

## The Catalytic Subunit of Phosphatase 2A Dephosphorylates Phosphopsin<sup>†</sup>

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**ABSTRACT:** Rod cell outer segments were found to contain a protein phosphatase activity toward phosphopsin with properties very similar to those of protein phosphatase 1 or 2A. The opsin phosphatase activity was stable to ethanol precipitation, had a  $M_r$  of 35 000–38 000 as determined by gel filtration, and was not dependent on divalent cations for activity. The chromatographic properties on DEAE-cellulose of the rod outer segment protein phosphatase were also similar to those reported for protein phosphatase 1 or 2A. In order to distinguish between these two protein phosphatases, we tested homogeneous preparations of protein phosphatases 1 and 2A from skeletal muscle for activity toward phosphopsin. Protein phosphatase 2A dephosphorylated phosphopsin at approximately 10% of its rate toward phosphorylase  $\alpha$ , whereas protein phosphatase 1 had no activity toward phosphopsin. We conclude that protein phosphatase 2A is present in the rod cell outer segment and that it is a likely candidate to perform the *in vivo* dephosphorylation of rhodopsin in the visual cycle.

**R**hodopsin is the photoreceptor protein in rod cells of the vertebrate retina. Following light absorption, rhodopsin activates a G-protein, transducin, which in turn activates a cGMP phosphodiesterase. This leads to hydrolysis of cGMP, which causes closing of cation channels in the plasma membrane, *i.e.*, transduction of the original energy of light into a neural event. One mechanism by which the receptor protein rhodopsin becomes deactivated following light activation is via phosphorylation. Rhodopsin kinase phosphorylates specific serine and threonine residues on the cytoplasmic surface of light-activated rhodopsin (Hargrave *et al.*, 1980; Pappin *et al.*, 1984). Phosphorylated rhodopsin has a decreased ability to activate

G-protein and an enhanced ability to bind a 48-kDa protein, arrestin, which completes rhodopsin inactivation (Kühn *et al.*, 1984; Miller *et al.*, 1986). In order for phosphorylated rhodopsin to again participate effectively in the process of phototransduction, it must be dephosphorylated. Such dephosphorylation has been observed to occur both *in vivo* (Kühn, 1974) and in isolated retina (Kühn & Bader, 1976), but the phosphatase involved has not been identified.

Four enzymes account for the majority of protein phosphatase (PrP)<sup>1</sup> activity toward proteins phosphorylated on serine and threonine residues (Ingebritsen & Cohen, 1983). The enzymes have been grouped into two classes (type I, PrP-1, and type 2, PrP-2A, PrP-2B, and PrP-2C) on the basis of their substrate specificities and their response to heat-stable inhibitor proteins. PrP-1, PrP-2A, and PrP-2C have broad and overlapping substrate specificities whereas PrP-2B is only active toward a very limited set of substrates. Each of the protein phosphatases is a distinct gene product, although the catalytic subunits of PrP-1 and PrP-2A show significant sequence homology (Berndt *et al.*, 1987). The four protein phosphatases are present in a wide variety of cell types and appear to be

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<sup>1</sup> Abbreviations: DTT, dithiothreitol; PrP, protein phosphatase; ROS, rod cell outer segments; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCA, trichloroacetic acid.

strongly conserved during animal evolution (Pondaven & Cohen, 1987).

We report here that the catalytic subunit of protein phosphatase 2A can carry out the dephosphorylation of phosphopsin and that the enzyme is present in rod outer segments. Since rhodopsin is the best-studied member of the family of seven transmembrane helix receptors that act via G-proteins (Applebury & Hargrave, 1986), this may have general significance for understanding the functions of other members of that receptor family.

#### MATERIALS AND METHODS

ATP disodium salt, poly(L-lysine) hydrobromide (MW 17 300), Tris-HCl, and other chemicals were purchased from Sigma Chemical Co. [ $\gamma$ - $^{32}$ P]ATP was obtained from New England Nuclear, Boston, MA. Histone H1 was purchased from Boehringer Mannheim Biochemicals.

