Cyclic Nucleotide Dependent Protein Kinase and the Phosphorylation of Endogenous Proteins of Retinal Rod Outer Segments†

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ABSTRACT: Cyclic nucleotide dependent protein kinase has been extracted with Tris or Lubrol PX from purified rod outer segments (ROS) of bovine retina. The activity of the enzyme is unaffected by light but is stimulated by either cyclic guanosine 3′,5′-monophosphate (cGMP) or cyclic adenosine 3′,5′-monophosphate (cAMP). Most of the solubilized enzyme elutes from DEAE-cellulose with about 0.18 M NaCl (type II protein kinase). An endogenous 30 000 molecular weight protein of the soluble fraction of ROS as well as exogenous histone are phosphorylated by the protein kinase in a cyclic nucleotide dependent manner. The Tris-extracted enzyme can be reassociated in the presence of Mg^{2+} with ROS membranes that are depleted of protein kinase activity. The reassociated protein kinase is insensitive to exogenous cyclic nucleotides, and it catalyzes the phosphorylation of the membrane protein, bleached rhodopsin. While the soluble and membrane-associated protein kinases may be interchangeable, they appear to be modulated by different biological signals; soluble protein kinase activity is increased by cyclic nucleotides whereas membrane-bound activity is enhanced when rhodopsin is bleached by light.

The visual response of rod photoreceptor cells is initiated when light absorbed by the visual pigment, rhodopsin, isomerizes its 11-*cis*-retinal chromophore to the all-trans configuration. In conjunction with this isomerization, the permeability of the rod outer segment (ROS)[1] plasma membrane to sodium is reduced, resulting in hyperpolarization of the photoreceptor cell. The mechanisms that link rhodopsin photochemistry in the membrane of the ROS disk to receptor potential changes in the ROS plasmalemma are still unresolved. However, biochemical and physiological findings suggest that cGMP acting alone or in conjunction with Ca^{2+} may modulate ion permeability of the ROS plasmalemma. Dynamic changes in cGMP levels upon exposure to light suggest further that cGMP-mediated biochemical reactions may link the bleaching of rhodopsin to light-induced polarization changes in the ROS. The following observations are particularly relevant in establishing cGMP as a possible component of the visual transducer mechanism:

(1) Dark-adapted ROS contain a high concentration of cGMP and lower levels of cAMP (Krishna et al., 1976).

(2) Low intensity of illumination causes a rapid (100–300 ms) decrease in cGMP content, with $1-2 \times 10^3$ molecules of cGMP hydrolyzed per molecule of rhodopsin bleached; cAMP levels are unchanged (Woodruff et al., 1977; Fletcher & Chader, 1976).

(3) Bleaching of rhodopsin activates, six- to tenfold, the cyclic nucleotide phosphodiesterase of ROS (Miki et al., 1973).

(4) cGMP modulates the activity of a soluble protein kinase from ROS, which phosphorylates preferentially one endogenous protein (mol wt 30 000) in a cyclic nucleotide dependent manner (Lolley et al., 1977).

(5) Brief exposure of isolated retinas to dibutyryl-cGMP or methylisobutylxanthine (MIX) causes depolarization of the membrane potential of the visual cells, an increase in response amplitude and some changes in wave form; but, under dark-adapted or partially light-adapted conditions, receptor sensitivity is virtually unaffected (Lipton et al., 1977).

In living animals or in isolated ROS (Kühn et al., 1977), phosphorylation of bleached visual pigment by ATP or GTP is catalyzed by a protein kinase that is associated with ROS membranes (Chader et al., 1975, 1976; Weller et al., 1976). The activity of this enzyme is independent of cyclic nucleotides (Goridis & Weller, 1976; Chader et al., 1975; Weller et al., 1975; Pannbacker, 1973; Frank & Bensinger, 1974). During the bleaching process, the conformation of the retinal–opsin complex is apparently modified so that the protein moiety is an acceptable substrate for phosphorylation (Goridis & Weller, 1976; Frank & Buzney, 1975); the protein kinase does not appear to be affected by illumination (Goridis et al., 1976). The phosphorylation of bleached rhodopsin and its slow dephosphorylation in the dark by endogenous phosphoprotein phosphatase implicate these phosphorylation/dephosphorylation processes in the visual mechanisms of light/dark adaptation (Kühn et al., 1977; Goridis & Weller, 1976).

In this paper, we present evidence that the soluble, cyclic nucleotide dependent protein kinase of ROS is activated by either cGMP or cAMP and is unaffected by light. The soluble protein kinase can be reassociated with protein kinase-depleted ROS membranes and, associated with the membranes, it phosphorylates bleached rhodopsin in a cyclic nucleotide independent manner. Our observations suggest that the activity of soluble and membrane-associated protein kinase of ROS may be modulated independently by cyclic nucleotides and light.

Materials and Methods

Preparation of ROS from Bovine Retina. Fresh bovine eyes were obtained from Globe Packing Co., San Fernando, Calif., immediately placed on ice, and dark-adapted for at least 60 min before dissection. Thereafter, all procedures for the isolation of ROS were carried out under darkroom conditions,

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[1] Abbreviations used: ROS, rod outer segment(s); cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; PDE, phosphodiesterase; MIX, methylisobutylxanthine; $NaDodSO_4$, sodium dodecyl sulfate.

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as previously described (Lolley et al., 1977). Briefly, 30 retinas were suspended in 60 mL of 1.13-density sucrose containing 5 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 62 mM NaCl, shaken vigorously 15 times and spun for 12 min at 10000*g*. The supernatant was separated, diluted with 1.5 volumes of 50 mM Tris buffer, pH 7.6, containing 5 mM $MgCl_2$, and centrifuged at 27000*g* to pellet the ROS.

Soluble and Particulate Fractions from ROS. The ROS pellet was resuspended in 4 mL of 50 mM Tris, pH 7.6, and the suspension was frozen and thawed, followed by homogenization and subsequent centrifugation at 100000*g* for 60 min. The Tris–supernatant and –particulate fractions were separated and stored frozen in the dark at −70 °C. To test the influence of illumination on the samples, they were exposed, after thawing, to laboratory fluorescent light.

For some experiments, ROS were extracted with 0.1% Lubrol PX and centrifuged at 100000*g*. It is important that the Tris– and Lubrol PX–supernatant fractions be assayed as quickly as possible and certainly within 2 to 3 days of preparation.

Preparation of "Purified Rhodopsin" Membranes. The procedure of Weller et al. (1975) for preparing "purified rhodopsin" membranes was followed without modification; all centrifugation steps were carried out at 100000*g*. The pellet remaining after extraction with Tris buffer was extracted twice with 0.1% sodium dodecyl sulfate ($NaDodSO_4$) in 10 mM Tris, pH 7.0. The resulting pellet was washed three times with 66 mM sodium phosphate buffer, pH 7.0. This procedure removed almost all of the protein kinase from the membranes and left opsin as the major protein in the preparation, as revealed by polyacrylamide gel electrophoresis. When required, a sample was bleached by laboratory lighting.

