Inorganic Pyrophosphatase from Bovine Retinal Rod Outer Segments*

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Rod outer segments from bovine retina contain a higher level of intracellular inorganic pyrophosphatase (EC 3.6.1.1) activity than has been found in any other mammalian tissue; the specific activity in extracts of soluble outer segment proteins is more than 6-fold higher than in extracts from bovine liver and more than 24-fold higher than in skeletal muscle extracts. This high activity may be necessary to keep inorganic pyrophosphatase concentrations low in the face of the high rates of pyrophosphate production that accompany the cGMP flux driving phototransduction. We have begun to explore the role of inorganic pyrophosphatase in photoreceptor cGMP metabolism by 1) studying the kinetic properties of this enzyme and its interactions with divalent metal ions and anionic inhibitors, 2) purifying it and studying its size and subunit composition, and 3) examining the effects of pyrophosphate on rod outer segment guanylyl cyclase. $K_a$ for magnesium pyrophosphate was 0.9–1.5 μM, and the purified enzyme hydrolyzed >885 μmol of PPi, min$^{-1}$ mg$^{-1}$. The enzyme appears to be a homodimer of 36-kilodalton subunits when analyzed by gel electrophoresis and density gradient centrifugation, implying that $k_{cat}$ = 10$^3$ s$^{-1}$, and $k_{cat}/K_m$ = 0.7–1 × 10$^3$ M$^{-1}$ s$^{-1}$. The enzyme was inhibited by Ca$^{2+}$ at submicromolar levels: 28% inhibition was observed at 138 nM [Ca$^{2+}$], and 53% inhibition at 700 nM [Ca$^{2+}$]. Imidodiphosphate acted as a competitive inhibitor, with $K_I$ = 1.2 μM, and fluoride inhibited half-maximally ~20 μM. Inhibition studies on rod outer segment guanylyl cyclase confirmed previous reports that pyrophosphate inhibits guanylyl cyclase, suggesting an essential role for inorganic pyrophosphatase in maintaining cGMP metabolism.

Inorganic pyrophosphate (PPi) is produced in living cells by numerous metabolic pathways (Stetten, 1960), including synthesis of polymers such as DNA, RNA, protein, and polysaccharides, as well as synthesis of small molecules such as cGMP and cAMP. PPi is also produced by oxidative phosphorylation and glycolysis. Consequently, the rate of PPi production varies with the metabolic activity in cells (Klemme, 1976) and has to be counterbalanced by efficient catabolism. In all cells studied, there is an inorganic pyrophosphatase (PPase, EC 3.6.1.1) activity that catalyzes rapid conversion of PPi into Pi (reviewed by Cooperman (1982) and Cooperman et al. (1992)). It has been suggested that PPi may regulate many enzyme activities without actually participating in the reaction (Khandelwal and Kamani, 1980; Wheeler and Lowenstein, 1980). In spite of its importance in metabolism and the fact that PPase activity varies widely among mammalian tissues (Shatton et al., 1981), almost nothing is known about how this enzymatic activity is regulated in mammals. It has been suggested that inhibition of PPase by Ca$^{2+}$ may represent a mechanism for hormonal control of PPase activity (Davidson and Halestrap, 1987; Hachimori et al., 1983), but this kind of regulation has not been clearly demonstrated. While PPase from rat liver mitochondria has the requisite Ca$^{2+}$ sensitivity ($K_i$ = 67 nM for CaPPi) (Davidson and Halestrap, 1989), cytoplasmic PPase from porcine brain has been reported to require non-physiological levels of Ca$^{2+}$ for inhibition (Hachimori et al., 1983).

In rod outer segments (ROS) of the vertebrate retina, the second messenger cGMP plays a pivotal role in phototransduction (reviewed by Stryer (1981) and Pugh and Lamb (1990)). Light initiates an enzymatic cascade that leads to hydrolysis of cGMP and, in turn, to closure of cGMP-gated cation channels in the plasma membrane. A secondary effect of this closure is a reduction in intracellular [Ca$^{2+}$] (Yau and Nakatani, 1986; McNaughton et al., 1986; Ratto et al., 1986) that appears to be essential for normal kinetics of recovery from the light response (Lamb et al., 1986) and for the phenomenon of light adaptation (Matthews et al., 1988; Nakatani and Yau, 1988). Lowering Ca$^{2+}$ markedly enhances the activity of the ROS guanylyl cyclase (Pepe et al., 1986; Koch and Stryer, 1988; Dizhoor et al., 1991). Studies of $^{32}$O incorporation into guanine nucleotides in intact retinas have demonstrated that cGMP is produced in the dark at a rate of 29 μM s$^{-1}$, and in the light the rate increases to 130 μM s$^{-1}$ (Ames et al., 1986).

