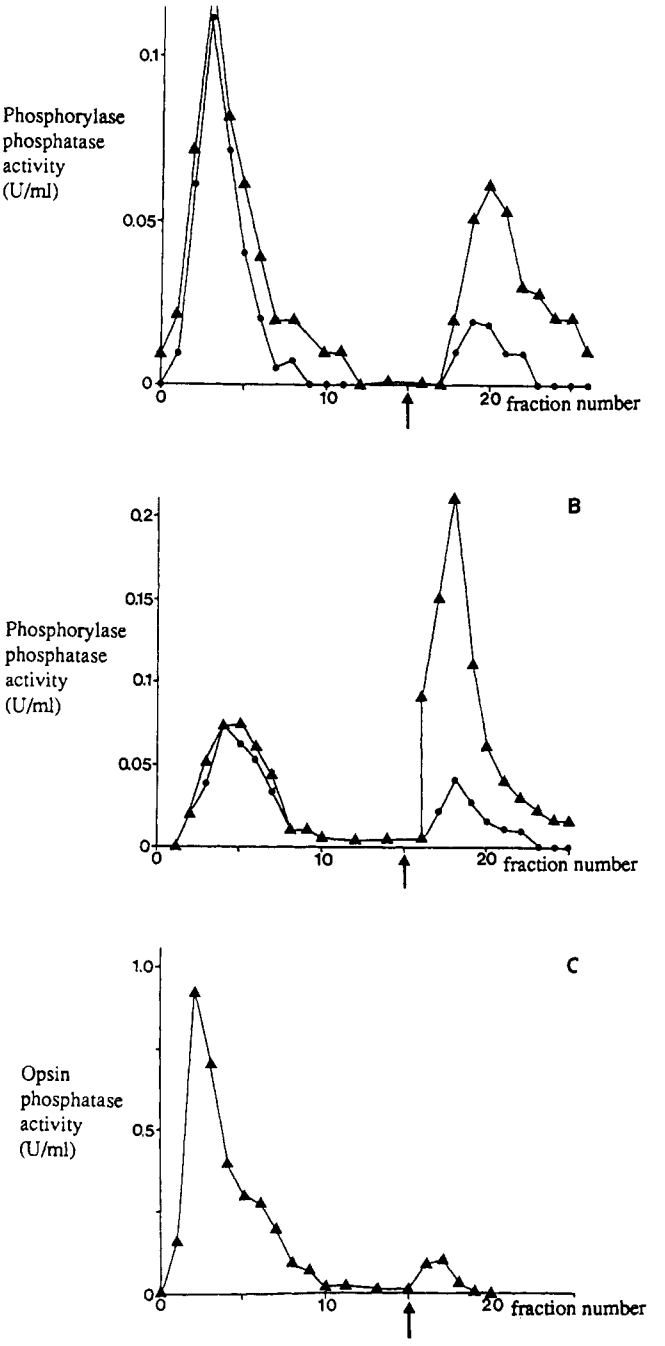

FIGURE 3: Separation of type 1 and type 2 protein phosphatases on heparin–Sepharose. ROS extracts (1–4 mL) were made 5% (v/v) in glycerol and 100 mM in NaCl and loaded onto a 3 × 2 cm heparin–Sepharose column (heparin CL-GB, Pharmacia) preequilibrated in buffer E at 4 °C. After being washed with 45 mL of buffer E, the column was further eluted with 45 mL of buffer E + 0.5 M NaCl. Fractions (3 mL) were collected and 0.1-mL aliquots assayed for phosphatase activity at a 3-fold final dilution. The 0.5 M NaCl eluate was dialyzed to decrease [NaCl] to a level (<30 mM) that did not interfere with the assays. The "medium salt extract" was chromatographed in (A) and (C) and the "high salt extract" in (B). The closed triangles show activity in the absence, and the closed circles activity in the presence, of inhibitor 2. In (C) the phosphatase activity was measured in the presence of 1 mg/mL protamine.

