Protein Kinases of Retinal Rod Outer Segments: Identification and Partial Characterization of Cyclic Nucleotide Dependent Protein Kinase and Rhodopsin Kinase†

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ABSTRACT: Protein kinase activity of dark-adapted bovine rod outer segments is partitioned by centrifugation into soluble and membrane-bound fractions. The soluble kinases are separated by DEAE-cellulose chromatography into three peaks of activity, which can be classified by substrate specificity and cyclic nucleotide dependence into two categories. One peak of protein kinase activity has the characteristics reported for rhodopsin kinase (category one); it phosphorylates only bleached rhodopsin, and its activity is not affected by light, exogenous adenosine cyclic 3′,5′-monophosphate (cAMP), guanosine cyclic 3′,5′-monophosphate (cGMP), or a protein kinase inhibitor from skeletal muscle. Rhodopsin kinase has an apparent molecular weight of 68 000. The second category of kinase includes two peaks of activity which are stimulated severalfold by cAMP or cGMP but not by light. These protein kinases phosphorylate soluble proteins including histones and a protein kinase substrate prepared from rat intestine but not rhodopsin. The two peaks elute from DEAE-cellulose with 0.09 and 0.20 M KCl, suggesting that they are similar respectively to type I and type II cyclic nucleotide dependent protein kinases that have been characterized in other tissues. The activity of type I kinase is variable and much less than that of the type II enzyme; its molecular weight was not determined. The type II protein kinase has an apparent molecular weight of 165 000. This study confirms that different protein kinase enzymes catalyze selectively the phosphorylation of bleached rhodopsin and soluble proteins, and it repudiates the speculation in a previous publication [Farber, D. B., Brown, B. M., & Lolley, R. N. (1979) *Biochemistry 18*, 370–378] that a single protein kinase might catalyze both phosphorylation reactions.

Rod outer segments (ROS)[1] of dark-adapted retinas contain high levels of cGMP (Krishna et al., 1976). Upon bleaching of rhodopsin, the level of cGMP is reduced rapidly due to hydrolysis by a light-activated phosphodiesterase (Woodruff & Bownds, 1979; Yee & Liebman, 1978; Miki et al., 1973). A role has been ascribed to cGMP in the visual process of normal rod photoreceptors (Woodruff & Bownds, 1979) and in the initiation of visual cell pathology in three inherited disorders that cause blindness (Lolley & Farber, 1976; Farber & Lolley, 1976; Chader et al., 1980; Acland et al., 1980). Cyclic GMP is a biological effector in many tissues, acting upon cyclic nucleotide dependent protein kinase enzymes to control the rate of protein phosphorylation (Goldberg & Haddox, 1977). Both in the normal and in the diseased retinas, changes in the concentration of cGMP are thought to modulate the metabolism of specific phosphoproteins that, in turn, regulate the metabolism or function of visual cells (Farber et al., 1978).

In order to understand the role of cGMP as a biological effector in rod photoreceptors, it is imperative that we identify and characterize the cyclic nucleotide dependent protein kinases (CNPK) that regulate the phosphorylation of receptor proteins. Our earlier studies (Farber et al., 1979) and those of Pannbacker (1973) have shown the presence in bovine ROS of soluble CNPK, but, until now, CNPK activity of ROS membranes was not demonstrable. We have also identified in ROS a soluble protein (molecular weight 30 000) that is phosphorylated in a cyclic nucleotide dependent manner (Lolley et al., 1977). Proteins of lower molecular weight (12 000–13 000) are reportedly phosphorylated in a cyclic nucleotide dependent manner in frog ROS, but the kinase which catalyzes this reaction has not been evaluated (Polans et al., 1979).

It is known that ROS contain a membrane-associated and extractable protein kinase, rhodopsin kinase (RK), that phosphorylates bleached rhodopsin in a cyclic nucleotide independent manner (Shichi & Somers, 1978; Frank & Buzney, 1975; Weller et al., 1975; Kühn et al., 1973). Recent studies by Kühn (1978) have identified procedures for extracting RK from bovine ROS, and his work has further characterized the involvement of light in the regulation of rhodopsin phosphorylation.

In this report, we compare the properties of soluble CNPK of bovine ROS with those of soluble RK and show that the enzymes are distinctly different in terms of cyclic nucleotide dependence, substrate specificity, molecular weight, and chromatographic property on the ion-exchange column.

Experimental Procedures

Materials

The ATP, cAMP, mixed histone (type II), and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. [γ-^{32}P]ATP (2.5–4 Ci/μmol) and [^{3}H]cAMP (32–36 Ci/mmol) were purchased from New England Nuclear Corp. Alum (aluminum potassium sulfate) was purchased from J. T. Baker Co. Cetyltrimethylammonium bromide (CTAB) was

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[1] Abbreviations used: ROS, rod outer segment(s); cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; CNPK, cyclic nucleotide dependent protein kinase; CNPK I, type I CNPK; CNPK II, type II CNPK; PKS, protein kinase substrate; RK, rhodopsin kinase; CTAB, cetyltrimethylammonium bromide; NaDodSO$_4$, sodium dodecyl sulfate.