**Isolation of Rod Outer Segments.** Rod outer segments (ROS) were prepared from frozen bovine retinas (Lawson, Inc., Omaha, NB) following the procedure of Wilden and Kühn (1982). The preparation was performed either under dim red light to prepare a substrate for rhodopsin kinase or under room lights for the preparation of rhodopsin kinase (Palczewski et al., 1988a). The substrate for the rhodopsin kinase assay, ROS washed with 5 M urea, was prepared according to the method of Shichi and Somers (1978).

To prepare crude ROS, 50 retinas were shaken with 50 mL of 50 mM 3-(*N*-morpholino)propionic acid, Na<sup>+</sup> salt buffer, pH 7.0, containing 20% sucrose and 100 mM KCl, and then centrifuged for 5 min at 5000 rpm. The supernatant, containing the ROS, was collected and used to prepare the phosphatase 2A catalytic subunit as described below. To measure the concentration of rhodopsin, ROS were solubilized in 50 mM Tris-HCl, 100 mM hydroxylamine, and 0.3 M tridecyltrimethylammonium bromide, pH 7.0. The concentration was determined from the light-sensitive OD<sub>498</sub>, assuming a molar extinction coefficient of 40 600 at 498 nm (Wald & Brown, 1953).

ROS used for study of phosphatase activity were also prepared from frozen retinas by the method of Wilden and Kühn (1982) and from fresh retinas by the method of Schnetkamp and Daemen (1982).

**Preparation of Rhodopsin Kinase.** Rhodopsin kinase was extracted from ROS prepared under room light as described by Palczewski et al. (1988b). The kinase extract was loaded on a DEAE-cellulose column (1.6 × 20 cm) equilibrated with 75 mM Tris-HCl, 1 mM DTT, and 1 mM Mg(OAc)<sub>2</sub>, pH 7.8. A KCl gradient from 0 to 250 mM in the same buffer was used to elute rhodopsin kinase. Fractions containing the kinase were diluted with the Tris-HCl buffer to reduce the KCl concentration, and the enzyme was concentrated by rechromatography on DEAE-cellulose. The kinase was eluted with 110 mM KCl in the above buffer. About 35  $\mu$ g of rhodopsin kinase in 2 mL was obtained from 400 retinas. The purity of the enzyme was greater than 80% as judged by SDS-PAGE.

**Preparation of Protein Phosphatase 1, Protein Phosphatase 2A, and Phosphorylase a.** Phosphorylase *b* (Fischer & Krebs, 1958), phosphorylase kinase (Cohen, 1973), and the catalytic subunits of PrP-1 and PrP-2A (Resink et al., 1983; Tung et al., 1984) were purified to homogeneity from rabbit skeletal muscle. Phosphorylase *a* was prepared by phosphorylation of phosphorylase *b* with phosphorylase kinase (Shenolikar & Ingebritsen, 1984).

**Preparation of Phosphorylated Opsin and Rhodopsin.** Phosphorylated opsin was prepared from urea-washed ROS,

partially purified rhodopsin kinase, and [ $\gamma$ - $^{32}$ P]ATP. ROS containing 1 mg of rhodopsin were incubated with about 10  $\mu$ g of rhodopsin kinase in 67 mM potassium phosphate buffer containing 1 mM DTT, 0.1 mM EDTA, and 100  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (100–1000 cpm/pmol) at 25 °C. After 60-min exposure to light, the reaction was stopped by 1000-fold dilution with cold 50 mM Tris-HCl buffer, pH 7.0, containing 50 mM  $\beta$ -mercaptoethanol. Membranes containing phosphorylated opsin were collected by centrifugation (20 000 rpm, 60 min), washed twice with 50 mL of the above Tris-HCl buffer, and then resuspended in 1 mL of Tris-HCl buffer.

**Regeneration of Phosphorylated Opsin.** Regeneration of phosphorylated opsin was performed as described by Palczewski et al. (1988), except that 9-*cis*-retinal was used instead of 11-*cis*-retinal.

