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GTPase from Rod Outer Segments: Characterization by Photoaffinity Labeling and Tryptic Peptide Mapping

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Summary: The photoaffinity label [γ-^{32}P]8-N_3GTP has been used to identify GTP-binding components in highly purified preparations of GTPase from bovine rod outer segments. These preparations contain two major polypeptides of 37,000 and 39,000 daltons. In the presence of photolyzing radiation, [γ-^{32}P]8-N_3GTP is covalently attached to the 37,000 dalton polypeptide. Tryptic peptide mapping of this polypeptide indicates that it is highly related to the 39,000 dalton species that has been previously identified as a GTP-binding component.

Introduction

It is generally accepted that bleaching of the rhodopsin molecule is the initial event in the visual excitation process occurring in retinal ROS (1). However, subsequent biochemical events leading to eventual hyperpolarization of the retinal plasma membrane are still less well understood. Studies of various ROS enzymes have demonstrated that a GTPase and a cGMP phosphodiesterase are both activated upon bleaching of rhodopsin (2-4). Activation of these enzymes occurs extremely rapidly (5,6), and is proportional to the amount of bleached rhodopsin in the ROS membrane (4). It is therefore highly possible that activation of these enzymes may be necessary events in the propagation of the visual excitation process in the retina.

Efforts to purify GTPase have demonstrated that activity is associated with peripherally-bound components that can be selectively eluted from bleached ROS

Abbreviations

ROS, rod outer segments; Buffer B, 10 mM 4-morpholinepropanesulfonic acid, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, pH 7.5, DPCC, diphenyl carbamyl chloride.

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membranes with micromolar concentrations of GTP (7,8). This preparation consists of major components of 37K and 39K (7-9). In this report, we have used photoaffinity labeling and tryptic peptide mapping to further characterize this GTPase preparation. Our results indicate that the substrate analogue [γ-^{32}P]8-N_3GTP selectively binds to the 37K component, and that both the 37K and 39K components are highly related in amino acid sequence.

Methods

Purification of GTPase - Retinas were dissected under dim red light from freshly killed bovine eyes that had been dark-adapted for 3-4 hrs. ROS were prepared from these retinas according to Baehr et al. (10). The GTPase was eluted from bleached ROS membranes with 40 µM GTP according to Kuhn (7), then further purified by elution from hexylagarose with 300 mM NaCl (9). One hundred microliters of the eluted fractions was assayed for GTPase in the presence of bleached rhodopsin vesicles as previously described (8,9), and the peak fractions pooled and used for radioiodination and photoaffinity labeling.

Photoaffinity labeling - 8-Azidoguanosine 5'-triphosphate (0.5 nmoles, 2 x 10^6 cpm), labeled in the γ position with [^{32}P] phosphate (11), was dissolved in methanol and dried onto the walls of 12 x 75 mm disposable test tubes. To these tubes was added 100 ul of the hexylagarose-purified material in a solution of Buffer B containing 300 mM NaCl and 4 mM $MnCl_2$. The tubes were vortexed vigorously and incubated at 0° C for 1 min. The contents were transferred to the bottom half of 35 x 10 mm plastic dishes (Falcon Plastics) and irradiated at a distance of 1 cm for 2 min. with the long wavelength mode of a mineral lite (Ultra-Violet Products, Model UVSL-25). The samples were then dissolved in sample buffer, and resolved on 7.5% polyacrylamide gels according to Laemmli (12). Human erythrocyte membrane proteins were used as molecular weight markers (13). Following electrophoresis, gels were stained, destained, dried and exposed to Kodak XRP-1 film and DuPont Cronex intensifying screens (14).

Radioiodination and Tryptic Peptide Mapping - The material eluting from the hexylagarose column was dialyzed overnite against 0.01M sodium phosphate, pH 7.5. The dialysate was dissolved in sodium dodecyl sulfate and radioiodinated with 200 µCi of ^{125}I (Amersham) according to Takemoto et al. (14). This material was resolved on a 7.5% polyacrylamide gel, and the 37K and 39K components excised from the gel and mapped in two-dimensions (14).