These high levels of cGMP production must also generate high levels of PPi, a by-product of the cyclization. This ion can inhibit cellular enzymes (Khandelwal and Kamani, 1980) including a cyclic nucleotide phosphodiesterase (Cheung, 1966), guanylyl cyclase (Hakki and Sitaramayya, 1990; Hayashi and Yamazaki, 1991) and adenylyl cyclase (Johnson and Shoshani, 1990), and its export from cells can lead to pathological conditions (Fritzker, 1988), so it seems likely that the PPase that catalyzes its hydrolysis is either maintained at high levels of activity at all times, or is regulated in synchrony with guanylyl cyclase. A high level of PPase activity has in fact been reported to be present in bovine ROS (Hakki and Sitaramayya, 1990). Because very little is known about retinal PPase or about PPase regulation in animals in general, we

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decided to purify and study the enzyme(s) from bovine ROS. Because Ca\(^{2+}\) plays an important role in the recovery phase of the rod cell's light response when PP\(_\text{P}\) production is expected to be maximal, and because Ca\(^{2+}\) is a potent inhibitor of yeast PP\(_\text{ase}\) (Ridlington and Butler, 1972), we have tested the sensitivity of ROS PP\(_\text{ase}\) to Ca\(^{2+}\) in the physiologically relevant concentration range, and have studied the effects of PP\(_\text{ase}\) on ROS guanylyl cyclase. We have also examined the effects on both PP\(_\text{ase}\) and guanylyl cyclase of imidodiphosphate, a nonhydrolyzable PP, analogue that has been reported to reduce a current component attributed to activation of guanylyl cyclase by low Ca\(^{2+}\) in gecko rods (Detwiler and Rispal, 1989).

**Materials and Methods**

Reagents—MOPS and DTT were obtained from Boehringer Mannheim. \(\alpha\)-pCITP (800 Ci/mol), \(\beta\)-PPi (2 Ci/ml), and \([8-\text{H}]\) cGMP (16 Ci/ml) were from Du Pont-New England Nuclear. Electrophoresis reagents were from Bio-Rad. Hydroxyapatite (last flow) was from Calbiochem. Polyethyleneimine cellulose TLC plates, Teflon bags, and glass tubing were purchased from Fisher Scientific Co. Buffers for hydroxylapatite chromatography included either 10 mM phosphate and 6 mM EGTA, or 10 mM Tris-base and 5 mM EGTA. The pH value of the DEAE-HPLC buffers was either 8.0 or 8.5 as indicated. Buffers for DEAE-HPLC contained 10 mM Tris-base, 3 mM MgCl\(_2\), 3 mM Na\(_2\)EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. The standard PP\(_\text{ase}\) assay buffer contained 50 mM Tris-Cl (pH 7.5), 3 mM MgCl\(_2\), 3 mM Na\(_2\)PO\(_4\), and 0.2 mM EGTA. The concentrations of individual components were varied as indicated to examine the effects of [Mg\(_2\)], [Ca\(^{2+}\)], [PP\(_\text{i}\)], [Pi], and pH. Buffers for hydroxyapatite chromatography included either 10 mM (buffer A) or 250 mM (buffer B) potassium phosphate (pH 6.8), 3 mM MgCl\(_2\), 1 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Buffers for DEAE-HPLC contained 20 mM Tris-Cl, 3 mM MgCl\(_2\), 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and either 0 (buffer C) or 1 M (buffer D) NaCl. The pH value of the DEAE-HPLC buffers was either 8.0 or 8.5 as indicated. For reverse phase HPLC, buffer E was water with 1.5% (v/v) trifluoroacetic acid, and buffer F was isopropanol:acetonitrile:water (3:2:2) with 0.08% trifluoroacetic acid.

**Rod Outer Segments and Protein Preparation**—For preparative purposes, ROS were prepared from frozen dark dissected bovine retinas by the sucrose gradient method as described (Fung and Stryer, 1980). For analysis of PP\(_\text{ase}\) localization, purer ROS were prepared by the sucrose gradient method of Papermaster and Dryer (1974). The ROS band was collected, washed with ROS buffer, resuspended in buffer containing 24% (w/w) sucrose, and loaded onto a second sucrose gradient. Fractions were dried under vacuum, redissolved in PP\(_\text{ase}\) assay buffer, and assayed for PP\(_\text{ase}\) activity.