**Partial Purification of Protein Phosphatase 2A from Crude ROS or from an Extract of Gradient-Purified Rod Outer Segments.** The crude rod outer segment suspension from 50 retinas was added at room temperature to 100% ethanol to a final concentration of 80% ethanol. The proteins were collected by centrifugation (15 000 rpm, 20 min). The catalytic subunit of the phosphatase was extracted with 50 mL of Tris-HCl buffer, pH 7.0 (25 °C), containing 50 mM  $\beta$ -mercaptoethanol. This procedure was also applied to the isolation of the catalytic subunit of PrP-2A from the cytosolic extract from ROS purified by the step sucrose gradient method (Papermaster & Dreyer, 1974). Purification of the phosphatase subunit on DEAE-cellulose was performed essentially as described by Ingebritsen et al. (1980).

**Gel Filtration of Phosphatases.** Gel filtration of protein phosphatase 2A and the extract of the catalytic subunit from crude ROS after ethanol precipitation was performed on a Bio-Sil TSK-125 column (Bio-Rad). The column had been calibrated by using the following proteins: thyroglobulin, IgG, ovalbumin, carbonic anhydrase, myoglobin, and cyanocobaltamine. The proteins were dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The column effluent was monitored at 280 nm. The flow rate was 0.8 mL/min and 240- $\mu$ L fractions were collected.

**Assay of Protein Phosphatase Activity.** Assays were carried out in a total volume of 150  $\mu$ L at 25 °C in a mixture containing 50 mM Tris-HCl buffer, pH 7.0, 50 mM  $\beta$ -mercaptoethanol, 0.1 mM EDTA, 1 mg/mL bovine serum albumin, and 0.3  $\mu$ M  $^{32}$ P-labeled opsin or  $^{32}$ P-labeled phosphorylase *a*. Assays on extracts from gradient-purified ROS employed the same conditions except that the concentration of  $^{32}$ P-labeled opsin was 2.5  $\mu$ M. As indicated in the text, some reaction mixtures contained 0.05 mg/mL histone H1, 50 mM NaF, or 0.5 mg/mL poly(L-lysine) (MW 17 300). Immediately prior to the assay, protein phosphatases 1 and 2A were diluted with 50 mM Tris-HCl buffer, pH 7.0 (25 °C), containing 50 mM  $\beta$ -mercaptoethanol, 1 mg/mL bovine serum albumin, and 0.005% Brij 35 and incubated for 5 min at 25 °C. The reactions were terminated by the addition of 100  $\mu$ L of 20% TCA, and the soluble  $^{32}$ P radioactivity was determined.

Protein determination was performed either by measuring OD<sub>280</sub> or by using the Bio-Rad protein assay with bovine serum albumin as the standard.

#### RESULTS

Extracts from ethanol-precipitated rod cell outer segments were found to dephosphorylate phosphopsin in a concentration-dependent manner (Figure 1). The opsin phosphatase activity was further characterized by DEAE-cellulose chromatography (Figure 2) and by HPLC gel permeation chro-

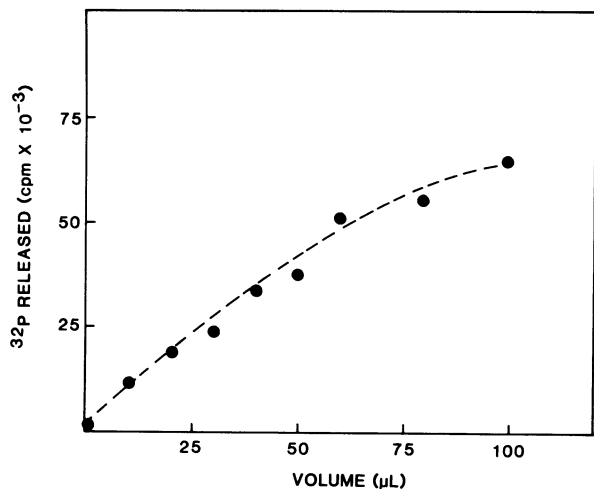


FIGURE 1: Dephosphorylation of phosphopsin by an extract from ethanol-precipitated crude rod outer segments. Various amounts of extract, described under Materials and Methods, were incubated with phosphopsin for 5 min. Free phosphate was determined from the radioactivity remaining soluble following TCA precipitation.