Preparation of Supernatant Fraction from Rat Muscle. Skeletal muscle from an adult Fischer rat was homogenized (5 g/37.5 mL of 50 mM Tris buffer, pH 7.6) in a Waring blender for 2 min at low speed. The suspension was filtered through two layers of cheesecloth and spun at 27000*g* for 20 min. The separated supernatant was spun at 100000*g* for 60 min, and the soluble fraction was separated and stored frozen at −70 °C.

Determination of Protein Kinase Activity. The protein kinase reaction was carried out with or without added cyclic nucleotides in a total volume of 110 μL containing 50–100 μg of protein (measured by the method of Lowry et al., 1951) in 50 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, and 0.6 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP (2–3 × 10^6 cpm/tube; New England Nuclear). When exogenous substrate was used, histone (calf thymus, Type II-S, Sigma; 75 μg/tube) was added; otherwise, the endogenous proteins of the different samples were the substrates of the reaction. Where indicated, MIX (1 mM) was added to the reaction mixture. All steps were carried out in dim red light. After incubation at 30 °C for 5 min, the reaction was terminated by adding 1 mL of cold 20% trichloroacetic acid. The precipitated protein containing incorporated ^{32}P was spun at 3500*g* and the supernatant fraction decanted. The precipitate was resuspended in 0.5 mL of 20% trichloroacetic acid, collected on glass fiber filters (Whatman GF/C), washed with 10 mL of 20% trichloroacetic acid, followed by 10 mL of chloroform–methanol (2:1), and counted using PCS:xylene scintillation cocktail (2:1; Amersham Corp.). Blanks were prepared by adding trichloroacetic acid to the tube prior to the addition of sample.

When detergent was present in the reaction mixture, the material precipitated with trichloroacetic acid stuck to the glass tubes. In these cases, after the precipitation, tubes were spun at 3500*g* and the supernatant fraction was decanted. The pellets were washed with 1 mL of 20% trichloroacetic acid and recentrifuged, repeating the procedure five times. The final pellet was dissolved in 0.5 mL of BBS-2 solubilizer (Beckman) and counted after addition of PCS:xylene scintillation cocktail.

To determine the cyclic nucleotide activation of the soluble protein kinase, cAMP and cGMP were added to the reaction mixture in concentrations ranging from 10^{-8} to 10^{-3} M.

Phosphate Incorporation into Protein. Experiments were carried out to determine whether the phosphorylation reaction involved formation of acyl or of ester bonds. The Tris–supernatant fraction was incubated with 5 mM $MgCl_2$ and 1 μM [^{32}P]ATP (2.5 × 10^6 cpm) in 50 mM Tris buffer, pH 7.6, at 30 °C for 15 min. The reaction was stopped with 1 mL of 10% trichloroacetic acid. The precipitate, separated after centrifugation, was suspended in 1 mL of 0.8 M hydroxylamine hydrochloride in 0.1 M sodium acetate buffer, pH 5.3. A blank was prepared by replacing hydroxylamine with 0.8 M NaCl. The tubes were incubated at 30 °C for 30 min and then 0.33 mL of ice-cold 50% trichloroacetic acid was added. After 30 min on ice, the tubes were centrifuged at 3500*g* and processed as usual for scintillation counting.

In addition, the protein kinase reaction was carried out with retinal proteins of the 100000*g* supernatant fraction in the presence of 10^{-5} M cAMP, and it was stopped with $NaDodSO_4$–β-mercaptoethanol (3.3% final concentration of each). Pronase (0.04 mg; Sigma) was added, and the mixture was incubated overnight at room temperature. Treated samples and controls (no Pronase) were applied to polyacrylamide gels, electrophoresed as described below, and processed for protein staining and for counting of radioactivity.

Measurement of Protein Dephosphorylation. The 100000*g* supernatant fraction of Tris-extracted ROS was incubated under the conditions described for the protein kinase reaction. After 7 min at 30 °C, an aliquot was precipitated with trichloroacetic acid; the rest of the reaction mixture was passed through a Sephadex G-200 column (1.5 × 14 cm) eluted with 50 mM Tris buffer, pH 7.6, and 1-mL fractions were collected. Aliquots from each fraction were counted for radioactivity. This procedure separated the [^{32}P]ATP from the phosphorylated proteins. The fractions containing the radioactive proteins were pooled, and one aliquot was precipitated with trichloroacetic acid in order to determine the level of ^{32}P in protein at the start of the dephosphorylation experiment. The remaining sample was quickly divided into two fractions. To one of them, cAMP was added to reach a final concentration of 10^{-3} M. The fractions were incubated at 23 °C and aliquots from each fraction were removed at different times, precipitated with trichloroacetic acid, and processed as usual for scintillation counting.

DEAE-Cellulose Chromatography. The Tris–supernatant fraction of ROS containing about 2 mg of protein was applied to a column (3 × 0.7 cm) of DEAE-cellulose (Cellex D; Bio-Rad Laboratories). The column had been treated previously with bovine serum albumin, washed thoroughly with 1 M NaCl in 50 mM Tris buffer, pH 7.6, and equilibrated with 50 mM Tris buffer. After application of the sample, the column was washed with 5 mL of buffer, and a linear gradient of NaCl (30 mL, 0–0.4 M) in the same buffer was started. Fractions (1.0 mL) were collected and each one was assayed for basal and cAMP- (10^{-5} M) and cGMP- (10^{-4} M) activated protein kinase activities.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of Endogenous Proteins. The phosphorylation reaction was carried out as described above, and it was terminated by the addition

of $NaDodSO_4$ and β-mercaptoethanol (final concentration of 1% for each). The mixture was incubated at 37 °C for 2 h and, subsequently, the proteins were separated as described earlier (Farber & Lolley, 1973) by electrophoresis on 10% polyacrylamide gels. Gels were stained with Coomassie brilliant blue and, after scanning at 570 nm on a Gilford spectrophotometer, were cut into 1.5-mm slices and counted using PCS:xylene scintillation cocktail.

In some experiments, electrophoresis was carried out on 8.34% polyacrylamide gel slabs, containing 0.1% $NaDodSO_4$ according to the method of Laemmli (1970). The phosphorylation reaction was terminated by addition of 20% trichloroacetic acid. The precipitate was washed twice with 5% trichloroacetic acid and three times with water; it was then suspended in 60 mM Tris buffer, pH 6.8, containing 3% $NaDodSO_4$, 0.7 M β-mercaptoethanol, and 10% glycerol, and dissolved by heating at 90 °C for 10 min. After electrophoresis, the gels were fixed in a mixture of 3.5% sulfosalicylic acid, 11.5% trichloroacetic acid, and 45% methanol for 30 min. Thereafter, they were stained with 0.5% Amido black in methanol–acetic acid for 30 min, destained electrophoretically, and dried. Autoradiography was carried out on Du Pont Cronex X-ray film that was exposed for 14 days.