**PP\(_\text{ase}\) Assay**—PP\(_\text{ase}\) activity was determined by measuring phosphate production from substrate PP\(_\text{i}\) generated from substrate PP\(_\text{P}\). For most routine purposes, PP\(_\text{ase}\) assays were performed by using a molybdate-blue-based colormetric assay (Bartlett, 1959). The standard assay was performed in a 100-µl total volume containing PP\(_\text{ase}\) assay buffer and sufficient enzyme to yield total PP\(_\text{ase}\) activity of less than 10% during a 10-min incubation at 37°C. The reaction was terminated by adding 40 µl of 10 N H\(_2\)SO\(_4\). For kinetics, K\(_m\) and K\(_i\) determinations or assays in the presence of Pi, \([\beta\text{PPi}]\) was used as substrate with 1 mM MgCl\(_2\) (for K\(_m\) and K\(_i\)) or 3 mM MgCl\(_2\) and PP\(_\text{P}\), and Pi were separated either by polyethyleneimine cellulose thin layer chromatography (1 M KH\(_2\)PO\(_4\)) or by isobutanol extraction of their molybdate complexes as described by Springs et al. (1981). One unit of PP\(_\text{ase}\) activity corresponds to 1 µmol of Pi, hydrolyzed in 1 min.

**Inhibition of ROS PP\(_\text{ase}\) by fluoride** was measured using the standard assay conditions and 0–10 mM NaF. Because imidodiphosphate interferes with the colorimetric assay, \([\beta\text{PPi}]\) was used for measuring inhibition of PP\(_\text{ase}\) by imidodiphosphate. Both imidodiphosphate and PP\(_\text{ase}\) concentrations were varied, using 1 mM Mg\(_2\), 0.2 mM Mg\(_2\), and 0.1 mM EDTA, 50 mM Tris-base (pH 7.5).

**Guanylyl Cyclase Assays**—Guanylyl cyclase activity was determined using the method of Koch and Stryer (1988) with the following modifications. ROS membranes stripped with low salt buffer to remove cGMP phosphodiesterase and recoverin were used as the source of enzyme. 2 mM Mg\(_2\) or 5 mM Mg\(_2\) was substituted for 12 mM Mg\(_2\), and isotubutylmethylxanthine and EGTA were not included, as they were found under these conditions not to increase cGMP production significantly. To examine the effect of PP\(_\text{ase}\) on guanylyl cyclase, PP\(_\text{P}\) was added to give a final concentration ranging from 0 to 12 mM in the case of Mg\(_2\) or 0 to 6 mM in the case of Mg\(_2\). Activity is reported as pmol of GMP synthesized as indicated to exceed an excess of divalent metal ions over PP\(_\text{ase}\).

**Determination of Free Ca\(^{2+}\)**—The concentration of free Ca\(^{2+}\) was determined using the indicator dye Fura-2 by the method of Grynkiewicz et al. (1985) using either an Amicon-Bowman spectrofluorimeter or a SLM 8000 spectrofluorimeter.

**Purification of PP\(_\text{ase}\)**—ROS extract from 50 retinas was diluted 2-fold with water and loaded onto a hydroxyapatite column (2.4 × 7 cm), which had been equilibrated with buffer A. The column was washed with 50 ml of buffer A and then with a linear 400-ml gradient from buffer A to buffer B, and the eluent was collected in 5-ml fractions. Fractions 35–52, which contained the peak of PP\(_\text{ase}\) activity, were pooled and concentrated to 5 ml with an ultrafiltration apparatus using Amicon YM-30 membranes. The sample was desalted by dilution to 50 ml with buffer C and repeated reconstitution (three times, final volume 3 ml) in the same apparatus, and then injected onto a weak anion exchange HPLC column (Waters Protein Pak DEAE 5 PW, 7.5 × 75 mm) and eluted with a 60-min linear gradient (1 ml/min) from buffer C to 10% buffer D, 90% buffer C, both at pH 8.0. The fractions containing the main peak of PP\(_\text{ase}\) activity were pooled, concentrated, and desalted as described above. The sample was then injected onto the same column and eluted using conditions identical to those for the first injection, except that the gradient was prolonged to 80 min. This concentration/desalting/injection procedure was repeated a third time, using an 80-min gradient from buffer C to 10% buffer D, both at pH 8.5. The PP\(_\text{ase}\)-containing fractions from the second chromatographic step were analyzed by SDS-polyacrylamide gel electrophoresis to examine the purity of the preparation. Reverse phase HPLC was carried out using a Vydac Protein C4 column and a linear gradient from 30% buffer F to 80% buffer F. Fractions were dried under vacuum, redissolved in PP\(_\text{ase}\) assay buffer, and assayed for PP\(_\text{ase}\) activity.

**Size of Native PP\(_\text{ase}\)**—Sucrose density centrifugation analysis was performed as described (Baehr et al., 1979). Crude PP\(_\text{ase}\) (ROS extracts, 500 µg of total protein), was applied to a linear gradient of 5–15% sucrose (w/v) in a total volume of 3 ml of ROS buffer, along with molecular weight marker proteins: hemoglobin, fluorescein isothiocyanate labeled carbonic anhydrase, and trypsin-activated phosphodiesterase (purified as described by Wensel and Stryer (1990)). After centrifugation at 400,000 × g for 3 h (Beckman TL-100 rotor), the gradient was collected in thirteen 220-µl fractions which were assayed for PP\(_\text{ase}\) activity, as well as for trypsin-activated phosphodiesterase (Liebman and Evanczuk, 1982; Wensel and Stryer, 1986), for 409-nm hemoglobin absorbance, and for carbonic anhydrase fluorescence.