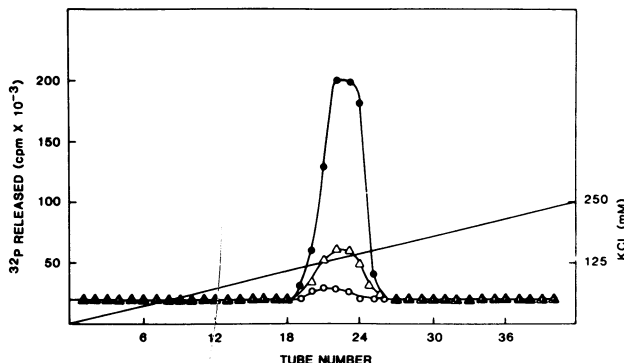


FIGURE 2: Ion-exchange chromatography of the protein phosphatase activity in the extract from ethanol-precipitated rod outer segments. Aliquots were assayed for protein phosphatase activity with phosphopsin as substrate in the presence (●) or absence (Δ) of histone and in the presence (○) of fluoride.

matography on a TSK G3000 SW column (Figure 3A). In each case, a single peak of activity was observed. Several properties of the opsin phosphatase activity suggested that it was due to the catalytic subunit of PrP-2A. The activity was stable to treatment with 80% ethanol at room temperature, was stimulated 4–5-fold by histone H1 (Figures 2 and 3A), and was inhibited by NaF (Figure 2). Additionally, the elution position from DEAE-cellulose (0.14 M KCl) and the apparent molecular weight on gel permeation chromatography (35–38 kDa) were consistent with this interpretation.

The catalytic subunits of PrP-1 and PrP-2A have many properties in common (Ingebritsen & Cohen, 1983; Resink et al., 1983; Tung et al., 1984), and they are not fully separated by the two chromatography steps used to characterize the opsin phosphatase from rod cell outer segments. Of the properties described, the stimulation by histone H1 is most diagnostic for PrP-2A (Mellgern & Schlender, 1983); however, histone H1 also stimulates the activity of PrP-1 toward some substrates (Pelech & Cohen, 1985). Because of this we tested homogeneous preparations of PrP-1 and PrP-2A from rabbit skeletal muscle for activity toward phosphopsin. PrP-1 had little activity toward this substrate whereas PrP-2A dephosphorylated phosphopsin (0.6  $\mu$ M) at  $\sim$ 10% of the rate observed with phosphorylase *a* (0.6  $\mu$ M) (Figure 4). The opsin phosphatase activity of skeletal muscle PrP-2A coeluted with

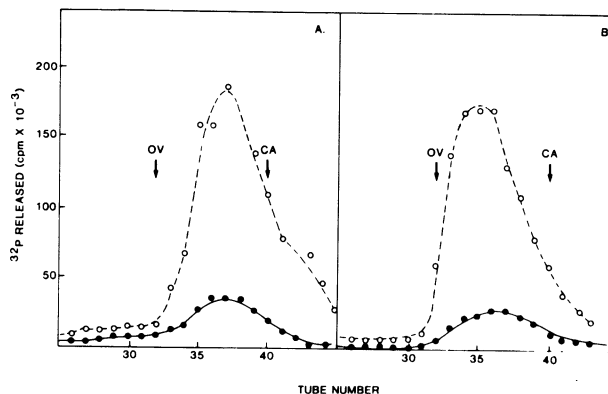


FIGURE 3: TSK-HPLC gel filtration of protein phosphatase activity toward phosphopsin: (A) extract from ethanol-precipitated rod outer segments in the presence (○) and absence (●) of 0.05 mg/mL histone; (B) PrP-2A from rabbit skeletal muscle with (○) and without (●) 0.05 mg/mL histone. OV and CA indicate the positions of elution of ovalbumin (43 kDa) and carbonic anhydrase (30 kDa), respectively.