Gel Filtration of Endogenous Proteins. The protein kinase reaction was carried out using the Tris–supernatant fraction of ROS (about 1 mg of protein). Without stopping the reaction, the total volume of 1.1 mL was applied to a Sephadex G-200 column (1.6 × 80 cm), which had been equilibrated with 50 mM Tris buffer, pH 7.6. Proteins were eluted using the same buffer. Fractions (2 mL) were collected and 100-μL aliquots counted for radioactivity. Fractions corresponding to separated peaks of radioactivity were pooled and concentrated on an Amicon MMC ultrafiltration system using UM20E membranes. Calibration of the column for molecular weight determination was carried out using aldolase (158 000), ovalbumin (45 000), and ribonuclease A (13 700).

cGMP Phosphodiesterase. Activity was measured as reported previously (Lolley & Farber, 1975).

Results

Extraction of Protein Kinase from ROS. We have reported previously that about 10–15% of the total protein kinase of dark-adapted ROS is present in the 50 mM Tris extracts of ROS and that further extractions with Tris buffer do not release significant additional protein kinase from the ROS membranes (Lolley et al., 1977).

Detergents were tested for their ability to solubilize protein kinase of ROS membranes; we found Lubrol PX to be the most effective. Lubrol PX (0.1%) solubilizes 50% of protein kinase, and this concentration of the detergent was used routinely for extracting ROS membranes. However, soluble protein kinase of Tris extracts of ROS made 0.1% with Lubrol PX was inhibited 20–40% by the detergent. With higher concentrations of Lubrol PX, extraction of the enzyme and inhibition of protein kinase both increased. From measured activity of protein kinase in extracted ROS membranes and from activities corrected for inhibition of Lubrol PX supernatants, it was estimated that about 90% of the total protein kinase of ROS is solubilized with 0.5% Lubrol PX. Lubrol WX (0.1%) or $NaDodSO_4$ (0.01%) solubilizes only 10% and 6%, respectively, of the total protein kinase (basal activity) of isolated ROS.

The concentration of divalent cations in the suspending medium influences to some degree the extraction of protein kinase from ROS membranes. For example, when aliquots of Tris-extracted ROS pellet are resuspended in 50 mM Tris, pH 7.6, containing 0.1 mM EDTA, about 10–15% of additional protein kinase is found in the 100000*g* supernatant fraction. With 1.0 mM EDTA, the apparent amount of protein kinase removed from the membranes doubled, but higher concentrations of EDTA did not facilitate further extraction of the enzyme.

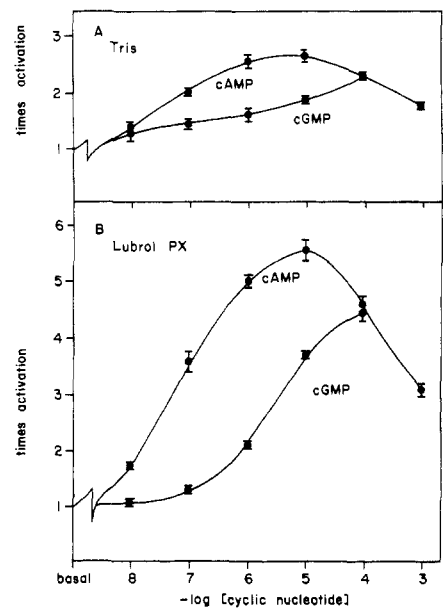

FIGURE 1: Cyclic nucleotide activation of soluble protein kinase of ROS. Protein kinase activity was determined in the presence of MIX (1 mM) as described in Materials and Methods, using either histone or soluble endogenous proteins as substrates. The values represent the mean ±SE of eight to ten individual assays. (A) Activation of protein kinase from Tris extracts of ROS. (B) Activation of Lubrol-solubilized protein kinase; ROS were extracted directly with 0.1% Lubrol PX without prior treatment with 50 mM Tris.

Characteristics of the Phosphorylation Reaction. Protein kinase of ROS catalyzes the phosphorylation of histone or endogenous proteins. Using either substrate and tissue concentrations of 150 μg of protein/tube, the incorporation of ^{32}P by the membranous or soluble fractions of ROS into components precipitable with trichloroacetic acid was linear for 5 min (cf. Figure 2). Treatment of these precipitates with chloroform–methanol (2:1) or hydroxylamine did not remove ^{32}P, excluding the incorporation of radioactivity into lipids or the formation of an acyl phosphate bond.

Pronase digestion of the products of the protein kinase reaction indicated that phosphate was incorporated into protein since, after electrophoresis on polyacrylamide gel, no detectable bands were obtained when staining with Coomassie brilliant blue, nor was radioactivity measured in the serial slices of the gel.

(1) *Cyclic Nucleotide Activation.* Tris- or Lubrol PX-solubilized protein kinase is activated by either cAMP or cGMP, whereas the enzyme associated with the membranes is cyclic nucleotide insensitive (Lolley et al., 1977). It is important to note that the reported values for cyclic nucleotide activation were obtained within a few hours after preparation of the sample. We observed that storage for 24 h at −70 °C reduced to about one-third the effect of cyclic nucleotides on the soluble kinase and, after 3 days, the enzyme was nearly unresponsive to cyclic nucleotides.

The activation of Tris- or Lubrol-solubilized protein kinase by cGMP or cAMP is shown in Figure 1. Protein kinase activity with histone or endogenous proteins of the Tris extract was enhanced by cyclic nucleotides. The rates of phosphorylation with histone or endogenous proteins differed (see Table

Table I: Differential Activation of Membrane and Supernatant Protein Kinase of ROS by Cyclic Nucleotides and Light[a]

	dark		light	
	histone	endogenous	histone	endogenous
membrane protein kinase				
basal	0.267 ± 0.012	0.033 ± 0.007	0.392 ± 0.004	0.276 ± 0.002
cAMP activated	0.278 ± 0.003	0.031 ± 0.01	0.391 ± 0.019	0.274 ± 0.004
cGMP activated	0.284 ± 0.006	0.031 ± 0.01	0.400 ± 0.049	0.291 ± 0.014
soluble protein kinase				
basal	0.243 ± 0.025	0.024 ± 0.002	0.261 ± 0.030	0.024 ± 0.004
cAMP activated	0.690 ± 0.057	0.064 ± 0.011	0.665 ± 0.098	0.057 ± 0.001
cGMP activated	0.558 ± 0.035	0.053 ± 0.012	0.570 ± 0.064	0.049 ± 0.011

[a] The protein kinase assay was carried out under darkroom conditions or laboratory lighting using either histone or endogenous proteins of the respective fractions as substrates. cAMP (10^{-5} M) or cGMP (10^{-4} M) was added to the basic reaction mixture (basal), and the reported activity values are means (± SE), expressed as nanomoles of ^{32}P incorporated (mg of protein)$^{-1}$ min^{-1}, of four to six experiments. The membrane and soluble fractions were obtained by extraction of ROS dark-adapted preparations with 50 mM Tris, pH 7.6, and centrifugation at 100000*g* for 1 h.