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from Eastman Kodak. All protein standards used in the calibration of the gel filtration columns were purchased from Pharmacia Fine Chemicals.

Methods

Preparation of ROS from Bovine Retina. About 100–150 fresh bovine eyes, obtained from Quality Meat Packing Co., Vernon, CA, were immediately placed on ice and dark-adapted for at least 1 h before dissection under dim red light. Twelve retinas were shaken vigorously for 1 min in a centrifuge tube containing 15 mL of sucrose (*d* 1.13), 5 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 62 mM NaCl and subsequently centrifuged at 10000*g* for 12 min. The supernatants containing detached ROS were pooled and stored on ice in the dark. Additional ROS were obtained by resuspending the pellet in the same volume of sucrose buffer medium and centrifuging again after several passages of a loosely fitting Teflon pestle. The first and second supernatant fractions were, on occasion, processed separately but were routinely combined, diluted with 2 volumes of 50 mM Tris-HCl, pH 7.6, containing 5 mM $MgCl_2$, and centrifuged at 27000*g* for 30 min to sediment the ROS. The ROS pellets obtained from six retinas were wrapped individually with aluminum foil and stored at −70 °C until needed for the preparation of soluble protein kinase. In some experiments, ROS were further purified with a discontinuous sucrose density gradient according to the method of Papermaster & Dreyer (1974). The purity of ROS was estimated by the spectral ratio of A_{280}/A_{498} with use of CTAB to solubilize the ROS membrane as described by McDowell & Kühn (1977).

Microdissection of ROS from Frozen Bovine Eyes. Four freshly enucleated eyes were frozen in dry ice, and the anterior segments were removed with a cold chisel. The resulting globes were hemisected, and half of a frozen eye was mounted with the sclera facing the microtome knife. Ten-micron sections of the eye were cut under white light with a Cryo-Cut microtome at −14 °C. Sections were discarded until tangential sections of the pigmented choriocapallaris were observed. Each pigmented section was inspected until the ROS of the neural retina were detected as a pink area within the pigmented tissue. The pink area was manually dissected on the microtome knife with cold tools and transferred to a homogenizer precooled on dry ice. One additional section (10 μm) was cut and the ROS area was dissected. The microtome was then realigned to a new position on the hemisectioned eye, and the operation was repeated. ROS from all areas of the retina were pooled and homogenized in extraction buffer, and the soluble extract was applied to a DEAE-cellulose column for separation of protein kinase. Since bovine ROS are about 20 μm in length, we have ensured, by limiting the depth of sectioning into the retina to 20 μm or less, that the microdissected preparation contained primarily ROS and the apical extensions of the pigmented epithelium, with some possible inclusion of photoreceptor inner segments.

Extraction of Soluble Protein Kinase from ROS. All extraction procedures were performed under dim red light at 4 °C. Frozen samples of ROS were thawed and homogenized in a glass-to-glass homogenizer. For each ROS pellet from six retinas, 1 mL of buffer A (20 mM Tris-HCl, pH 7.6, 2 mM EDTA, and 50 mM β-mercaptoethanol) was used. Following homogenization, the samples were centrifuged at 100000*g* for 1 h, and the supernatant fraction was collected. A second supernatant fraction was obtained by rehomogenizing the pellet in the same volume of buffer A and centrifuging again. The two extracts were combined and used for later studies. The extracted pellet was stored in the dark at −70 °C for use in the preparation of alum-treated ROS membranes.

Preparation of "Protein Kinase Substrate" (PKS) from Rat Small Intestine. Flushed and minced intestine of adult Fischer rats was suspended in 3 volumes of ice-cold 5 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and homogenized in a cooled Waring blender. The homogenate was immediately boiled for 10 min and cooled on ice before centrifugation at 27000*g* for 20 min. Trichloroacetic acid (Cl_3AcOH; 50% w/v) was added dropwise to the supernatant until the final concentration of Cl_3AcOH was 5%. After being stirred in an ice bath for 30 min, the suspension was centrifuged again. The Cl_3AcOH precipitate was resuspended in a small volume of phosphate buffer, and the pH was adjusted to 7.0 with the addition of 1 N NaOH. The preparation was dialyzed overnight against 4 L of 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The content of the dialysis bag was divided into small aliquots and stored at −70 °C. In a typical preparation, 30 g of rat small intestine yielded about 180 mg of protein.

Preparation of cAMP-Dependent Protein Kinase Inhibitor from Rabbit Skeletal Muscle. Protein kinase inhibitor was prepared according to the method of Ashby & Walsh (1974) up to the stage of heat treatment and acid precipitation. The inhibitory activity of the preparation was tested with CNPK prepared from bovine ROS and rat brain. Under our regular assay conditions, where 5–20 μg of ROS protein was used as enzyme, 4 μg of inhibitor protein suppressed protein kinase activity by 50%, and 20 μg caused 100% inhibition. In routine assays of protein kinase activity where kinase inhibitor was included, 5-μL aliquots containing 39 μg of protein were used to ensure 100% inhibition.