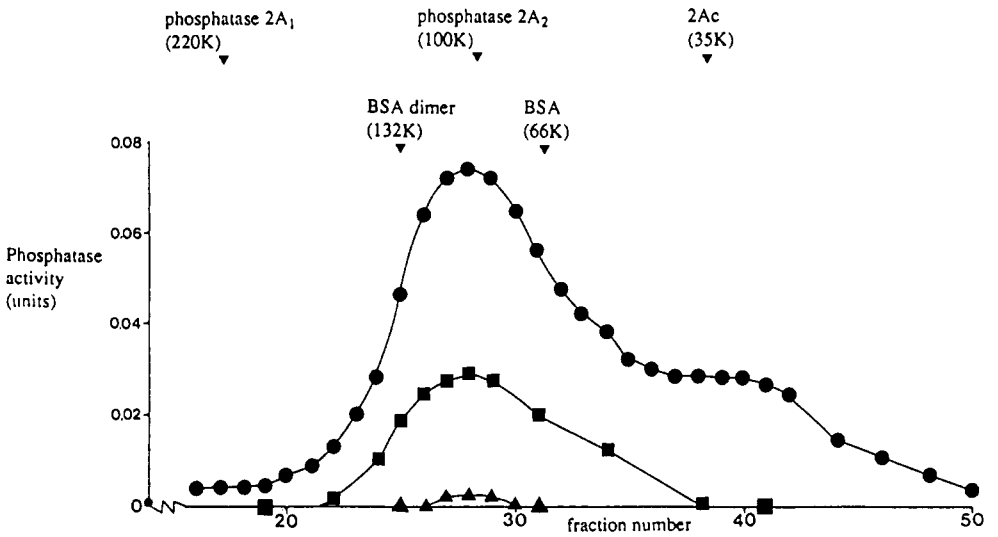

FIGURE 4: Gel permeation chromatography of ROS opsin phosphatase on Superose 12. The active fractions from chromatography of "medium salt ROS extract" on the heparin–Sepharose column (Figure 3C) were pooled and concentrated to 1 mL by vacuum dialysis, and an aliquot was applied to a column of Superose 12 equilibrated in buffer C + 150 mM NaCl. Fractions of 0.2 mL were collected at a flow rate of 0.5 mL/min and assayed for opsin phosphatase activity in the presence (■) and absence (▲) of 1 mg/mL protamine, and for phosphorylase phosphatase activity (●). The elution positions of the high molecular mass forms of protein phosphatase 2A ($2A_1$ and $2A_2$), the free catalytic subunit ($2A_c$), and bovine serum albumin (BSA) are marked. Results are presented as nanomoles of P_i released per minute per fraction.

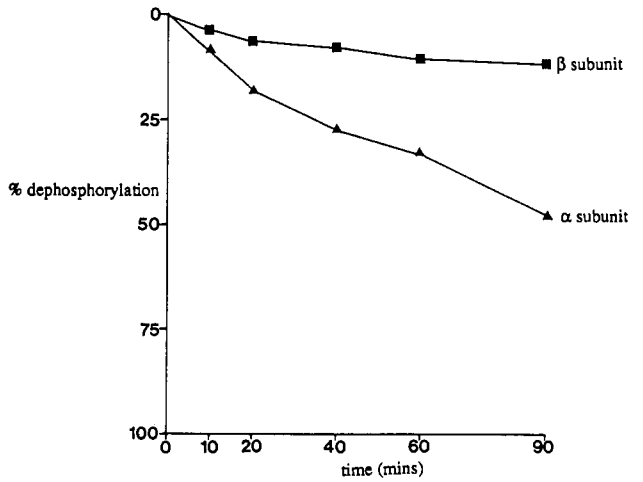

FIGURE 5: Dephosphorylation of the α- and β-subunits of phosphorylase kinase by opsin phosphatase. Opsin phosphatase from gel filtration on Superose 12 (fractions 26–29, Figure 4) was incubated with phosphorylase kinase, and released phosphate from the α- and β-subunits was quantitated at various times as described in Stewart et al. (1981).