I) but the percentage of activation by the cyclic nucleotides was comparable and both sets of data were combined in Figure 1. In the Tris extracts (Figure 1A), activation is apparently maximal with cAMP at 10^{-6} M, causing a 2.7-fold increase in levels of phosphorylation, whereas cGMP, at 10^{-4} M, causes a 2.3-fold activation. In the Lubrol PX extracts (Figure 1B), the stimulation produced by both cyclic nucleotides is higher than that observed in the Tris extracts. cAMP increases basal activity 5.7-fold at 10^{-5} M and cGMP, 4.4-fold at 10^{-4} M. At higher concentrations of cyclic nucleotides, the apparent activation of protein kinase is decreased; perhaps this is due to activation by cyclic nucleotides of a protein phosphatase in the ROS soluble fraction (see Figure 2). The concentrations of cAMP and cGMP required for half-maximal activation are approximately 1×10^{-7} and 4×10^{-6} M, respectively. Thus, in vitro, the protein kinase is responsive to both cyclic nucleotides but it has a higher affinity for cAMP.

(2) *Light Activation.* We tested the effects of light on protein phosphorylation both with the soluble and the particulate fractions of ROS, using histone or the respective endogenous proteins as substrates (Table I). In agreement with what has been reported by other laboratories (Weller et al., 1975; Kühn et al., 1973; Bownds et al., 1972; Frank et al., 1973; Miller & Paulsen, 1975), the membrane-associated kinase phosphorylates preferentially histone in the dark. (Some phosphorylation of rhodopsin takes place, probably due to incomplete dark adaptation of our ROS preparations.) Illumination significantly increases the incorporation of ^{32}P into the endogenous membrane protein. Histone increases phosphorylation in dark-adapted outer segments to the level seen with light-adapted samples without histone, and it enhances the incorporation of ^{32}P in bleached preparations 1.4-fold. Light does not make the membrane protein kinase sensitive to either cAMP or cGMP. In contrast, the soluble kinase phosphorylates either histone or endogenous substrates in a cyclic nucleotide dependent manner (Table I), and its activity is independent of light.

(3) *Nucleotide Specificity.* In agreement with what has been reported by Chader et al. (1976), we found that the protein kinase of ROS membranes uses GTP as substrate (GTP-kinase) and that the GTP-kinase is activated to a greater extent by light than is ATP-kinase (data not shown). For example, phosphorylation of rhodopsin using GTP as phosphate donor is lower in the dark (0.1-fold) and higher in the light (1.8-fold) than that found with ATP as substrate. However, with histone as substrate, GTP phosphorylation is approximately 20 times lower than the ATP reaction.

The protein kinase of the soluble fraction of ROS uses GTP also as a phosphate donor but much less effectively than ATP.

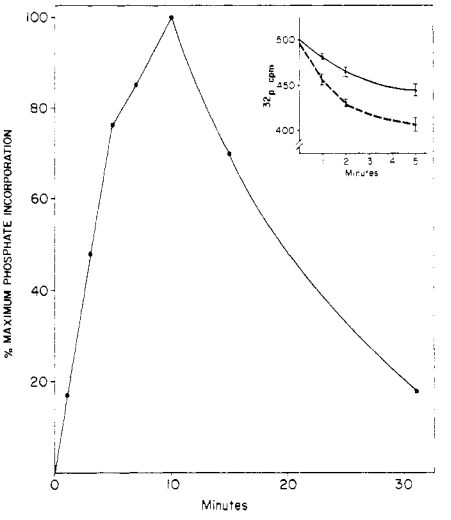

FIGURE 2: Phosphorylation and subsequent dephosphorylation of soluble proteins of Tris extract of ROS. Incubations were carried out as described in Materials and Methods for protein kinase reaction. At the times indicated, the reaction was stopped with 10% trichloroacetic acid, and the precipitated material was collected on filters before counting for ^{32}P radioactivity. Maximum incorporation of ^{32}P (2500 cpm above blank) was observed after 10-min incubation, and this value was considered arbitrarily to be 100%. In other experiments (see inset), the protein kinase reaction was carried out for 7 min, and the reaction mixture was applied directly to a gel filtration column as described in Materials and Methods. The fractions containing ^{32}P-labeled protein were pooled, and the aliquots were sampled for the rate of protein dephosphorylation in the absence (—) or in the presence (---) of 10^{-3} M cAMP. Values represent the mean of two experiments, with duplicate samples for each.

GTP-kinase activities obtained with either histone or endogenous proteins as substrates are about one-tenth of those observed with ATP (Table I). Cyclic nucleotides also stimulate this GTP-kinase. Since we obtain better incorporation of ^{32}P into protein using [γ-^{32}P]ATP rather than [γ-^{32}P]GTP, our studies were done with the former nucleotide as donor.

Dephosphorylation. The soluble fraction of ROS contains phosphoprotein phosphatase (Figure 2). In the presence of 5 mM ATP, maximum incorporation of ^{32}P is reached at 10 min of incubation and, thereafter, dephosphorylation predominates. There may be some phosphorylation occurring concurrently with dephosphorylation, since efforts were not made in these experiments to remove ATP. However, this was not the case in an experiment where protein kinase activity was prevented by the chromatographic removal of ATP (Figure 2, inset).

Our findings presented in Figure 1 suggest that high concentrations of cyclic nucleotides may favor dephospho-

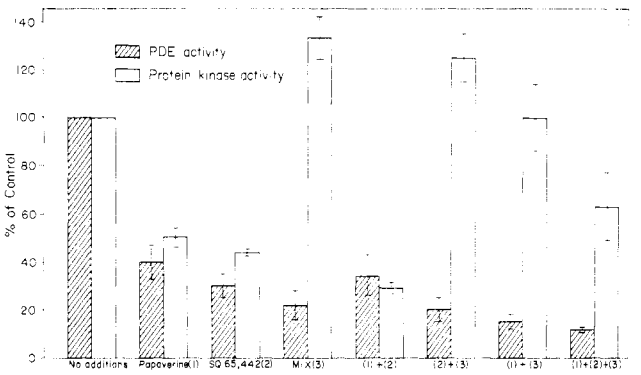

FIGURE 3: PDE and protein kinase activity of Tris extract of ROS after treatment with PDE inhibitors. The PDE and protein kinase reactions were carried out as described in Materials and Methods. Histone was used as substrate for the protein kinase and, where indicated, the PDE inhibitors (papaverine, SQ 65,442, and MIX) were added to the reaction mixture, singly or in combination, at 1 mM concentration. Values represent the mean ±SE of four to six experiments, and control values (100%) correspond to 23.7 nmol of cGMP hydrolyzed (mg of protein)$^{-1}$ min^{-1} for PDE activity and 0.025 pmol of ^{32}P incorporated (mg of protein)$^{-1}$ min^{-1} for protein kinase activity.

rylation of proteins in Tris or Lubrol extracts from ROS. For example, at 10^{-3} M, cAMP and cGMP both considerably reduce incorporation of ^{32}P into soluble protein. This observation is consistent with that of the experiment of Figure 2 (inset), which shows that the addition of 10^{-3} M cAMP increases the rate of dephosphorylation, when compared with that observed in the absence of the cyclic nucleotide.

Effect of Phosphodiesterase Inhibitors on the Activity of Solubilized Protein Kinase from ROS. In order to ensure that the concentrations of cAMP and cGMP in the protein kinase reaction mixture are unchanged during the incubation period, several phosphodiesterase inhibitors were tested for their effect both on phosphodiesterase and protein kinase activities. Squibb inhibitor (SQ 65,442), papaverine, and MIX were added at concentrations of 10^{-3} M, alone or in combinations.