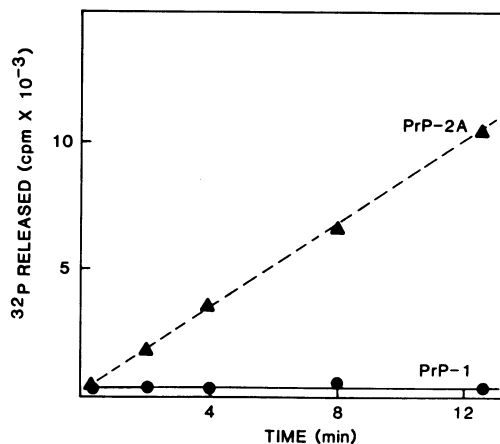


FIGURE 4: Dephosphorylation of phosphopsin by protein phosphatase 2A (▲) and protein phosphatase 1 (●). Activity is expressed as the radioactivity released as a function of time. Under the same conditions PrP-2A removed 10 times more phosphate from a different substrate, phosphorylase *a*. PrP-1 used in this experiment was 50% more active against phosphorylase *a* than was PrP-2A; however, this phosphatase does not have the ability to dephosphorylate phosphopsin.

the phosphorylase phosphatase activity of the preparation on HPLC gel permeation chromatography (data not shown). Additionally, the opsin phosphatase activity was stimulated 4–5-fold by histone H1 (Figure 3B) and was inhibited by NaF (data not shown). We conclude that PrP-2A or a closely related isozyme accounts for the opsin phosphatase activity of the rod outer segment protein phosphatase preparation.

PrP-2A from skeletal muscle or rod cell outer segments was able to remove 80–95% of the [ $^{32}$ P]phosphate from phosphopsin. In order to confirm that the TCA-soluble  $^{32}$ P radioactivity released by PrP-2A was inorganic phosphate rather than small TCA-soluble peptides, the TCA supernatants from the reactions were treated with molybdate and the resulting inorganic phosphate–molybdate complexes were extracted into organic solvent (Antoniw & Cohen, 1976). On the basis of these measurements, >85% of the TCA-soluble radioactivity was due to inorganic phosphate. When the phosphopsin-containing membranes were frozen and thawed several times prior to use as a substrate for PrP-2A, the maximal amount of  $^{32}$ P that could be released by the protein phosphatase was decreased to 55–70%. The maximal amount released could be increased somewhat (10–25%) by sonication of the frozen–thawed membrane preparations. This suggests that a portion of the  $^{32}$ P-labeled opsin is sequestered in the interior

of vesicles and is inaccessible to the phosphatase.

To ensure that the protein phosphatase activity measured is in fact derived from rod cell outer segments, density gradient purified ROS were prepared and extracts from these highly purified ROS were tested for protein phosphatase activity. The activity in these extracts behaved exactly as that of PrP-2A; i.e., it was activated by histone, was inhibited by fluoride, was stable to ethanol, and showed the same molecular weight by gel filtration. We find the amount of phosphoopsin phosphatase activity in gradient-purified ROS to be 0.3 nmol of  $P_i$ /min per 50 retinas. (This amount is  $\sim 10\%$  that of the activity of rhodopsin kinase present, assuming that all of our conditions of extraction and assay are optimal.) These data indicate that PrP-2A or a closely related enzyme is present in the rod cell outer segment and that it can dephosphorylate phosphoopsin.

Rhodopsin only becomes a substrate for rhodopsin kinase upon bleaching. We tested phosphorylated rhodopsin under three conditions to see if bleaching has any effect on the ability of phosphorhodopsin to serve as a substrate for the protein phosphatase activity in the extract from crude ROS. Phosphorhodopsin samples that were unbleached, freshly bleached, or had been bleached previously all served equally well as substrates. This was observed for the phosphatase activity in the presence or absence of histone (data not shown).

#### DISCUSSION

The *phosphorylation* of rhodopsin has been studied extensively since its discovery in 1972 (Kühn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973). The reverse reaction, *dephosphorylation*, has received little attention due to the inability to reproducibly dephosphorylate phosphoopsin *in vitro*. Weller et al. (1975) reported phosphoopsin phosphatase activity in retinal homogenates; however, the dephosphorylation was slow. Miller and Paulsen (1975) also found evidence for dephosphorylation of phosphoopsin in crude ROS preparations. They observed that addition of ATP shortly after dephosphorylation became evident led to an increase in the extent of phosphorylation, suggesting that phosphorylation and dephosphorylation were occurring simultaneously. Wilden and Kühn (1982) found dephosphorylation of highly phosphorylated opsin using density gradient purified ROS in the absence of phosphate buffer. While it was evident that a protein phosphatase activity that acted on phosphoopsin was present and necessary in the visual cycle, it was not clear whether this protein phosphatase was very specific (one which had a restricted specificity for phosphoopsin) or more general in its substrate requirements. In fact, since it appeared that rhodopsin *kinase* was very specific, phosphorylating only freshly bleached rhodopsin and no other typical kinase substrates, many workers assumed that the protein *phosphatase* would also be very specific.