Assay of CNPK. The reaction was carried out at 30 °C in a final volume of 0.1 mL containing 50 mM potassium phosphate, pH 7.0, 10 mM magnesium acetate, 0.1 mM isobutylmethylxanthine, 400 μg of PKS protein, and sufficient protein kinase to incorporate ^{32}P to levels at least 2-fold above blank values. Basal and CNPK activities were determined in the absence and in the presence of 10^{-6} M cAMP. In some instances, 39 μg of rabbit skeletal muscle inhibitor was included in the reaction mixture. The reaction was started by the addition of 1 nmol of [γ-^{32}P]ATP (sp act. about 2000 cpm/pmol) to temperature-equilibrated tubes. The reaction was terminated after 1–7 min of incubation by removing 50-μL aliquots which were immediately applied to premarked Whatman 3MM filter disks. The disks were dropped into a basket that was immersed in 20% ice-cold Cl_3AcOH. The basket was kept in a bouncing motion in order to ensure thorough washing of the disks, and the Cl_3AcOH was circulated with a magnetic stirrer located beneath the basket. The disks were washed for 10 min in 20% Cl_3AcOH and for 10-min intervals in four changes of fresh 5% Cl_3AcOH before the individual disks were transferred to counting vials containing 5 mL of PCS–xylene scintillation cocktail (2:1 mixture; Amersham Corp.). Blanks were prepared by using either water or boiled tissue to substitute for enzyme. The amount of phosphate incorporated into Cl_3AcOH-insoluble material was calculated from the known specific activity of the [γ-^{32}P]ATP.

The CNPK assay of the soluble enzymes was regularly carried out under laboratory lighting, but darkroom conditions were used in the measurement of the CNPK activity of ROS homogenates and ROS membranes using either endogenous proteins or PKS as the substrate.

[^3H]cAMP Binding Assay. The binding of [^3H]cAMP to CNPK was determined according to Gilman (1970). The

binding reaction mixture, in a final volume of 200 μL, contained 50 mM sodium acetate, pH 4.0, 1 mM EDTA, 0.5 mM isobutylmethylxanthine, and 20–25 nM [^{3}H]cAMP (sp act. 32–36 Ci/mmol). Binding was initiated by addition of 80 μL of eluate from a Sephadex G-100 column and was allowed to reach equilibrium by incubation at 4 °C for 90 min. The reaction mixture was then diluted with 1 mL of potassium phosphate, pH 6.0, and, 3 min later, the sample was filtered through a Millipore HAWP filter. The filter was washed twice with 3 mL of the phosphate buffer and then put into a scintillation vial containing 5 mL of PCS–xylene (2:1). The vial was shaken for 30 min before counting for radioactivity.

Preparation of Alum-Treated ROS Membranes. ROS membranes that had been extracted previously for soluble protein kinase were used in the preparation of alum-treated ROS membranes (McDowell & Kühn, 1977). In a typical preparation, membranes from the ROS of 12 bovine retinas were homogenized in 5 mL of water and repelleted by centrifugation at 27000*g* for 30 min. The pellet was treated with 4% alum (w/v) and, subsequently, washed twice with water (8 mL). The alum-treated ROS membranes were homogenized in 1.8 mL of buffer A, and they were used as the protein substrate in the assay of RK. The alum-treated membranes possessed no measurable level of endogenous protein kinase activity. Routinely, freshly prepared alum-treated ROS membranes were used in the assay of RK. However, they can be stored in the dark at 4 °C for up to 5 days without significant loss in their ability to serve as the substrate in this reaction. Freezing of the preparation results in loss of activity.

Assay of RK. The selective phosphorylation of bleached rhodopsin is unique to RK, and it was used as a marker for the presence of the enzyme. For measurement of RK in the soluble fraction of ROS, alum-treated ROS membranes that had been depleted of protein kinase activity were used as a source of rhodopsin. A standard assay mixture of 0.1 mL containing 50 mM potassium phosphate, pH 7.0, 10 mM magnesium acetate, 20 μL of alum-treated ROS membranes (~400 μg of protein), and an appropriate amount of enzyme was prepared and immediately incubated at 30 °C for 5 min in the dark (dark controls) or under laboratory illumination (bleached rhodopsin samples). [γ-^{32}P]ATP was added to a final concentration of 10 μM to start the reaction. The reaction was terminated by transferring an aliquot of the reaction mixture to Whatman filter disks. The subsequent procedures are identical with those described for CNPK.

Rhodopsin kinase activity in ROS homogenates or ROS membranes was assayed by phosphorylation of the endogenous rhodopsin.

Determination of Molecular Weight by Gel Filtration Column Chromatography. The molecular weight of CNPK was determined by a Bio-Gel A-1.5m column (1.5 × 75 cm), which had been preequilibrated in buffer A containing 0.1 M KCl and calibrated with ferritin (440000), catalase (232000), aldolase (158000), bovine serum albumin (67000), and ovalbumin (45000). One milliliter of the 100000*g* supernatant, which was adjusted to 0.1 M KCl, was applied, and the column was eluted under 60 cm of H_2O pressure at 4 °C. Fractions of 1.5 mL were collected and assayed for phosphorylation of PKS in the presence of cAMP or cAMP plus the skeletal muscle inhibitor. The molecular weight of RK was determined by a Sephadex G-100 column (1.6 × 71 cm), preequilibrated in buffer A, containing 0.1 M KCl and 10% sucrose (w/v). The sample was pretreated with KCl as described above, and the elution profiles of RK and CNPK were determined by light-dependent phosphorylation of alum-treated ROS membrane and binding of [^{3}H]cAMP, respectively. Standards used for the calibration of the column included bovine serum albumin (67000), ovalbumin (43000), and chymotrypsinogen (25000).