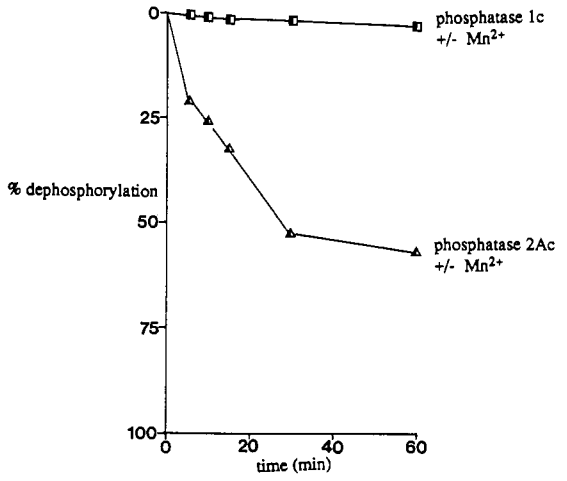

FIGURE 6: Dephosphorylation of phosphoopsin by the catalytic subunits of protein phosphatases 1 (1_c) and 2A ($2A_c$). Samples of phosphoopsin were incubated with each catalytic subunit (final concentration 10 units phosphorylase phosphatase activity/mL) in the presence (closed symbols) or absence (open symbols) of 1 mM Mn^{2+}. Samples (0.3 mL) were removed at various times and analyzed for the release of $[^{32}P]P_i$.

phatase activity (fractions 26–29, Figure 4) were also active toward phosphorylase kinase and dephosphorylated the α-subunit much faster than the β-subunit (Figure 5).

Dephosphorylation of Opsin by the Purified Catalytic Subunits of Protein Phosphatases 1 and 2A from Rabbit Skeletal Muscle. The purified catalytic subunit of protein phosphatase 1 from rabbit muscle showed little or no activity toward opsin (Figure 6) in the absence or presence of Mn^{2+}, which is required for the dephosphorylation of certain substrates by this enzyme (Foulkes et al., 1983). However, the purified catalytic subunit of protein phosphatase 2A showed significant opsin phosphatase activity, dephosphorylation reaching a plateau when approximately 50% of the counts had been removed (Figure 6). Addition of a further aliquot of phosphatase at this point did not result in further dephosphorylation, a phenomenon also observed with the ROS phosphatase in the absence of protamine (not shown). The opsin phosphatase activity of protein phosphatase 2A was also unaffected by Mn^{2+} (Figure 6).

Discussion

The pivotal role of metarhodopsin II (Rho*) in signal transmission and termination is illustrated in Figure 1 which also shows the phosphorylation of Rho* and highlights that in order to return $(phospho)_n$-Rho* to its dark-adapted form, the protein must undergo various reactions. It is convenient to assume that $(phospho)_n$-Rho* decays via intermediates until *all-trans*-retinal dissociates from the protein. The protein must subsequently be regenerated with 11-*cis*-retinal and the covalently bound phosphate groups removed to re-form rhodopsin.

Dephosphorylation of rhodopsin has been observed by various groups to differing extents. Kühn (1974) performed studies in living frogs and demonstrated both the light-dependent phosphorylation of rhodopsin and the subsequent dephosphorylation reaction in the dark. However, many groups have only observed low levels of dephosphorylation in vitro. Weller et al. (1975) reported poor dephosphorylation