In this study we report the identification of a protein phosphatase activity in rod cell outer segments that dephosphorylates highly phosphorylated opsin. The properties of this opsin phosphatase are very similar to those of the catalytic subunit of PrP-2A. These similarities include the stability to treatment with 80% ethanol at room temperature, the apparent molecular weight on gel permeation chromatography, the elution position during DEAE-cellulose chromatography, stimulation of the opsin phosphatase activity by histone H1, and inhibition of the opsin phosphatase activity by NaF. Additionally, a homogeneous preparation of the PrP-2A catalytic subunit from rabbit skeletal muscle was able to dephosphorylate phosphoopsin at 10% the rate observed with phosphorylase *a*, whereas homogeneous skeletal muscle PrP-1

was unable to dephosphorylate phosphoopsin.

Our results differ from those of Yang et al. (1988), who reported that PrP-2A from pig brain was unable to dephosphorylate rhodopsin. The most significant difference between the two studies is that Yang and co-workers used the  $\beta$ -adrenergic receptor kinase to phosphorylate rhodopsin to a stoichiometry of 0.05 mol of phosphate/mol of rhodopsin whereas in the present study we used rhodopsin kinase to phosphorylate rhodopsin to a stoichiometry of 4 mol of phosphate/mol of rhodopsin. While rhodopsin and the  $\beta$ -adrenergic receptor belong to the same family of receptors, the low level of phosphorylation of rhodopsin by the  $\beta$ -adrenergic receptor kinase suggests that it may phosphorylate different sites on rhodopsin than does rhodopsin kinase. The sites phosphorylated by the  $\beta$ -adrenergic receptor kinase may not serve as substrates for PrP-2A.

Yang and co-workers (1988) report the dephosphorylation of phosphoopsin with a pig brain protein phosphatase termed latent phosphatase 2 (Yang et al., 1986). This protein phosphatase has an apparent  $M_r$  of  $\sim 350\,000$  under non-denaturing conditions and appears to contain a  $M_r$  49 000 catalytic subunit. These properties clearly distinguish latent phosphatase 2 from the opsin phosphatase activity of PrP-2A (either from rod cell outer segments or from skeletal muscle).

In intact cells the catalytic subunit of PrP-2A is entirely associated with other regulatory subunits to form two high molecular weight species, termed PrP-2A<sub>0</sub> and PrP-2A<sub>1</sub> (Ingebritsen & Cohen, 1983). The two species have the structures AB'C<sub>2</sub> and ABC<sub>2</sub>, respectively, where A is a 60-kDa subunit, B' is a 54-kDa subunit, B is a 55-kDa subunit, and C is the catalytic subunit (Tung et al., 1985). The free catalytic subunit can be isolated from these complexes by a number of treatments (e.g., treatment with 80% ethanol at room temperature) that appear to selectively denature the regulatory subunits. PrP-2A<sub>0</sub> is an inactive species whose activity is only expressed upon dissociation of the catalytic subunit from the regulatory subunits (see before) or in the presence of basic polypeptides (e.g., histone H1, protamine, polylysine) (Ingebritsen & Cohen, 1983; Tung et al., 1985). The activity of PrP-2A<sub>1</sub> is also stimulated by these treatments but to a much smaller extent. Although the relative amount of these two species in rod cell outer segments is not known, we find a relatively low level of opsin phosphatase activity in extracts from rod cell outer segments prior to ethanol treatment [less than 20% (data not shown)]. This suggests that the majority of the activity may be in the PrP-2A<sub>0</sub> form.

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