Table I: CNPK and RK Activities in Bovine ROS[a]

	endogenous		PKS	
	dark	light	dark	light
basal	0.023	0.169	0.078	0.249
cAMP	0.025	0.151	0.216	0.355
cAMP + I	0.029	0.195	0.069	0.219

[a] Dark-adapted ROS from six retinas were homogenized in 1 mL of buffer A; protein kinases in the homogenate were assayed, as described under Methods, in the absence (basal) and in the presence of cAMP (cAMP) or cAMP plus skeletal muscle inhibitor (cAMP + I). The activities were expressed as nanomoles of phosphate incorporated per minute per milligram of protein. Experiments with at least three ROS preparations gave consistent results. Values in this table give results from one representative experiment.

Protein Determination. The protein content of ROS homogenates and other samples where reducing agents were absent was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. However, since buffer A contained 50 mM β-mercaptoethanol, a modified Lowry method as described by Ross & Schatz (1973) was used, wherein the sulfhydryl reagent was first removed by alkylation with iodoacetic acid.

Results

Evidence for Two Protein Kinases in Bovine ROS: CNPK and RK. (1) Bovine ROS Contain Two Protein Kinase Activities Which Differ in Substrate Specificity and Mechanism of Activation. Bovine ROS were homogenized in buffer A and analyzed for protein kinase activity in the light or dark by using endogenous or exogenous protein as phosphate acceptors (Table I). The level of phosphorylation of endogenous proteins in the dark was low in the absence of added cAMP (basal activity), and the level was neither enhanced by the addition of 10^{-6} M cAMP (cAMP activity) nor inhibited by the addition of rabbit skeletal muscle inhibitor (cAMP + I). Upon exposure to light, the basal level of endogenous (rhodopsin) phosphorylation was increased by 7.3-fold, and the increase was not significantly affected by the presence of either cAMP or inhibitor.

The basal protein kinase activity of dark-adapted ROS homogenate was enhanced 3.4-fold by the addition of exogenous substrate PKS. The phosphorylation of PKS was stimulated an additional 2.8-fold by 10^{-6} M cAMP. The response to the cyclic nucleotide was abolished by the presence of the inhibitor, and the level of phosphorylation was about 15% lower than that of the basal activity, suggesting slight dissociation of the CNPK in the ROS. After the endogenous phosphorylation was subtracted, it was estimated that the phosphorylation of PKS by dark-adapted ROS was stimulated 4.4-fold by cAMP.

When the ROS homogenate was exposed to light, the levels of phosphorylation measured in the presence of PKS, under either basal, cAMP-stimulated, or cAMP + I inhibited conditions, were increased from the dark levels by the amounts which correspond to the light-stimulated endogenous phosphorylation under the respective assay conditions. The additivity of the cyclic nucleotide dependent phosphorylation of PKS and light-dependent phosphorylation of rhodopsin suggests the presence in ROS of two protein kinase activities which differ in substrate specificity and mechanism of acti-

Table II: Distribution of CNPK and RK in the Soluble and Membrane Fractions of Bovine ROS[a]

	endogenous		PKS		alum-treated ROS	
fraction	dark	light	dark	light	dark	light
soluble						
basal	0.073	0.061	0.117	0.072	0.025	1.875
cAMP	0.127	0.109	0.967	0.673	0.043	1.836
cAMP + I	0.095	0.073	0.132	0.079		
membrane						
basal	0.016	0.074	0.030	0.112		
cAMP	0.013	0.058	0.135	0.193		
cAMP + I	0.014	0.076	0.029	0.087		

[a] The soluble and membrane fractions of bovine ROS were obtained by centrifuging the homogenate of dark-adapted ROS at 100000*g* for 1 h. After removal of the supernatant (soluble fraction), the pellet was resuspended in 1 mL of buffer A and used as the membrane fraction. The protein kinase activities were determined as described under Methods and expressed as nanomoles of phosphate incorporated per minute per milligram of protein. Experiments run in duplicate with four to six ROS preparations gave consistent results. Values in this table represent results from one representative experiment.

vation.