Figure 3 shows that, of the three compounds tested individually, MIX inhibits phosphodiesterase activity of ROS homogenates more effectively (80%) than papaverine (60%) or Squibb 65,442 (70%). Combinations of MIX and Squibb 65,442 or of MIX and papaverine inhibit phosphodiesterase activity by 80% and 85%, respectively, and maximum inhibition (90%) is observed when the three compounds acted together.

We found that Squibb 65,442 and papaverine both are quite strong inhibitors of the soluble protein kinase from ROS (Figure 3) and that, in combination, they reduce phosphorylation to about 30% of the control. In contrast, MIX stimulates soluble protein kinase activity, increasing it 33% above the control. When in combination, MIX counteracts partially the inhibitory effects of papaverine or Squibb 65,442.

DEAE-Cellulose Chromatography of Solubilized Fraction from ROS. We observed three fractions of eluted material that show protein kinase activity (Figure 4). Most of the solubilized enzyme chromatographs as a peak of cAMP- or cGMP-dependent activity, eluting at approximately 0.18 M NaCl (type II protein kinase). A minor peak of activity that elutes at a lower NaCl concentration (0.07 M) is activated 3.5-fold by cAMP and 2.5-fold by cGMP (type I protein kinase). The third peak, which appears in the flow-through portion of the profile, is not stimulated by cyclic nucleotides and is probably free catalytic subunit (Corbin et al., 1975).

Apparent Reassociation of Solubilized Protein Kinase with ROS Membranes. In order to determine if the solubilized

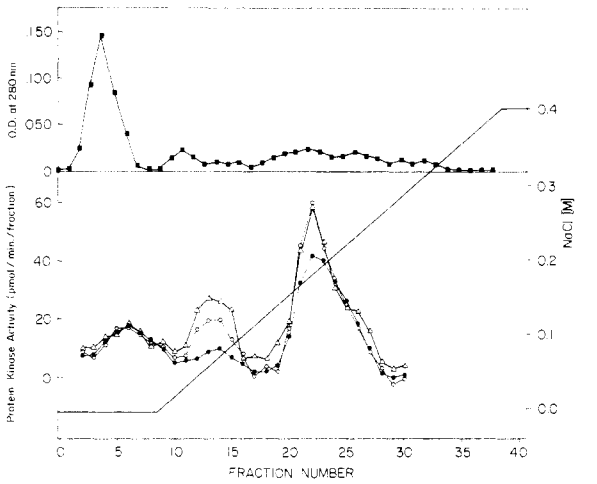

FIGURE 4: Separation of protein kinase of Tris extract of ROS by DEAE-cellulose chromatography. About 2 mg of protein was applied to a DEAE-cellulose column, and it was eluted with a linear gradient of NaCl in 50 mM Tris buffer, pH 7.6, as described in Materials and Methods. Fractions (1.0 mL) were assayed for protein (■) and for protein kinase activity, using histone as substrate, in the absence of exogenous cyclic nucleotide (●) or in the presence of 10^{-5} M cAMP (△) or 10^{-4} M cGMP (○).

Table II: Apparent Reassociation of Soluble Protein Kinase with "Purified Rhodopsin" Membranes[a]

	protein kinase act. of washed membranes (pmol of ^{32}P incorp (mg of protein)$^{-1}$ min^{-1})			
	dark		light	
	basal	+cAMP	basal	+cAMP
purified rhodopsin membranes	8.5	7.7	9.2	10.2
purified rhodopsin membranes + Tris extract of ROS + 5 mM EDTA	8.5	10.9	11.8	15.5
purified rhodopsin membranes + Tris extract of ROS + 5 mM $MgCl_2$	8.3	11.5	59.4	64.8

[a] Tris extracts of purified ROS were prepared under darkroom conditions, and the particulate material was extracted with 0.1% $NaDodSO_4$ as described in Materials and Methods for preparation of purified rhodopsin membranes. Tris extract (500 μL containing about 0.4 mg of soluble protein) was mixed with 1.2 mg of "purified rhodopsin" membranes and incubated in an ice bath for 1 h. After the incubation, the suspension was centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was removed and the particulate was resuspended in 500 μL of 50 mM Tris buffer, pH 7.6, containing 5 mM $MgCl_2$. The centrifugation procedure was repeated, and the particulate material (washed membranes) was resuspended in the Tris-Mg buffer before it was assayed for protein kinase activity. The washed membranes were maintained in darkness and, where indicated, bleached by laboratory fluorescent lighting immediately before the assay for protein kinase activity. Values are the means of two experiments, each run in duplicate.

protein kinase could be reassociated with ROS membranes, we mixed the Tris-extracted soluble enzyme with the "purified rhodopsin" membranes that had only minimal protein kinase activity (Table II). Since the removal of divalent cations from the membranes facilitated Tris extraction of the enzyme, we considered that addition of divalent cations could favor its reassociation. Therefore, one-half of the protein kinase-membrane mixture was incubated at 0 °C for 60 min with $MgCl_2$ (5 mM) and the other half was incubated in the same conditions with EDTA (5 mM). The samples were then

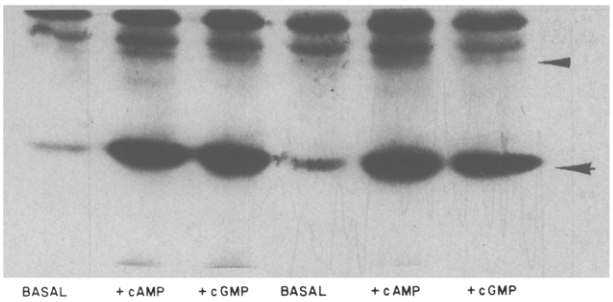

FIGURE 5: Separation of phosphorylated proteins of Tris extract of ROS by electrophoresis on 8.34% polyacrylamide gel slabs. The proteins were phosphorylated using [γ-^{32}P]ATP and endogenous protein kinase in reaction mixtures, without added cyclic nucleotides (gel slots 1 and 4 from left), or in those containing 10^{-5} M cAMP (slots 2 and 5) or 10^{-4} M cGMP (slots 3 and 6). The kinase reactions were stopped with trichloroacetic acid and the samples prepared for electrophoresis and autoradiography as described in Materials and Methods. The arrow identifies a protein (apparent molecular weight of 30 000) that is phosphorylated in a cyclic nucleotide dependent manner, and the pointer shows the position of a second protein (apparent molecular weight of 60 000) that is phosphorylated to a lesser extent but also in a cyclic nucleotide dependent manner. The molecular weights were interpolated from the migration of purified proteins of known molecular weight.