**Electrophoresis**—SDS-polyacrylamide gel electrophoresis was carried out essentially according to the method of Laemmli (1970) using 12% acrylamide. Protein bands were visualized with Coomassie Blue stain.

**Results**

**PP\(_\text{ase}\) Activity in ROS**—The PP\(_\text{ase}\) activity detected in ROS varied from preparation to preparation, as might be expected for a soluble enzyme within a membrane organelle.
that is fairly leaky because of preparation from frozen retinas. The highest specific activity of PPase that we have observed in ROS-derived samples is 1.0 unit/mg rhodopsin in ROS suspensions, or 2.4 units/mg in extracts, but values as low as 0.7 unit/mg have also been observed in some extracts. Because PPase activity is ubiquitous, we were concerned initially that the activity observed could be due to contamination from other cells in the homogenate, even though the high level of activity suggested that contamination was not likely to be the major source of PPase. As a test of the origin of the activity, we prepared somewhat purer ROS using small scale sucrose gradient centrifugation according to the method of Papermaster and Dreyer (1974) and then centrifuged the purified ROS on a second sucrose gradient and analyzed the activity in each fraction. The results (Fig. 1) indicated that the enzyme copurified with rhodopsin, and not with soluble proteins nor with more rapidly sedimenting membranes. Although the ROS tend to be rather leaky under our assay conditions, we observed an increase (about 2-fold) in apparent specific activity when the membranes were sonicated, as expected for an enzyme trapped within membranes.

Because the PPase activity in ROS is soluble (approximately 20% of the activity remains on the membranes after washing in either isotonic or low salt buffers; data not shown) the activity copurifying with rhodopsin must be trapped within sealed membrane compartments. PPase activity is present in all the supernatants obtained at those stages of our standard ROS preparation at which sucrose is diluted to allow the ROS to sediment. This activity is presumably derived from other cells in the retina, as well as from leaky ROS. Importantly, the specific activity of PPase was always higher within sealed membrane compartments. PPase activity trapped within membranes.

Extracts of soluble proteins from other bovine tissues expressed lower levels of PPase mRNA present in retina as compared to brain (Yang and Wensel, 1992). The activity in liver extracts (0.36 unit/mg) was somewhat lower than in brain, but, as found in rodents (e.g. ~0.8 unit/mg in rat or mouse liver extracts (Shatton et al., 1981) and 0.3 unit/mg in mouse liver extracts (Irie et al., 1970)), much higher than the activity we found in extracts from bovine skeletal muscle (≤0.1 unit/mg). Thus, the level of PPase activity expressed in different bovine tissues can vary by more than 24-fold.

Dependence of Activity on Pyrophosphate and Divalent Cations—The [PPi] dependence of the initial hydrolytic velocity is shown in Fig. 2. In this experiment the calculated \( K_a \) was 1.47 \( \mu M \), and in a number of experiments values between 0.9 and 1.5 \( \mu M \) were consistently obtained, using either ROS extracts or highly purified PPase. This value is significantly lower than the \( K_a \) values reported for other PPases which range from 5 \( \mu M \) (Yoshida et al., 1982) to 700 \( \mu M \) (Chen et al., 1973), and it is also much lower than the value of 26 \( \mu M \) previously reported for ROS PPase (Hakki and Sitaramayya, 1990). The discrepancy in \( K_a \) for ROS PPase may be due to the reported use of concentrations of PPi between 50 \( \mu M \) and 1 mM for the \( K_a \) determinations in the previous study. When

![Fig. 1. Copurification of PPase and ROS on sucrose gradient.](image)

**Fig. 1.** Copurification of PPase and ROS on sucrose gradient. Rod outer segments prepared from bovine retinas by sucrose density gradient centrifugation were washed and applied to a second (identical) sucrose gradient. After centrifugation, fractions were collected and assayed for rhodopsin content (absorbance at 500 nm: open circles) and for pyrophosphatase activity, before (triangles) and after (filled circles) sonication. A value of 1 corresponds to 0.014 unit of activity.