(*2*) *CNPK and RK Activities Are Distributed in the Soluble and in the Membrane Fractions of Bovine ROS.* ROS homogenate was fractionated into soluble and membrane fractions by high-speed centrifugation, and Table II depicts the protein kinase activities present in the respective fractions. The soluble fraction contained endogenous proteins that served as the substrate for protein kinase. $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography of the phosphorylated endogenous proteins revealed that cAMP stimulated primarily the incorporation of phosphate into a 33 000 protein (results not shown). Owing to the low level of endogenous substrate, cAMP did not significantly increase the level of ^{32}P incorporation into this fraction when assessed by filtration techniques. Addition of exogenous PKS increased basal activity by 1.6-fold, and it enhanced CNPK activity by about 7.6-fold. Neither the endogenous nor the PKS phosphorylation by the soluble protein kinase was affected by light. On the other hand, while little phosphorylation was observed when alum-treated ROS membranes (as a source of rhodopsin) were added to the soluble fraction in dark, bleaching of the ROS membranes increased the level of phosphorylation by 7.5-fold. The phosphorylation of alum-treated ROS membranes was not affected by cAMP.

The dark-adapted membrane fraction of ROS contained little protein kinase activity that phosphorylated endogenous proteins. Exposure to light increased the phosphorylation of endogenous protein (bleached rhodopsin) by 4.6-fold. The presence of PKS enhanced the basal level of protein kinase in the dark-adapted membrane fraction 3.2-fold, and an additional 4.5-fold stimulation by cAMP was observed. Addition of the skeletal muscle inhibitor reduced PKS phosphorylation to the basal level. Thus, the membrane-bound CNPK activity appears to have the same characteristics as the soluble form. The level of phosphorylation, measured in the light and in the presence of PKS, equals the PKS phosphorylation measured in the dark plus the endogenous phosphorylation measured in the light. The additivity was found in the basal, cAMP, and cAMP + I conditions, suggesting that the membrane-bound CNPK and RK activities react independently.

(*3*) *Soluble CNPK and RK Can Be Separated by DEAE-cellulose Chromatography.* Three peaks of protein kinase activity were observed when the 100000*g* supernatant fraction

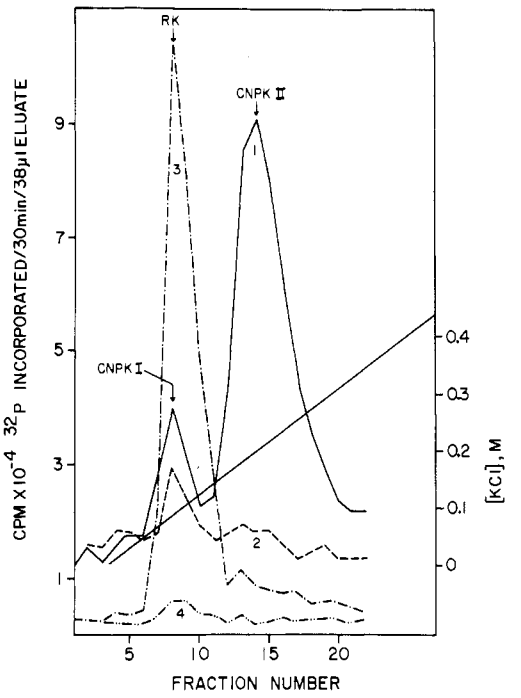

FIGURE 1: Soluble protein kinase of bovine ROS: fractionation by DEAE-cellulose. A soluble fraction from ROS, containing about 4.0 mg of protein, was applied to a DEAE-cellulose column (0.6 × 3.5 cm), which had been preequilibrated in 20 mM Tris-HCl, pH 7.6, containing 2 mM EDTA and 50 mM β-mercaptoethanol. After being washed with 1.0 mL of buffer, the column was developed with a linear gradient of KCl (30 mL; 0–0.4 M), and fractions of 1.3 mL were collected. Each fraction was assayed for the following protein kinase activities: phosphorylation of PKS in the presence [curve 1 (—)] or absence [curve 2 (- - -)] of 10^{-6} M cAMP; phosphorylation of alum-treated ROS membranes under light [curve 3 (---)] or dark [curve 4 (----)] conditions.

of bovine ROS was fractionated on a DEAE-cellullose column (Figure 1). The first and third peaks of activity eluted at 0.09 and 0.20 M KCl, respectively (curves 1 and 2). The protein kinases in both peaks phosphorylated PKS and histone (profile not shown) in a cyclic nucleotide dependent manner, but they did not phosphorylate the ROS membrane protein, rhodopsin. Phosphorylation of the soluble proteins was not affected by light or dark conditions under which the assay was carried out. According to Corbin et al. (1975), the first and third peaks of activity are designated to contain type I and type II cyclic nucleotide dependent protein kinases (CNPK I and CNPK II), respectively. CNPK II appears to be the predominant isoenzyme in bovine ROS, and it was further characterized. The amount of CNPK I activity varied somewhat with different preparations of ROS. The activity of CNPK II is stimulated by increasing concentrations of cAMP, with maximal activation achieved in the presence of 10^{-6} M cAMP. Higher concentrations of cGMP (10^{-5} M) than of cAMP were needed for the same extent of activation (unpublished experiments). The cyclic nucleotide stimulated activities can be totally inhibited by the skeletal muscle inhibitor (result not shown).

The second peak of protein kinase activity eluted at 0.10 M KCl (curves 3 and 4); it phosphorylated alum-treated ROS membranes in a light-dependent manner. However, it did not phosphorylate PKS or histone, and its activity was neither stimulated by cyclic nucleotides nor inhibited by skeletal muscle inhibitor. The characteristics of this enzyme are consistent with those reported for RK by Shichi & Somers (1978).

