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ISOLATION AND RECOMBINATION OF BOVINE ROD OUTER SEGMENT cGMP PHOSPHODIESTERASE AND ITS REGULATORS

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SUMMARY: cGMP phosphodiesterase extracted from rod outer segments can be activated by GTP in the presence of phospholipid vesicles containing bleached rhodopsin. I have separated the phosphodiesterase from a phosphodiesterase inhibitory protein and a GTPase also present in the crude extracts from rods. The GTPase can be activated by bleached rhodopsin. However, in the absence of the GTPase and inhibitor, the phosphodiesterase was not activated by GTP in the presence of bleached rhodopsin. Recombination with these proteins partially restored the activation by GTP and bleached rhodopsin.

Rod outer segments contain several light activated enzymes, in particular a cGMP phosphodiesterase and a GTPase. Recent evidence suggests that activation of the phosphodiesterase by the bleaching of rhodopsin by light may be an important step in the visual transduction process (1,2). However, the detailed molecular mechanism of this process has not been elucidated. I report here the isolation and recombination of several proteins which appear to play an essential role in the regulation of bovine rod outer segment phosphodeisterase by light (3). Shinozawa et al. (4) have previously tried such a reconstitution from components isolated from frog rods, but they were not aware of the presence of the inhibitor.

MATERIALS AND METHODS

cGMP, GTP, trypsin, trypsin inhibitor, and snake venom (Ophiophagus hannah) were obtained from Sigma Chemical Company; Cellex D(DEAE-cellulose) from Bio-Rad Laboratories; Sephadex G200 and Sepharose 6B from Pharmacia Fine Chemicals. (^{3}H) cGMP, (^{14}C) 5'GMP and (γ-^{32}P) GTP were purchased from Amersham Corporation; (γ-^{32}P) GTP was also purchased from New England Nuclear. Dodecyltrimethylammonium bromide was obtained from Eastman. Protein concentration was accomplished using a single hollow fiber concentrator obtained from Bio-Med Instruments, Chicago, Illinois.

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cGMP phosphodiesterase was assayed as described elsewhere (5). GTPase was assayed by measuring ^{32}P release from (γ-^{32}P) GTP using activated charcoal to isolate the free phosphate from nucleotides (6).

Extraction of the rod outer segment phosphodiesterase and DEAE-cellulose chromatography were carried out as described elsewhere (6). Bovine retinae obtained from Hormel Co., Austin, Minn. were isolated by flotation on 42% sucrose, followed by a 15% sucrose wash. Phosphodiesterase was extracted by homogenization of the rods under dim red light in 10 mM Tris pH 8.0 and 1 mM dithiothreitol. The extract was applied to a 1.5 x 15 cm DEAE-cellulose column at 40 mM Tris pH 8.0, 1 mM $MgCl_2$ and 1 mM dithiothreitol. Inhibitor was eluted by washing with 40 mM Tris pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol and 100 mM NaCl. Phosphodiesterase and GTPase were then eluted together with a gradient from 100 mM to 500 mM NaCl.

Rhodopsin solubilized in dodecyltrimethylammonium bromide was purified from rod outer segment (7) by conconavalin A affinity chromatography (8). Vesicles were prepared by adding purified egg phosphotidyl choline (9) to purified rhodopsin in the ratio 100:1 and dialyzing 4 days to remove the detergent.

RESULTS

As reported elsewhere (3), a trypsin sensitive phosphodiesterase inhibitor is present in extracts from bovine rod outer segments which can be separated from the phosphodiesterase and the GTPase in the extracts by DEAE-cellulose chromatography. Here I first show that inhibitor free phosphodiesterase and GTPase from the DEAE-cellulose column can be quickly and efficiently separated by gel filtration chromatography. I isolated these proteins by the following procedures so that recombination experiments, described below, could be carried out.

Phosphodiesterase. Pooled phosphodiesterase from the DEAE-cellulose column was concentrated to a volume of 1 to 2 ml and applied to a Sephadex G200 column. A typical elution profile is shown in Fig. 1. GTPase activity is not recovered off this column but rather from a Sepharose 6B column; see below. The G200 column was used for phosphodiesterase isolation because it provided better resolution than the Sepharose column. The specific activity of purified phosphodiesterase from several preparations is concentration independent and ranges from 20 to 100 μmoles cGMP/min mg.