![Fig. 2. PPi and Mg²⁺ dependence of PPase activity.](image)

**Fig. 2.** PPi and Mg²⁺ dependence of PPase activity. The initial velocity of PPi hydrolysis by an ROS extract was measured using \[^{32}P\]PPi. The results were fit to the equation \( V = V_{max} [PPi] / (K_{m} + [PPi]) \), using a nonlinear least squares routine to determine both \( V_{max} \) and \( K_m \). The theoretical curve drawn is for the best fit values of \( K_m = 1.47 \mu M \) and \( V_{max} = 1.34 \) unit/mg. Inset: Activation of ROS PPase by Mg²⁺. PPase activity of an ROS extract was measured by the colorimetric assay using 3 mM PPi, and the indicated values of [Mg²⁺].
we measured the $K_m$ in extracts of bovine liver, values of 2–2.5 $\mu$m were observed.

When the effects of cations were tested, using 3 mM PPi as substrate and 3 mM added cation, it was found that Mg$^{2+}$ confers maximal activity (100%) on ROS PPase. Some activity is also found in the presence of the same concentration of Ca$^{2+}$ (14.2%), Al$^{3+}$ (9.9%), Ni$^{2+}$ (8.3%), Zn$^{2+}$ (7.3%), Cu$^{2+}$ (7.0%), or Mn$^{2+}$ (6.6%). Similar results have been reported for other PPases such as those from yeast (Butler and Sperow, 1977; Janson et al., 1979) and pig cartilage (Felix and Fleisch, 1975). Since Mg$^{2+}$ has maximum potential to activate the enzyme and is the probable cofactor in vivo, we further investigated the Mg$^{2+}$ concentration dependence of this activation, as shown in Fig. 2 (inset). At 3 mM PPi, with varying MgCl$_2$ concentrations, PPase had maximal activity at about 3 mM Mg$^{2+}$ and declined in activity at Mg$^{2+}$/PPi ratios greater than 1. This result is consistent with the finding for other PPases that both MgPP$_i$ and Mg$_2$PP$_i$ complexes are substrates, with a slightly lower maximal velocity for the dimagnesium species (Moe and Butler, 1972a; Felix and Fleisch, 1975; Pyne and Younathan, 1967).

**Inhibition by Ca$^{2+}$—**Submillimolar levels of EGTA enhanced the activity of PPase in ROS approximately 8-fold (data not shown). This effect may be partly due to removal of Ca$^{2+}$, and partly due to removal of trace heavy metal ions which also bind EGTA. Inhibition of the ROS PPase-activated enzyme by addition of Ca$^{2+}$ confirmed that Ca$^{2+}$ does inhibit ROS PPase. Fig. 3 shows the [Ca$^{2+}$] dependence of this inhibition, determined by using varying ratios of Ca$^{2+}$ to EGTA and measuring free [Ca$^{2+}$] with the indicator dye fura-2. The maximal activity of CaPP$_i$ under the latter condition is estimated to be ~70% that of free Ca$^{2+}$, while the concentration of MgPP$_i$ is slightly less than 2 mM. Inhibition by Ca$^{2+}$ seems to be an intrinsic property of the enzyme, as Ca$^{2+}$ effects were not significantly different with highly purified ROS PPase as compared to crude extracts of soluble proteins. When Ca$^{2+}$ sensitivity of PPase was examined in crude extracts of soluble proteins from other bovine tissues, it was found to be similar, although at every Ca$^{2+}$ concentration examined, inhibition of ROS PPase was slightly greater (data not shown).

**Anionic Inhibitors of PPase—**Imidodiphosphate, a nonhydrolyzable PP-analogue, was found to be a potent inhibitor of ROS PPase. Fig. 4A shows a Dixon (1953) plot of the inverse velocity as a function of imidodiphosphate concentration at three different substrate concentrations. The results indicate that inhibition is competitive with $K_i = 1.2 \mu$m.

Fluoride, an anion commonly used to stimulate G-proteins (including transducin) and adenylyl cyclase, is an inhibitor of many PPases (Felix and Fleisch, 1975). We have found that it inhibits ROS PPase with half-maximal inhibition at 20 $\mu$m (Fig. 4B), a much lower concentration of F$^{-}$ than is normally used for G-protein stimulation.

**Inhibition of Guanylyl Cyclase by Pyrophosphate—**PP$\alpha_i$ was an effective inhibitor of guanylyl cyclase when either Mg$^{2+}$ or Mn$^{2+}$ was used as cofactor, and the inhibition could not be explained simply by competition for metal ion cofactor (Fig. 5). This result confirms two previous reports that PP$\alpha_i$ inhibits ROS guanylyl cyclase. One (Hakki and Sitaramayya, 1990) reported competitive inhibition with $K_i = 30–45 \mu$m. Our results are not extensive enough to distinguish between the two types of inhibition but are consistent with competitive inhibition and a $K_i$ of 100 $\mu$m, assuming $K_m = 250 \mu$m for GTP (Koch, 1991; Hakki and Sitaramayya, 1990). We have also found that imidodiphosphate can directly inhibit guanylyl cyclase, but with much lower potency. Cyclase was 50% inhibited at 3–4 mM imido-

![Fig. 3. Inhibition of ROS PPase by Ca$^{2+}$.](image)

![Fig. 4. Inhibition of PPase by imidodiphosphate and fluoride.](image)
diphosphate (data not shown). ATP has been reported to inhibit ROS guanylyl cyclase (Sitaramayya et al., 1991), and we have confirmed this result as well (data not shown), in contrast to another report (Hayashi and Yamazaki, 1991) that ATP is not inhibitory.