Rhodopsin kinase, which was only partially resolved from CNPK I on the DEAE-cellulose column, can be clearly dis-

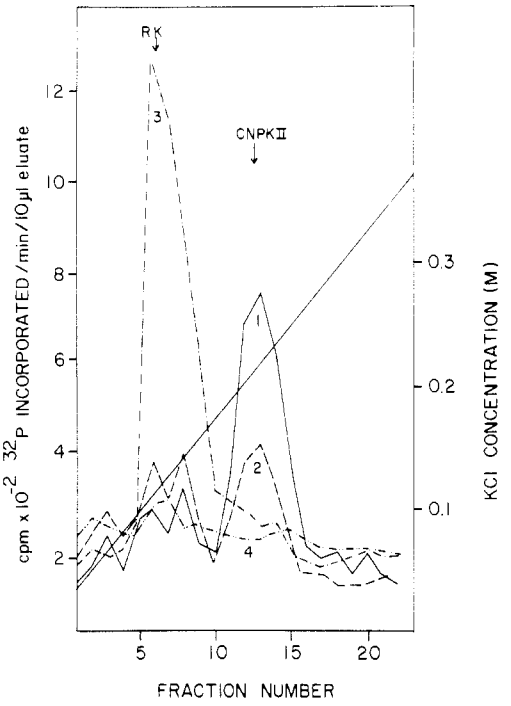

FIGURE 2: Fractionation of soluble protein kinases from a purified ROS preparation. The purification by gradient centrifugation of ROS and the determination of its purity are described under Methods. The soluble fraction was obtained and fractionated on a DEAE-cellulose column, as described in Figure 1.

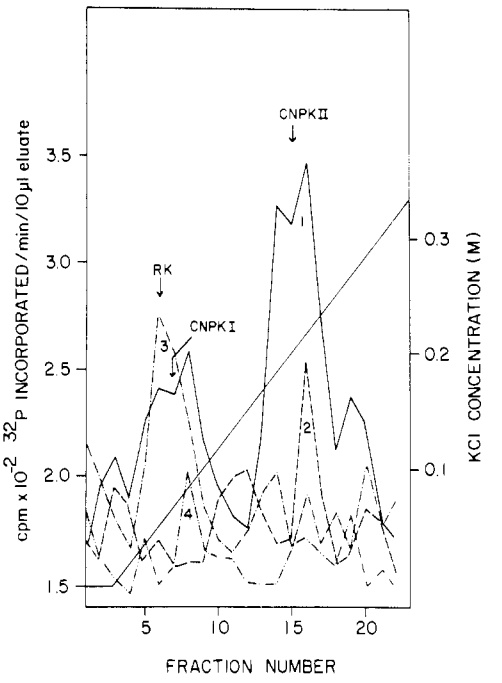

FIGURE 3: Fractionation of soluble protein kinases from microdissected ROS. ROS were prepared by microdissection of frozen bovine eyes, as described under Methods.

tinguished from the latter after rechromatography on a second DEAE column. It was found that the catalytic activity of CNPK I, characterized by PKS phosphorylation and by its inhibition in the presence of skeletal muscle inhibitor, was eluted in the void volume by 10^{-5} M cAMP, while the RK activity was eluted later by 0.1 M KCl (results not shown).

Identification of CNPK II in ROS Prepared by Gradient Centrifugation or Microdissection. The object of the studies described in Figures 2 and 3 was to verify that CNPK II was associated with bovine ROS and was not a contaminant from the inner layers of the retina or the basal portion of the visual cell. The enzymes obtained from the preparation described under Methods were compared to those from two other ROS preparations differing in technique and, therefore, differing probably in contaminating components.

The ROS prepared as described under Methods were further purified on a discontinuous gradient of sucrose (Papermaster & Dreyer, 1974). The ROS membranes had a spectral A_{280}/A_{498} ratio of 2.6 to 3.0. Analysis by DEAE chromatography of buffer A extracts of the purified ROS membranes (Figure 2) showed the presence of both CNPK II and RK; the extracts contained very little CNPK I activity.

ROS were prepared by microdissection of frozen sections of bovine eye as described under Methods. The ROS containing approximately 200 µg of protein were homogenized in buffer A, and a supernatant fraction was prepared by centrifugation at 100000*g* for 1 h. Analysis by DEAE chromatography of buffer A extracts (Figure 3) showed the presence of CNPK I, CNPK II, and RK activities.

Molecular Weights of CNPK II and RK. The molecular weights of CNPK and RK were estimated by gel filtration on Bio-Gel A-1.5m and Sephadex G-100 columns, respectively. Because of the instability of both enzymes, the 100000*g* supernatant of bovine ROS was used for the determination. The CNPK has an apparent molecular weight of 165 000 (Figure 4a) which agrees closely with that reported for pure CNPK II from bovine heart (170 000) (Erlichman et al., 1973). The CNPK contains not only catalytic activity for PKS phosphorylation but also binding activity for [^{3}H]cAMP and [^{3}H]-cGMP (results not shown). The molecular weight of RK, estimated as 68 000 by gel filtration (Figure 4b) and by Na-DodSO$_4$ gel electrophoresis (results not shown), is consistent with that reported by Kühn (1978) but different from that reported by Shichi & Somers (1978). As expected from its molecular weight, CNPK (characterized by its [^{3}H]cAMP binding activity) was eluted in the void volume of the Sephadex G-100 column.