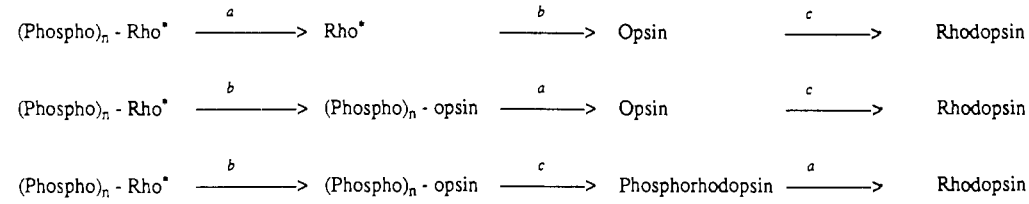

FIGURE 7: Alternative pathways for the regeneration of rhodopsin from (phospho)$_n$-Rho*. The three reactions are (a) release of nP$_i$ through the action of phosphatase, (b) hydrolysis of the Schiff base linkage, releasing *all-trans*-retinal, and (c) reaction with 11-*cis*-retinal.

of opsin in ROS, which was only slightly improved by addition of the supernatant obtained after centrifuging a homogenate of whole retinas. Wilden and Kühn (1982) reported that no dephosphorylation occurred in the presence of phosphate buffer and only slight dephosphorylation in the presence of Tris buffer. However, dephosphorylation increased at lower concentrations of ATP. Subsequently, Thompson and Findlay (1984) also demonstrated low levels of opsin dephosphorylation by the supernatant obtained after centrifuging a homogenate of whole retinas, and went on to show that total dephosphorylation by the supernatant was only observed in incubations containing a low concentration of a specific detergent (dodecyltrimethylammonium bromide). Interestingly, however, Miller et al. (1977) claimed that significant dephosphorylation of phosphoopsin was observed in ROS in which ATP was removed after a period of phosphorylation.

The enzymes which might participate in this dephosphorylation have been investigated in the present work, and two protein phosphatases (type 1 and type 2A) were detected in purified bovine ROS. A fraction containing predominantly the type 2A phosphatase was obtained from ROS extracted with "medium salt buffer", while further extraction of the resulting membrane pellet with "high salt buffer" solubilized predominantly a type 1 phosphatase. Several lines of evidence demonstrated that it was type 2A activity which effectively dephosphorylated opsin. Thus, opsin phosphatase activity copurified with phosphorylase phosphatase activity through chromatography on heparin–Sepharose and gel filtration on Superose 12, and like the type 2A phosphatase was active in the absence of divalent cations and unaffected by inhibitor 2. The opsin phosphatase from Superose 12 also dephosphorylated the α-subunit of phosphorylase kinase much faster than the β-subunit, as expected of a type 2A enzyme. The molecular mass of the ROS type 2A phosphatase (100 kDa) suggests that this enzyme is similar to protein phosphatase 2A$_2$, which is composed of the 36-kDa catalytic subunit complexed to a 60-kDa A subunit (Cohen, 1989). Protamine sulfate has previously been shown to stimulate the activity of certain protein phosphatases, notably protein phosphatase 2A (Tung et al., 1985). The dephosphorylation of opsin by ROS type 2A phosphatase was also stimulated ~10-fold by protamine, but not by Mn^{2+}. The involvement of the ROS type 2A phosphatase in the dephosphorylation of phosphoopsin was confirmed by showing that the homogeneous catalytic subunit of protein phosphatase 2A from rabbit muscle effectively released P$_i$ from phosphoopsin. By contrast, the ROS type 1 phosphatase and the pure catalytic subunit of protein phosphatase 1 from rabbit skeletal muscle were ineffective.