GTPase. A Sepharose 6B column was used to further purify the GTPase from the DEAE-cellulose column. GTPase yields appear to be significantly

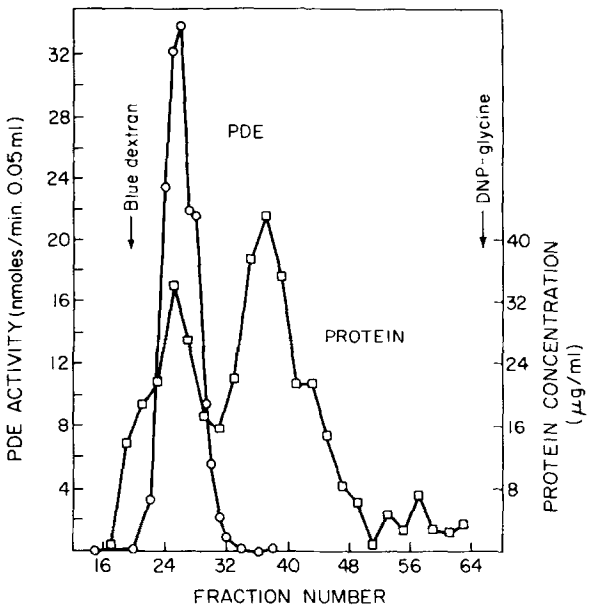

Figure 1. Typical elution profile of the phosphodiesterase on the Sephadex G200 column as described in the text. The column was 2.5 x 36 cm and was run at a flow rate of 5 ml/hr at 4°C with 50 mM Tris pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM phenylmethyl sulfonyl fluoride. Approximately 3 ml fractions were collected. Blue dextran was used to make the void volume and DNP-glycine to mark the included volume.

higher on agarose based columns than on dextran based columns. In addition, I find that the presence of 1 mM 5' GMP causes the GTPase to elute very late off the column, enhancing the separation of GTPase from phosphodiesterase. Nevertheless, as can be seen in Fig. 2, the GTPase is not completely free of phosphodiesterase activity. GTPase is typically recovered with about 20% yield. The GTPase fractions were pooled and dialyzed overnight against 50 mM Tris-Cl at pH 8.0, 1 mM $MgCl_2$ and 1 mM dithiothreitol to remove 5'GMP.

On SDS polyacrylamide electrophoresis gels, the major protein bands of the phosphodiesterase appear as a doublet at 84,000 and 88,000 daltons (Fig. 3b). The major bands associated with the GTPase appear at 36,000 and 39,000 daltons (Fig. 3c). The phosphodiesterase inhibitor of the DEAE-cellulose column appears as several major bands, none of which is yet positively identified as the inhibitor (3d). Purified rhodopsin vesicles show only rhodopsin both as a monomer at 39,000 and as a dimer at 80,000 daltons (3a)

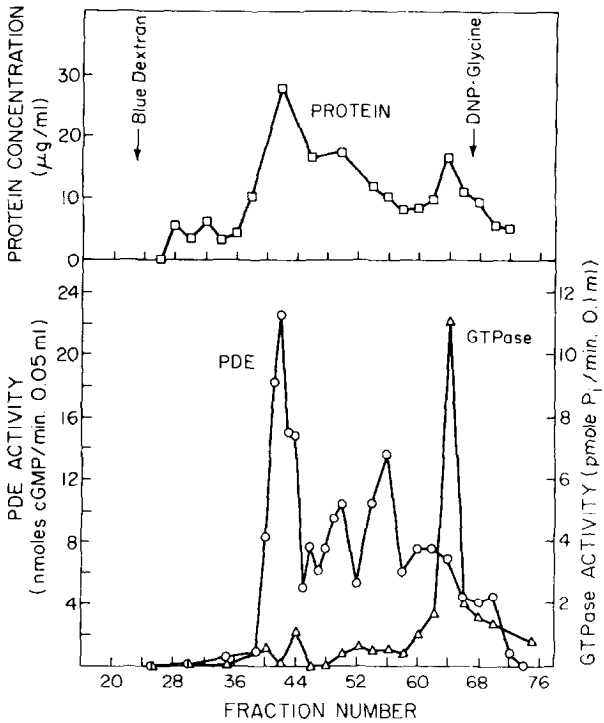

Figure 2. Typical elution profile of phosphodiesterase and GTPase off the Sepharose 6B column used to isolate GTPase for recombination experiments as described in the text. The column was 2.5 x 38 cm and was run at a flow rate of 7 ml/hr at 4°C with 40 mM Tris pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM phenylmethyl sulfonyl fluoride, and 1 mM 5' GMP. 3 ml fractions were collected. GTPase was assayed in the presence of bleached purified rhodopsin vesicles (5 μg rhodopsin).