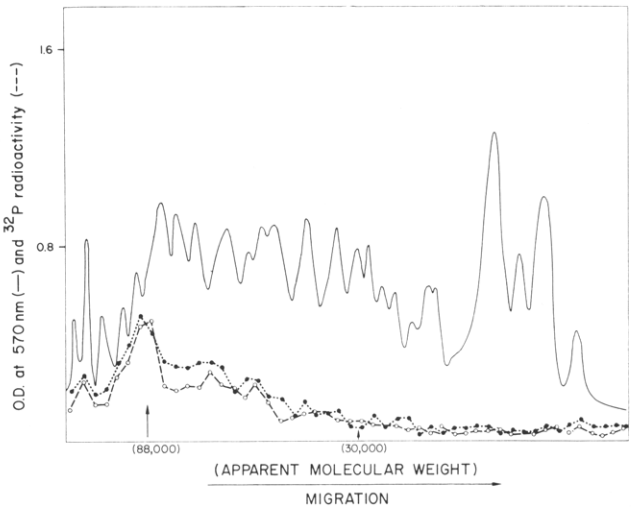

FIGURE 6: Phosphorylated proteins from Tris extract of retinal pellet, remaining after detachment of ROS. Phosphorylation and electrophoresis were carried out as described in Materials and Methods. (—) Absorbance at 570 nm of proteins stained with Coomassie brilliant blue; distribution of ^{32}P (---) for samples phosphorylated without cyclic nucleotides (○) or in the presence of 10^{-5} M cAMP (●).

centrifuged at 100000*g* for 1 h, and special care was taken to remove all of the supernatant fraction from the pelleted material. The pellets were washed with Tris (50 mM) containing $MgCl_2$ (5 mM) to ensure minimal contamination with soluble kinase. The effects of light and cAMP on the protein kinase activity of the membranes were investigated, and the results are shown in Table II. Protein kinase activity of the "purified rhodopsin" membranes was independent of exogenous cAMP, and it was stimulated minimally by light. Similar results for protein kinase activity were observed when the "purified rhodopsin" membranes were incubated with soluble protein kinase in the presence of EDTA.

When Mg^{2+} was incubated with the "purified rhodopsin" membranes and soluble kinase in the dark, the basal or cAMP-stimulated protein kinase activities were similar to those in the presence of EDTA. However, upon exposure to light, protein kinase activity was enhanced about six- to sevenfold. This experiment shows that the cyclic nucleotide dependent soluble protein kinase can reassociate apparently with ROS membranes and phosphorylate bleached rhodopsin. Moreover, the activity of the reassociated kinase is independent of cyclic nucleotides.

Specificity of the Reassociation of Soluble Protein Kinase from ROS with the ROS Membranes. In order to determine if another soluble protein kinase could associate with the ROS membranes and phosphorylate rhodopsin, we carried out the same kind of experiment described above: we mixed purified rhodopsin membranes with solubilized protein kinase obtained from rat skeletal muscle. Apparently, there is no association of this enzyme with the ROS membranes that resulted in phosphorylation of rhodopsin; protein kinase activities were similar with or without added cAMP and in the dark or in the light.

Endogenous Substrates of the Soluble Protein Kinase from ROS. We have shown previously that, when the protein kinase reaction is carried out with the Tris extract of ROS in the absence of cyclic nucleotides, several bands of phosphorylated proteins are obtained. The apparent molecular weights of these proteins are 22 000, 30 000, 64 000, and 70 000. The addition of cGMP (10^{-4} M) increases, selectively (about 10.6-fold), the incorporation of ^{32}P in the 30 000 molecular weight protein (Lolley et al., 1977). We now have found that cAMP (10^{-6} M) causes an even larger effect, increasing the basal phosphorylation of the same protein approximately 17- to 18-fold.

An autoradiograph of the products of the protein kinase reaction after gel slab electrophoresis is shown in Figure 5. Several bands of ^{32}P-labeled protein are revealed, and two show increased incorporation in the presence of exogenous cyclic nucleotides. The one with the greatest radioactivity has an apparent molecular weight of 30 000, whereas the other is estimated to be 60 000. The 30 000 molecular weight protein is apparently a soluble component of the ROS. It is easily extracted with Tris and remains after centrifugation in the Tris–100000*g* supernatant fraction of ROS. In addition, polyacrylamide gel electrophoresis of the products of the protein kinase reaction, obtained by Lubrol PX extraction of previous Tris-extracted ROS, does not show a radioactive band at 30 000. Furthermore, if the method of Basinger et al. (1976) is used to isolate the ROS, very pure preparations of ROS membranes are obtained in terms of rhodopsin (a 278 nm/498 nm of about 2), but the membranous disks are broken and the 30 000 molecular weight protein is lost as determined by gel electrophoresis. From such observations we conclude that the 30 000 molecular weight protein is a component of the ROS cytoplasm.

Localization of the 30 000 Molecular Weight Protein in ROS. We verified that the 30 000 molecular weight protein is specific for visual cells and intrinsic to ROS by means of the following experiment. The pellet of retina remaining after detachment of the ROS was homogenized in 50 mM Tris buffer, pH 7.6. This pellet contained all of the inner layers of the retina together with the photoreceptor cell soma without significant ROS contamination. The Tris–100000*g* supernatant fraction of this preparation was used for the protein kinase reaction, and the endogenous proteins were separated on polyacrylamide gels. Figure 6 shows that the ^{32}P radioactivity had been incorporated into proteins of higher molecular weight; a phosphorylated protein of molecular weight 30 000 is not present, suggesting that photoreceptor soma do not contain this protein. These results support the localization of the 30 000 molecular weight phosphoprotein in the visual cells and, more specifically, in their ROS.

Differential Conditions for Phosphorylation of 30 000 Molecular Weight Protein and Rhodopsin. We combined the