**Purification of ROS PPase**—We have tried a number of different procedures for purification of PPase extracted from ROS and have arrived at a scheme involving: 1) isotonic extraction of homogenized ROS, 2) chromatography on hydroxylapatite, and 3) three rounds of anion exchange HPLC using different gradients or pH conditions each time (Table I). Procedures used for purification of other PPases including ammonium sulfate precipitation (Irie et al., 1970) and ω-aminohexyl agarose chromatography (Yoshida et al., 1982) gave very poor recovery of activity. The protein obtained using the procedure outlined in Table I was typically 70%-95% pure, as estimated from Coomassie Blue staining of gels. In the purest preparations the specific activity was >885 units/mg. This specific activity is comparable to that of PPase purified from pig brain (93 units/mg, Hachimori et al., 1983) which has the highest specific activity reported for any PPase. This high specific activity rules out the possibility that the enzymatic activity observed is due to a minor protein not visible on stained gels. Fig. 5 shows the profiles of PPase activity and absorbance at 280 nm of the third DEAE-HPLC column as well as SDS-polyacrylamide gel electrophoresis of the activity peak fractions. Only one major band on the Coomassie Blue-stained gels correlates well with the relative activity in each fraction, so that we can identify this 36-kilodalton band as corresponding to PPase. Traces of a protein with an apparent molecular mass of 65-kDa were occasionally detected in the peak PPase fractions, but its presence or absence did not correlate well with PPase activity, and it is unlikely that such a small amount of protein could account for the observed activity. It may represent two covalently linked PPase subunits. A minor diffuse band migrating just below the main PPase band was also frequently observed. The intensity of this band increased with time after purification, so it is probably a fragment of the PPase subunit produced by proteolytic degradation. For analytical purposes, we chromatographed some of the partially purified PPase on a reverse phase (C-4) column. While most of the enzymatic activity was lost following denaturation by the reverse phase solvents (isopropanol, acetonitrile, and trifluoroacetic acid), enough was recovered upon addition of aqueous buffers to the dried fractions that they could be tested for activity. Only the fractions containing the 36-kDa band had detectable PPase activity (data not shown).

**Size of Native PPase**—To determine the native size of ROS PPase, sucrose density gradient centrifugation was employed, because PPase appeared to bind to two different HPLC gel filtration columns tried, eluting at a volume consistent with a 45-kDa protein (Superose 12) or a 12-kDa protein (Biosil TSK-250). Yeast PPase, known to be a homodimer of 32-kDa subunits, eluted slightly after the ROS PPase on these columns. Results of the sedimentation velocity experiment are shown in Fig. 7 and indicate a molecular mass of approximately 70 kDa, assuming a globular shape, consistent with ROS PPase being a homodimer of 36-kDa subunits. The distribution of activity in the gradient is broad enough, with enough of a shoulder below the main peak in the gradient, that we cannot rule out the possibility that some higher order aggregates (trimers and tetramers) may be present as well. The mobility of yeast PPase relative to the same standards on the same kind of gradient was consistent with its known molecular weight.

**DISCUSSION**

While the PPase activity level we observed (up to 2.4 units/mg soluble protein) in ROS extracts is higher than found in any other mammalian tissue studied, the actual activity inside intact ROS is probably even higher. While our preparations were all derived from frozen retinas, so that a significant fraction of soluble ROS proteins was lost, when PPase activity was measured in ROS prepared from retinas that had not been frozen, and therefore should retain a higher proportion of soluble proteins (Hakki and Sitaramayya, 1990), approximately 2-fold higher specific activities were found (2.2 units/mg rhodopsin; the activity of soluble extracts was not reported). Thus bovine rod outer segments contain significantly

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**TABLE I**

| Purification of ROS PPase |
|---------------------------|
| Step                      | Total protein | Total activity | Specific activity | Recovery | Purification |
|----------------------------|---------------|----------------|-------------------|----------|--------------|
|                            | mg            | units          | units/mg          | %        | -fold        |
| ROS supernatant            | 75.3          | 102            | 1.36              | 100      | 1            |
| Hydroxylapatite           | 13.4          | 75             | 5.6               | 73       | 4            |
| First DEAE-HPLC (pH 8.0)  | 0.262         | 58             | 222               | 57       | 163          |
| Second DEAE-HPLC (pH 8.0) | 0.067         | 39.4           | 588               | 39       | 434          |
| Third DEAE-HPLC (pH 8.5)  | <0.024        | 21.4           | >885              | 21       | >653         |