The β-adrenergic receptor exhibits a high degree of similarity with rhodopsin [e.g., see Dixon et al. (1986) and Weiss et al. (1987)]. Bovine rhodopsin contains an internal disulfide bond between Cys-110 and Cys-187, and these residues are conserved in all known visual pigments, as well as in β-adrenergic receptors (Al-Saleh et al., 1987, 1988). Site-directed mutagenesis experiments by Karnik et al. (1988) on bovine rhodopsin and by Dixon et al. (1987) on β-adrenergic receptor have confirmed the essential nature of these cysteines, emphasizing the close similarities which exist between the two classes of proteins. Furthermore, the G-proteins involved in both systems are also similar in both structure and activity [e.g., see Cerione et al. (1985)]. Yang et al. (1988) recently phosphorylated the β-adrenergic receptor to 6 mol/mol with β-adrenergic receptor kinase, whereas rhodopsin was only phosphorylated to 0.05 mol/mol using the same kinase. These two phosphorylated substrates were used to examine dephosphorylation by type 1 and 2B phosphatases, a high molecular mass form of type 2A phosphatase, and an enzyme termed latent phosphatase 2 (LP-2). The latter two enzymes, isolated from brain, required freezing and thawing in the presence of 0.2 M 2-mercaptoethanol to unmask their activities. Of these enzymes, only LP-2 was reported to be capable of dephosphorylating the β-adrenergic receptor and opsin. The lack of dephosphorylation of phosphoopsin by type 2A protein phosphatases in these studies (Yang et al., 1988) is in conflict with results presented here. The reason for this discrepancy is unclear, but one possibility is that the β-adrenergic receptor kinase phosphorylates sites on rhodopsin distinct from those phosphorylated by rhodopsin kinase, which are resistant to type 2A phosphatases. Our conclusions are, however, in qualitative agreement with a recent report (Palczewski et al., 1989) which appeared at about the same time as our original publication (Fowles, 1988). Palczewski et al. (1989) described the isolation of an opsin phosphatase from bovine ROS using ethanol precipitation as the initial step. On gel filtration, the activity eluted at 35–38 kDa and was ascribed to the catalytic subunit of protein phosphatase 2A (which would be dissociated from higher molecular mass complexes by ethanol precipitation). The method of isolation used by thse investigators gave an opsin phosphatase activity of 6 pmol of P$_i$ released per minute per retina, 14-fold lower than reported in the present work, even without stimulation by protamine (85 pmol of P$_i$ released min^{-1} retina^{-1}). It should be noted that the phosphatase activity determined by Palczewski et al. (1989) was determined with phosphoopsin prepared using 0.1 mM ATP, which we have shown gives low levels of phosphorylation and Figure 2 shows that the activity of the phosphatase is dependent on the level of phosphorylation of opsin/rhodopsin. The phosphatase activity obtained by us is about equal to that of rhodopsin kinase when the latter activity is measured in the presence of 0.1 mM ATP (100 pmol of phosphate transferred min^{-1} retina^{-1}). This suggests that ROS phosphatase 2A identified in the present work may play an important role in the regulation of the phosphorylation/dephosphorylation cycle during vision.

The rates of dephosphorylation of rhodopsin and opsin by the ROS type 2A phosphatase were identical, in agreement with the studies of Weller et al. (1975), who observed low levels of opsin dephosphorylation and reported that dephosphorylation was unaffected by regeneration with 11-*cis*-retinal [see also Palczweski et al. (1989)]. Inclusion of protamine in our incubations markedly stimulated the dephosphorylation of both opsin and rhodopsin. These results suggest that the recycling of (phospho)$_n$-Rho* to rhodopsin which requires three steps,

(1) dissociation of the chromophore, (2) reaction with 11-*cis*-retinal, and (3) dephosphorylation, may have no set order with respect to the dephosphorylation state (Figure 7). Thus, the reaction of 11-*cis*-retinal may occur before or after the process of dephosphorylation since the phosphatase is, at least in vitro, equally active with phosphoopsin and phosphorhodopsin as substrates. The use of "medium salt buffer" for solubilization of the ROS type 2A phosphatase suggests that the enzyme may be located in the interdiscal cytosolic compartment in vivo, that must also house the phosphorylated domain of opsin and rhodopsin, and by implication phospho-Rho*. Thus, the location of the phosphatase in ROS and its physiological function are mutally compatible.

Registry No. Protein phosphatase, 9025-75-6.

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