The gel filtration studies also reveal that in the soluble fraction of bovine ROS, there is an additional protein kinase (molecular weight 45 000) which phosphorylates PKS in a cyclic nucleotide independent manner (Figure 4a). This kinase is not a catalytic subunit of either CNPK I or CNPK II, because its enzymic activity is not inhibited by the skeletal muscle inhibitor. The source and the physiological significance of this kinase remain to be investigated.

Discussion

A role for protein phosphorylation in the control of cellular function has been proposed for several tissues including muscle, brain, and retina. The phosphorylation of proteins using ATP or GTP as the phosphate donor is catalyzed by several types of protein kinase. The rate of protein phosphorylation is dependent upon the availability of suitable protein substrates and, in many cases, upon intracellular effector molecules. In rod visual cells of the retina, the rate of protein phosphorylation appears to be regulated by light and cGMP.

Our previous studies (Farber et al., 1979) demonstrated that bovine ROS contained both soluble and membrane-associated protein kinase activities. It was found also that the soluble activity was modulated by cyclic nucleotides whereas the membrane-associated activity was independent of cyclic nucleotides. From data that were then available, it was suggested that the activity of the soluble and membrane-associated fractions was catalyzed by a single type of protein kinase that showed different characteristics in the soluble and membrane-associated state. Our current findings, however, show

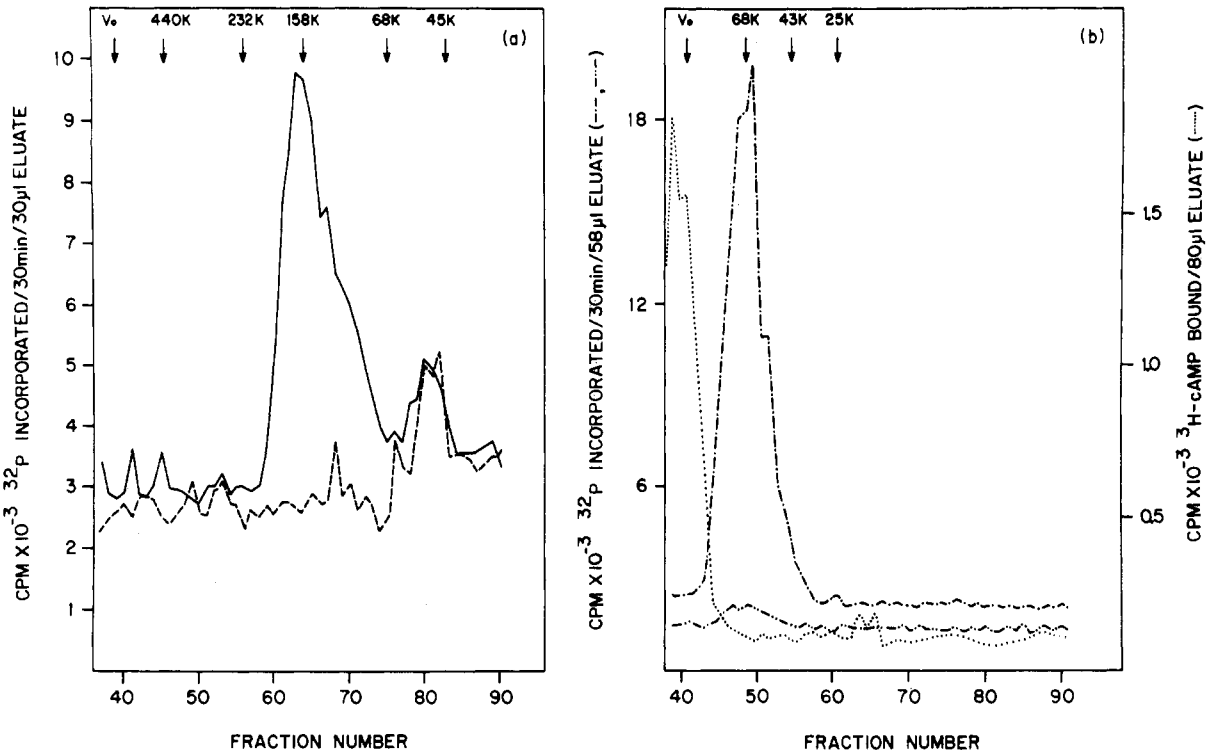

FIGURE 4: Determination of the molecular weights of CNPK II and RK by gel filtration. Procedures for the gel filtration determinations are described under Methods. (a) In the Bio-Gel A-1.5m column, CNPK activity was determined by phosphorylation of PKS in the presence of cAMP (—) and cAMP + I (---). (b) In the Sephadex G-100 column, RK activity was determined by phosphorylation of alum-treated ROS membrane under light (---) and dark (····) conditions; CNPK activity was determined by the binding of [^{3}H]cAMP (···). Vo, void volume.

conclusively that rod visual cells contain two categories of protein kinases that differ markedly in their physical and biochemical characteristics.