**Fig. 5. Inhibition of ROS guanylyl cyclase by pyrophosphate.** Guanylyl cyclase activity was measured in washed ROS membranes using 2 mM GTP and either Mg\(^{2+}\) (A) or Mn\(^{2+}\) (B) as cofactor at the indicated concentrations. PPi was added to the indicated concentrations.
Fig. 6. Anion exchange HPLC of ROS PPass. The absorbance profile at 280 nm and the PPass activity (filled circles) of corresponding 1-ml fractions. Inset, Coomassie Blue staining pattern following SDS-gel electrophoresis of the indicated fractions (numbers above the lanes). Positions are indicated for the following molecular mass standards, applied in an adjacent lane: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and soybean trypsin inhibitor. The position of yeast PPass, applied in an adjacent lane, is shown by the arrow.

Fig. 7. Sucrose density centrifugation analysis of PPass size. An ROS extract and marker proteins were centrifuged on a sucrose gradient as described in the text. A, protein assay results for each fraction plotted as a fraction of the value measured in the peak fraction for each protein. Open circles, cGMP phosphodiesterase (200 kDa) activity; open triangles, hemoglobin (64 kDa) absorbance at 409 nm; filled triangles, fluorescence of fluorescein isothiocyanate-labeled carbonic anhydrase (30 kDa); filled circles, PPass activity. B, plot of log M, values as a function of peak fraction for standards, and for PPass M, values expected for different aggregation states (monomer-tetramer) of the 36-kDa PPass subunit. PDE, cGMP phosphodiesterase; CA, carbonic anhydrase; Hb, hemoglobin.

higher levels of cytoplasmic PPass than has been found in any other mammalian tissue.

It seems likely that the very high level of PPass activity in ROS is related to their extraordinarily high levels of cGMP metabolism (Fig. 8). Even in the dark, production of 29 µM cGMP s⁻¹ could lead in less than 1 min to accumulation of sufficient PPi to inhibit guanylyl cyclase. However, the ROS PPass activity measured in vitro corresponds to hydrolysis inside ROS of 3–9 mM PPi, s⁻¹ at saturating [MgPPi] (assum- ing 3 mM rhodopsin) or to a PPi half-life of 77–350 µs under substrate-limited conditions. This should be more than enough activity to keep up with guanylyl cyclase, even when cGMP production increases 4-fold in the light.

With such high levels of PPass activity, it seems unlikely that PPi, or PPass plays a regulatory role unless under some conditions the activity in vivo is much less than estimated by extrapolation from in vitro measurements. One possible mechanism for lowering PPass activity is inhibition by Ca²⁺, which would allow for stimulation of PPass along with guanylyl cyclase when Ca²⁺ levels are reduced after exposure to light. Preliminary studies of the effects of Mg²⁺ and PPi on Ca²⁺ inhibition² suggest that the mechanism for inhibition may be similar to that found with yeast PPass (Ridlington and Butler, 1972; Moe and Butler, 1972b). In that case the principal inhibitory species is actually CaPPi, which binds tightly to PPass (Kd = 7 nM for CaPPase and Ke = 100 nM for Mg-PPase), and Ca²⁺ and CaPPi appear to compete with Mg²⁺ and MgPPi, respectively, for binding to sites essential in catalysis. While our results are fairly consistent with the effects of Ca²⁺ on yeast PPass and on rat mitochondrial soluble PPass (Davidson and Halestrap, 1989), our observation of inhibition at submicromolar Ca²⁺ levels contrasts with other reports for mammalian PPass (Baykov et al., 1983; Yoshida et al., 1982; Hachimori et al., 1983) that indicated much higher concentrations of Ca²⁺ were needed for inhibition. Although it is possible that there are species- and tissuespecific differences in Ca²⁺ sensitivity, it is also possible that the discrepancy is due to the fact that previous reports were based on total Ca²⁺ added rather than on free Ca²⁺. The similarity of the Ca²⁺ sensitivity of PPass in bovine liver and ROS extracts argues against any large tissue-specific differences in calcium sensitivity. Because the inhibitory potency of Ca²⁺ is a function of [PPi] and [Mg²⁺], further studies of the quantitative dependence of Ca²⁺ inhibition on the concentrations of these ions, as well as better estimates of their free concentrations in ROS, will be necessary to determine whether Ca²⁺ regulation of PPass has any functional significance in ROS.