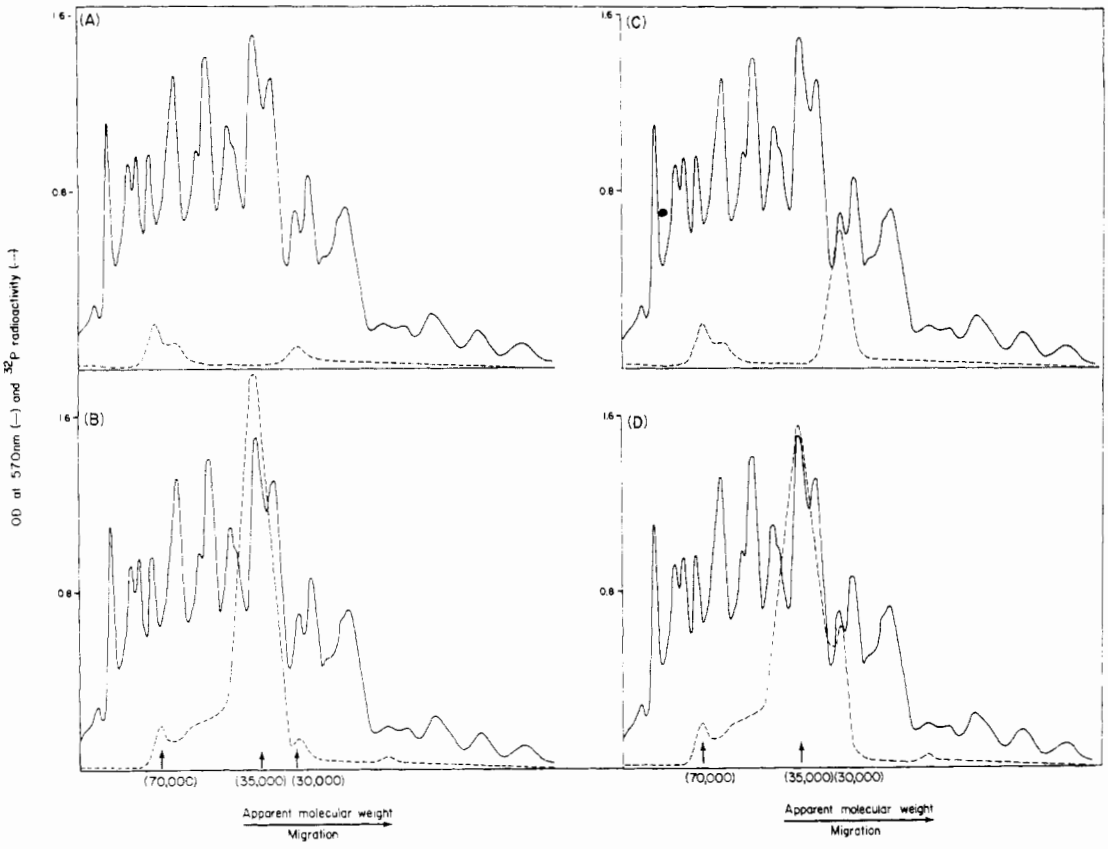

FIGURE 7: Cyclic nucleotides and light as modulators of protein phosphorylation. Tris extract of ROS and "purified rhodopsin" membranes were prepared as described in Materials and Methods, and the two fractions were combined as described in Table II. Four aliquots (A to D) were prepared for the protein kinase reaction, and each was treated differently before they were processed for polyacrylamide gel electrophoresis and scintillation counting of gel slices: (A) protein phosphorylation was carried out in darkness (red light) without added cyclic nucleotide; (B) protein phosphorylation was carried out after bleaching of the sample by laboratory lighting without added cyclic nucleotide; (C) protein phosphorylation was carried out in darkness in the presence of 10^{-5} M cAMP; (D) protein phosphorylation was carried out after bleaching of preparation in the presence of 10^{-5} M cAMP. Apparent molecular weights were interpolated from the migration of purified proteins. The 30 000 and 70 000 molecular weight proteins, indicated by arrows, are markers of the soluble fraction of ROS, and the 35 000 molecular weight protein is the membrane protein, opsin.

Tris-extracted soluble fraction of ROS with the purified rhodopsin membranes. Aliquots of the mixture were phosphorylated under different conditions, with or without cAMP, in the dark or in the light. The reaction was terminated by the addition of $NaDodSO_4$. The denatured samples were then electrophoresed on polyacrylamide gels and processed as usual.

Figure 7A shows that, in the presence of unbleached rhodopsin, in the dark, only the proteins found in the soluble extract of ROS are phosphorylated; Figure 7C verifies that the 30 000 molecular weight protein is phosphorylated under these conditions in a cyclic nucleotide dependent manner. When the combined fractions were illuminated before carrying out the protein kinase reaction (Figure 7B), rhodopsin and the 30 000 molecular weight protein both are phosphorylated. Figure 7D shows that, in the light, cAMP does not stimulate the phosphorylation of rhodopsin but, instead, increases the incorporation of ^{32}P into the soluble 30 000 molecular weight protein.

Molecular Weight of the Native Soluble Substrate. Gel filtration of the phosphorylated proteins from the 100000*g* Tris extract of ROS on Sephadex G-200 gave the profile shown in Figure 8. Two peaks of radioactive protein were separated, one eluting with the void volume of the column and the other between fractions 53 and 63. The estimated molecular weight of this native protein is 60 000. When cAMP was included in the phosphorylating media, the two separated peaks showed an increase in ^{32}P incorporation, indicating cyclic nucleotide dependency in their phosphorylation. The fractions corresponding to the void volume and the 60 000 molecular weight protein, in the presence and absence of cAMP, were pooled

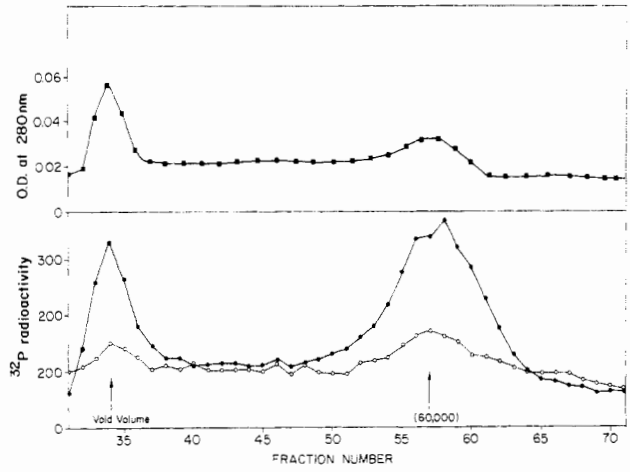

FIGURE 8: Separation of endogenous phosphoproteins of Tris extract of ROS by gel filtration, using Sephadex G-200. Proteins were phosphorylated by endogenous protein kinase and [γ-^{32}P]ATP, in the presence (●) or absence (○) of 10^{-5} M cAMP. About 1 mg of native protein was applied to individual Sephadex columns, eluted with 50 mM Tris buffer, pH 7.6, and collected in 2-mL fractions. An aliquot (100 μL) of each fraction was measured for ^{32}P radioactivity by scintillation counting or for absorbance at 280 nm (■) (relative protein content). Arrows indicate the void volume and apparent molecular weight (interpolated from standard proteins) of 60 000.

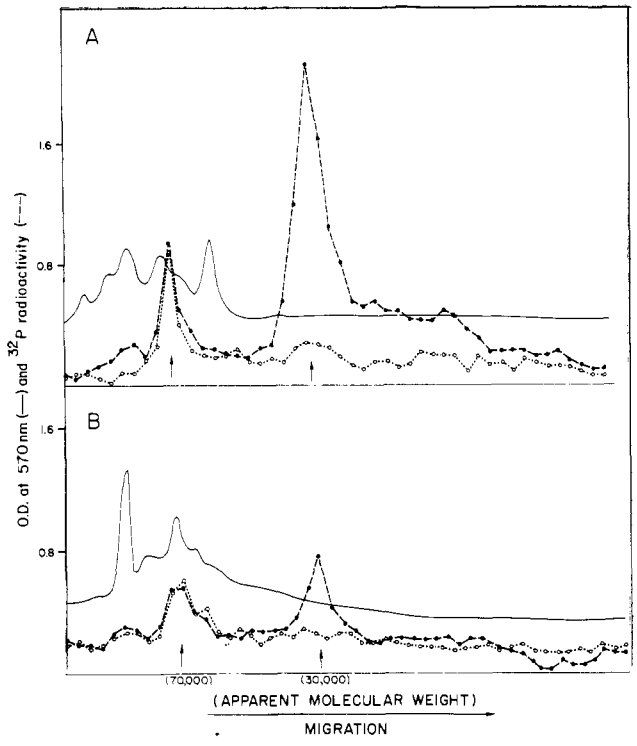

FIGURE 9: Evaluation of proteins from gel filtration fractions by polyacrylamide gel electrophoresis. (A) Fractions 53–63 of Sephadex G-200 columns of Figure 8, containing proteins phosphorylated in the absence or presence of cAMP, were concentrated separately and aliquots (approximately 30 μg of protein) were electrophoresed on 10% gels. (—) Protein distribution within the gels after Coomassie brilliant blue staining; (---) corresponding bands of radioactivity after slicing of the gels (O, no cAMP or ●, 10^{-5} cAMP). Arrows mark the apparent molecular weights of 30 000 and 70 000, interpolated from standard proteins. (B) Fractions 32–37 (void volume) were concentrated and processed for electrophoresis, protein staining, and counting, as described in A.