Bovine ROS possess a protein kinase enzyme with an apparent molecular weight of 68 000 that phosphorylates only bleached rhodopsin. Consistent with the findings of Kühn (1978) and Shichi & Somers (1978), we found that the enzyme which is designated RK can be readily solubilized from dark-adapted ROS, and the soluble enzyme does not phosphorylate various commonly used soluble protein kinase substrates. Rhodopsin kinase is equally inactive with dark-adapted ROS membranes that have been depleted of kinase activity, but it actively phosphorylates the same membrane preparation following illumination. Analysis of the phosphorylated membranes by $NaDodSO_4$–polyacrylamide gel electrophoresis demonstrates that rhodopsin is the only protein that is phosphorylated (Shichi & Somers, 1978). Moreover, the phosphorylation of rhodopsin is not modulated by cyclic nucleotides. Kühn (1978) has shown that RK is transferred from the cytosol to ROS membranes during illumination, and this observation has been confirmed by our findings that soluble RK reassociates with RK-depleted membranes to phosphorylate bleached rhodopsin. Our experiments provide an additional clue to the mechanism that regulates rhodopsin phosphorylation. We find that rhodopsin is phosphorylated in RK-depleted membranes only in the light and independent of whether the RK used for reassociation with the membranes was maintained in darkness or light. This suggests that rhodopsin phosphorylation is regulated by light-induced changes in the visual pigment molecule and that the activity of RK is limited in darkness by the lack of a suitable protein substrate for phosphorylation (Frank & Buzney, 1975).

The CNPK activity of bovine ROS is catalyzed by kinase enzymes (CNPK I and CNPK II) that are similar to those identified in other tissues. The CNPK II is the predominant enzyme with lesser amounts of CNPK I being present in most ROS preparations. The CNPK II appears to be localized in ROS because CNPK II actively sediments with RK during centrifugation of ROS on sucrose gradients, and RK and CNPK II both are present in microdissected samples that contain ROS predominantly. The CNPK II activity partitions after centrifugation of ROS homogenates with both the soluble and membrane fractions. The membrane-associated CNPK II can be solubilized from dark-adapted ROS almost completely during sequential extractions with low ionic strength buffers, and the extractability of CNPK II appears similar to that of RK. Illumination of ROS membranes does not appear to enhance the binding of CNPK II to the ROS membranes (result not shown) as it does for RK (Kühn, 1978).

The type II cyclic nucleotide dependent protein kinase (CNPK II) has an apparent molecular weight of 165 000 that phosphorylates several soluble proteins, including PKS and histone, in a cyclic nucleotide dependent manner. The endogenous substrates of this enzyme have not been evaluated fully. In bovine ROS, an endogenous protein, with an apparent molecular weight of 30 000–35 000, is phosphorylated in a cyclic nucleotide dependent manner (Lolley et al., 1977), whereas, in frog ROS, the smaller polypeptides (12 000 and 13 000) are phosphorylated in a cyclic nucleotide dependent fashion (Polans et al., 1979).

The study of CNPK II in bovine ROS has been facilitated by the fortuitous finding that PKS from rat small intestine served as a better substrate than histone in the phosphorylation reaction. When saturating amounts of either substrate are used, about 2–3-fold more ^{32}P is incorporated into PKS than into histone. The polypeptide in PKS that is phosphorylated by CNPK II has an apparent molecular weight of 14 000 on $NaDodSO_4$ gels (results not shown). This polypeptide may be favored over histone as a substrate for CNPK II because its structure resembles that of the endogenous substrate of the enzyme. The PKS was found while we were attempting, without success, to show that ROS contain a cGMP-specific

protein kinase. The PKS preparation is thought to contain the "stimulatory modulator" that Kuo et al. (1976) reported is necessary for the demonstration of cGMP-dependent protein kinase activity. In our experience, neither addition of PKS nor inclusion of high Mg^{2+} concentration in the reaction mixture revealed cGMP-specific protein kinase activity (Goldberg & Haddox, 1977). Our findings still support the hypothesis that light-induced changes in the cGMP concentration of ROS modulate the activity of CNPK II.

We initiated this study in order to identify the characteristics of the protein kinase(s) in bovine ROS. We have shown that there are two major types, RK and CNPK II. It is difficult to determine which enzyme is most active because they do not utilize the same protein substrate. They both appear to show substrate specificity, and, in the case of RK, the specificity for bleached rhodopsin appears to be absolute. The activity of both RK and CNPK II is apparently modulated indirectly by light-triggered visual pigment changes. The phosphorylation of bleached rhodopsin has been known for nearly a decade, but the functional significance of this event remains speculative (Kühn et al., 1977). The endogenous protein substrates of CNPK II are not clearly understood. They are believed to represent the ultimate effectors of cGMP activity in rod visual cells, and their isolation and characterization must rank as one of the important challenges of retinal biochemistry.

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