An unresolved question is whether the ROS PPass is specific for photoreceptors or some larger set of cell types, or is instead identical to a single type of cytoplasmic PPass expressed in all bovine tissues. A number of the properties of the bovine ROS enzyme are somewhat different from those previously reported for mammalian PPasses, including its lower Km and its greater Ca²⁺ sensitivity. It is not clear, however, to what extent these differences reflect species differences or different assay methods, as opposed to properties unique to PPass expressed in ROS. Our studies on PPass in other bovine tissues suggest that, while levels of activity expressed vary widely, differences in kinetic properties (e.g. Km and Ca²⁺ sensitivity) if any, may be fairly subtle. One study (Baykov et al., 1989) on bovine heart mitochondrial PPass indicated that its dependence on [PPi] and its inhibition by [Ca³⁺] appear to be quite different from those of the ROS enzyme. There have been reports from three groups

² Z. Yang and T. G. Wensel, unpublished results.

Anion exchange HPLC of ROS PPass. The absorbance profile at 280 nm and the PPass activity (filled circles) of corresponding 1-ml fractions. Inset, Coomassie Blue staining pattern following SDS-gel electrophoresis of the indicated fractions (numbers above the lanes). Positions are indicated for the following molecular mass standards, applied in an adjacent lane: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and soybean trypsin inhibitor. The position of yeast PPass, applied in an adjacent lane, is shown by the arrow.

Sucrose density centrifugation analysis of PPass size. An ROS extract and marker proteins were centrifuged on a sucrose gradient as described in the text. A, protein assay results for each fraction plotted as a fraction of the value measured in the peak fraction for each protein. Open circles, cGMP phosphodiesterase (200 kDa) activity; open triangles, hemoglobin (64 kDa) absorbance at 409 nm; filled triangles, fluorescence of fluorescein isothiocyanate-labeled carbonic anhydrase (30 kDa); filled circles, PPass activity. B, plot of log M, values as a function of peak fraction for standards, and for PPass M, values expected for different aggregation states (monomer-tetramer) of the 36-kDa PPass subunit. PDE, cGMP phosphodiesterase; CA, carbonic anhydrase; Hb, hemoglobin.

Proposed role of PPass in ROS cGMP metabolism. PPass removes PPi produced from GTP by guanylyl cyclase, preventing inhibition of cyclase by PPi. Reduction of [Ca²⁺] in response to light may act to stimulate PPass in addition to its stimulatory effects on cyclase.

FIG. 6. Anion exchange HPLC of ROS PPass. The absorbance profile at 280 nm and the PPass activity (filled circles) of corresponding 1-ml fractions. Inset, Coomassie Blue staining pattern following SDS-gel electrophoresis of the indicated fractions (numbers above the lanes). Positions are indicated for the following molecular mass standards, applied in an adjacent lane: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and soybean trypsin inhibitor. The position of yeast PPass, applied in an adjacent lane, is shown by the arrow.

FIG. 7. Sucrose density centrifugation analysis of PPass size. An ROS extract and marker proteins were centrifuged on a sucrose gradient as described in the text. A, protein assay results for each fraction plotted as a fraction of the value measured in the peak fraction for each protein. Open circles, cGMP phosphodiesterase (200 kDa) activity; open triangles, hemoglobin (64 kDa) absorbance at 409 nm; filled triangles, fluorescence of fluorescein isothiocyanate-labeled carbonic anhydrase (30 kDa); filled circles, PPass activity. B, plot of log M, values as a function of peak fraction for standards, and for PPass M, values expected for different aggregation states (monomer-tetramer) of the 36-kDa PPass subunit. PDE, cGMP phosphodiesterase; CA, carbonic anhydrase; Hb, hemoglobin.

FIG. 8. Proposed role of PPass in ROS cGMP metabolism. PPass removes PPi, produced from GTP by guanylyl cyclase, preventing inhibition of cyclase by PPi. Reduction of [Ca²⁺] in response to light may act to stimulate PPass in addition to its stimulatory effects on cyclase.
photoreceptor inorganic pyrophosphatase

(Thullier, 1978; Fisher et al., 1974a; Pynes and Younathan, 1967) that the subunits of human erythrocyte PPase are 20–23 kDa, implying significant structural differences between this enzyme and other eukaryotic PPases, all of which have been reported to have subunits of 30–36 kDa. Using the technique of enzyme assays in starch gels, humans and a number of other mammals have been found to have multiple (two to six) electrophoretically distinct PPase isozymes (Fisher et al., 1974b). This technique apparently did not detect a distinct mitochondrial enzyme in humans, although a distinct nuclear gene encoding mitochondrial PPase is presumably present in animals as it is in Saccharomyces cerevisiae (Lundin et al., 1991). It seems likely that mammals in general have more than one functionally important isozyme of PPase.

We have detected multiple chromatographically distinct forms of PPase in extracts of total bovine retina but have not yet determined their cellular origins nor compared them functionally to the ROS enzyme. While we have been able to obtain partial amino acid sequence data for one of these isozymes (see our companion study, Yang and Wensel, 1992), it seems likely that mammals in general have more than one functionally important form of PPase.

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