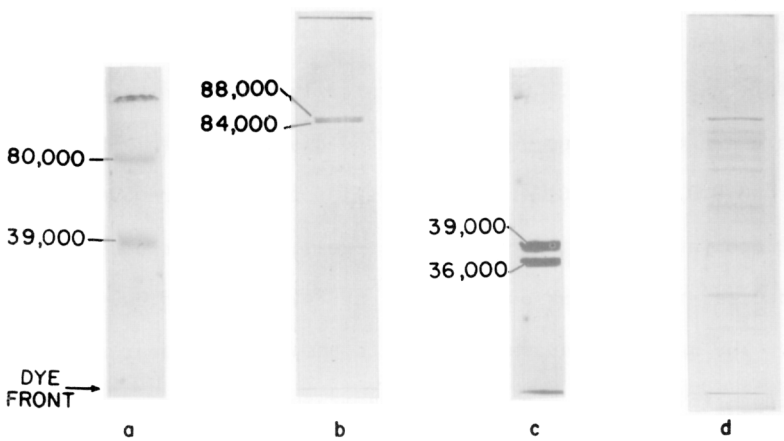

Figure 3. SDS polyacrylamide gel electrophoresis (10) of a) purified rhodopsin vesicles (~5 μg), b) gel filtration purified phosphodiesterase (2 μg), c) Sepharose 6B purified GTPase (~30 μg), d) DEAE-cellulose inhibitor (~50 μg). a), c), d) were run with 12.5% acrylamide; b) with 10.5%.

Table 1. RECOMBINATION OF PURIFIED ROD OUTER SEGMENT cGMP PHOSPHODIESTERASE WITH ITS REGULATORS. ~5 μg purified phosphodiesterase (PDE), ~6 μg purified GTPase (27 pmoles GTP/min), and ~30 μg DEAE-cellulose inhibitor were added where indicated. The numbers in parentheses are activities due to added PDE corrected for residual PDE activities in the isolated GTPase.

	Phosphodiesterase activity (nmoles cGMP/min)	
Recombination mixture	w/o GTP	with 100 μM GTP
Bleached rhodopsin + PDE	61	72
Bleached rhodopsin + PDE + inhibitor	35	31
Bleached rhodopsin + GTPase	14	19
Bleached rhodopsin + GTPase + inhibitor	2.6	3.8
Bleached rhodopsin + GTPase + PDE	68 (54)	74 (55)
Bleached rhodopsin + GTPase + PDE + inhibitor	29 (26)	59 (55)

The agarose purified GTPase is activated in the presence of bleached rhodopsin vesicles alone. However, Sephadex purified phosphodiesterase is not activated significantly by GTP in the presence of bleached purified rhodopsin vesicles (Table 1). In an attempt to restore this sensitivity to the phosphodiesterase, I have carried out recombination experiments. Addition of inhibitor to the phosphodiesterase in the presence of bleached rhodopsin vesicles decreases the phosphodiesterase activity in both the absence and presence of GTP; i.e., inhibitor alone does not restore bleached rhodopsin plus GTP sensitivity. However, when the GTPase is included in this recombination mixture, the inhibitor decreases phosphodiesterase activity in the absence of GTP but not when GTP is present. Thus, in the presence of bleached rhodopsin vesicles the combination of inhibitor and GTPase restores the GTP effect on the phosphodiesterase.

DISCUSSION

Hurley et al., (3,6) have recently reported the isolation of an inhibitor protein from crude extracts of bovine rod segments by DEAE-cellulose chromatography. This procedure allows recovery of inhibitor free phosphodiesterase and GTPase. Here I report that these enzymes can be separated by gel filtration chromatography. Furthermore, I provide evidence suggesting that the phosphodiesterase inhibitor and GTPase play an essential role in phosphodiesterase regulation by rhodopsin and GTP.

Isolated phosphodiesterase appears as an 84,000; 88,000 dalton doublet (3) on SDS polyacrylamide gels. Isolated GTPase appears as two major bands at 36,000 and 39,000 daltons. This GTPase fraction can be activated by phospholipid vesicles containing bleached rhodopsin. I have not yet assigned a molecular weight to the phosphodiesterase inhibitor.

The recombination experiments presented here are based on activation of phosphodiesterase by GTP in the presence of bleached rhodopsin vesicles as a criterion for successful recombination. Isolated phosphodiesterase is not significantly activated in this way (Table 1) suggesting the loss of regulatory proteins during purification. The results in Table 1 show that I have partially restored GTP and bleached rhodopsin activation to the isolation phosphodiesterase by recombination with tht GTPase and inhibitor. These results suggest a mechanism for phosphodiesterase regulation in which the inhibitor protein maintains the phosphodiesterase at low activity in the basal state. Activation occurs by bleached rhodopsin activating the GTPase, which in the presence of GTP, counteracts the effects of the inhibitor on the phosphodiesterase.

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