separately and concentrated and, after denaturation with $NaDodSO_4$, electrophoresed on polyacrylamide gels. The four samples showed identical distribution of radioactivity (Figures 9A and 9B); two bands corresponding to molecular weights of 70 000 and 30 000, respectively, were obtained in all cases, but they differed in their response to cyclic nucleotide activation. Only the 30 000 molecular weight protein had increased its incorporation of ^{32}P in the presence of cAMP.

The present experiments leave unresolved the question of what is the molecular weight of the native substrate of the cyclic nucleotide dependent protein kinase. Our findings can be interpreted to indicate that a native protein with a molecular weight of 60 000 is split into equal subunits by $NaDodSO_4$ or that a native protein with a molecular weight of 30 000 aggregates into 60 000 dimers or larger multiples. Additional work is needed to resolve this question and to identify the 70 000 molecular weight protein that is phosphorylated in a cyclic nucleotide independent manner and that appears to coaggregate with the 30 000 or 60 000 molecular weight protein.

Discussion

Protein kinase of bovine ROS is partitioned, after centrifugation in Tris buffer, into a soluble and a membrane-bound form, and the membrane kinase can be solubilized by detergent. Both the soluble and membrane-solubilized enzymes are cyclic nucleotide dependent, whereas the membrane-bound form is insensitive to cyclic nucleotides (Lolley et al., 1977). Observations presented in this paper indicate that the degree of activation by cyclic nucleotides is greatest in the solubilized kinase preparation, suggesting that detergents extract predominantly the holoenzyme from the ROS membranes. The soluble enzyme from dark-adapted ROS may be less responsive to cyclic nucleotides in vitro, as a result of partial dissociation into its regulatory and catalytic subunits. The dissociation could occur in situ, since cGMP levels are high in dark-adapted ROS.

When compared with protein kinases of other tissues, the soluble, cyclic nucleotide dependent protein kinase from ROS has the characteristics of a cAMP-dependent enzyme. For example, DEAE-cellulose chromatography of the Tris-extract of ROS resolves the two types of cAMP-dependent protein kinase that are present in most tissues (Corbin et al., 1975). Additionally, the soluble kinase responds to lower concentrations of cAMP than cGMP in vitro, and the apparent level of activation is greater with cAMP. But the maximal activation of the soluble protein kinase by cyclic nucleotides is still unresolved since, in these studies, it may be obscured by the action of a soluble phosphoprotein phosphatase.

The presence of a cAMP-dependent protein kinase in ROS appears to be paradoxical since cGMP is the major cyclic nucleotide of rod photoreceptor cells, with levels several-fold higher than those of cAMP (Krishna et al., 1976). Moreover, the concentration of cGMP in ROS in vitro or in vivo is regulated by light (Woodruff et al., 1977), whereas that of cAMP is unchanged. Therefore, we suggest that ROS possess a protein kinase that has cAMP-dependent characteristics but is modulated directly by light-induced changes in cGMP concentrations in vivo.

The soluble protein kinase can phosphorylate the exogenous substrate histone and also several proteins of the soluble fraction from ROS. Of these endogenous proteins, however, only one with an apparent molecular weight of 30 000 is phosphorylated in a cyclic nucleotide dependent manner. In the native state, this protein may exist as a dimer or, possibly, as a larger aggregate.

The soluble kinase can be reassociated apparently with ROS membranes that have been depleted of kinase activity but have retained their visual pigment content. Under these conditions, the reassociated kinase phosphorylates bleached rhodopsin (a membrane protein) independent of cyclic nucleotides; these characteristics are identical with those of the kinase from freshly prepared ROS membranes. Our findings suggest that the soluble and membrane-associated protein kinases of ROS are interchangeable. The mechanisms that regulate an exchange between a soluble and a membrane-associated form are still unknown but our preliminary studies show that the presence of magnesium ions favors reassociation of the soluble enzyme with ROS membranes.

By combining the soluble proteins of ROS with purified rhodopsin membranes, we demonstrate further that membranous and/or soluble proteins of ROS are phosphorylated selectively in response to biological signals. The signals correspond to those that may act within ROS in vivo; light promotes the phosphorylation of rhodopsin, independent of cyclic nucleotides, and cyclic nucleotides stimulate the phosphorylation of a 30 000 molecular weight protein, independent of light.

Acknowledgments

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Spin-Label Techniques for Monitoring Macromolecular Rotational Motion: Empirical Calibration under Nonideal Conditions†

Michael E. Johnson

ABSTRACT: Practical techniques are demonstrated for determining rotational correlation times of macromolecules from the first harmonic absorption electron spin reasonance spectra of tightly bound spin labels. The techniques are developed to compensate for such nonideal conditions as residual label motion, temperature dependence of rigid limit spectral parameters, and the presence of inhomogeneous line broadening. These effects are all shown to be of importance in monitoring the rotational motion of carbonmonoxyhemoglobin which is spin labeled with the tightly bound nitroxide label, 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy. Spin-label interactions with other paramagnetic agents are also shown to produce spectral changes which are qualitatively similar to, but quantitatively different from, those resulting from increases in the rate of rotational motion.

Over the last few years there has been increasing interest in the use of spin-label techniques to monitor macromolecular motional behavior. In those favorable cases where a spin label can be rigidly bound to a macromolecule, its electron paramagnetic resonance (EPR)[1] spectrum should directly reflect the motion of the macromolecule itself. Theoretical studies by McCalley et al. (1972) and Goldman et al. (1972) have shown that in the slow motion region the apparent hyperfine splitting can be quantitatively related to the spin-label rotational correlation time. More recently Mason & Freed (1974) have predicted that the hyperfine extremal line widths should be an even more sensitive monitor for rotational motion.

Thus far, however, only the hyperfine splitting measurement method appears to have been used in monitoring macromolecular motional behavior. Through application of this technique, McConnell and co-workers have measured the rotational correlation times of spin-labeled HbO_2 (McCalley et al., 1972) and spin-labeled α-chymotrypsin (Shimshick & McConnell, 1972) in solution and have shown that the spin-label measurements agree well with the behavior predicted for Brownian rotational diffusion. In an extension of this work, Kuznetsov et al. (1975) have used correlation time mea-

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[1] Abbreviations used: EPR, electron paramagnetic resonance; HbCO, carbonmonoxyhemoglobin; Tempo-maleimide, 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy.

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