Results

Figure 1a illustrates the polypeptide composition of the material eluted from the hexylagarose column with 300 mM NaCl. The major radioiodinated species were components of 37K and 39K. When assayed for enzymatic activity, this fraction possessed significant GTPase activity that was measurable only when reconstituted with bleached vesicles of phosphatidylcholine and purified rhodopsin (Table I).

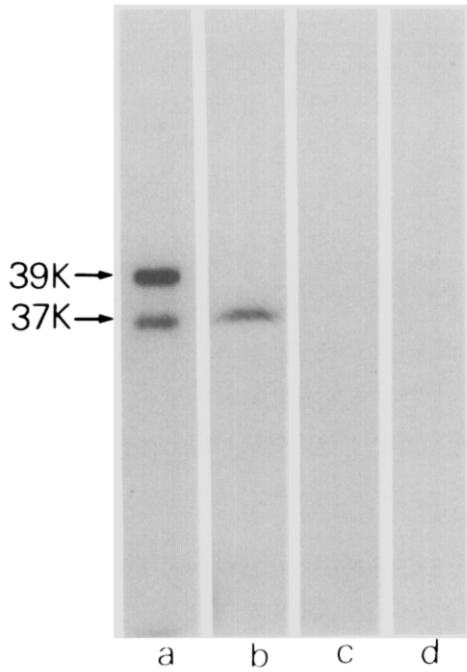

Figure 1: Autoradiography of purified GTPase treated with ^{125}I or [γ-^{32}P]8-N_3GTP. One-hundred microliters of material eluted by 300 mM NaCl was resolved after either a) radioiodination, b) photolysis with 5 uM [γ-^{32}P]8-N_3GTP, c) 5 uM [γ-^{32}P]8-N_3GTP with no photolysis, or d) photolysis with 5 uM [γ-^{32}P]8-N_3GTP after preincubation in 500 uM cold GTP. Following staining and destaining, the dried gel was exposed with Kodak XRP-1 film for 48 hours.

To ascertain which components actually bound GTP, the material eluted from hexylagarose was incubated in the presence of the photoaffinity analogue [γ-^{32}P] 8-N_3GTP. Only one component with an apparent molecular weight of 37,000 was covalently bound to the photoaffinity reagent under these conditions (Figure 1b). This binding was dependent upon photolyzing light (Figure 1c) and was absent when the GTPase preparation was preincubated in a 100X molar excess of cold GTP prior to addition of [γ-^{32}P]8-N_3GTP and photolysis (Figure 1d).

Based upon these results, it appears that at least the 37K component binds GTP and may therefore take part in the catalytic reaction. These results are reversed from those of Fung et al.(9), who found that only the 39K (and not the 37K) component bound guanosine 5'-[β- γ-imido]triphosphate, a nonhydrolyzable analogue of GTP. To better clarify the relationship between the 39K and 37K

Table I

GTPase Activity of Reconstituted Enzyme

Sample	GTPase Activity* (pmoles/3 min/assay tube)
Rhodopsin vesicles	N.D.
Hexylagarose-purified GTPase	N.D.
GTPase + Reconstituted Vesicles	200

* GTPase activity was measured as previously described (9) using 10 uM [γ-^{32}P]-GTP for 3 min at 37° C. Results are averages of duplicate samples. N.D., not detectable.

components, each radioiodinated band was excised from the polyacrylamide gel and digested with DPCC-treated trypsin.

Figure 2 illustrates the two-dimensional maps of the radioiodinated peptides from both these bands. It is apparent that the 39K and 37K components (Figure 2a and 2b) are highly related, with at least eight peptides (numbered 1-8) that co-migrate when a mixture of 39K and 37K peptides are resolved together (Figure 2c). In addition, both the 37K and 39K components possess unique radioiodinated peptides not found in the other species. For example, the 37K component possesses radioiodinated peptides a, b and c (Figure 2b) that are not found in the map of 39K (Figure 2a).

Discussion

Because of the possible importance of GTPase in the visual excitation process in the retina, numerous attempts have been made to identify the molecular species associated with this enzymatic activity. Recent reconstitution studies have strongly suggested that direct binding of GTPase and bleached rhodopsin is necessary for maximum enzymatic activity (7,8,15). Unambiguous identification of the catalytic moeity of GTPase would therefore be a necessary prerequisite to fully understanding the visual excitation process on a molecular level. All previous purification attempts have obtained major components of approximately

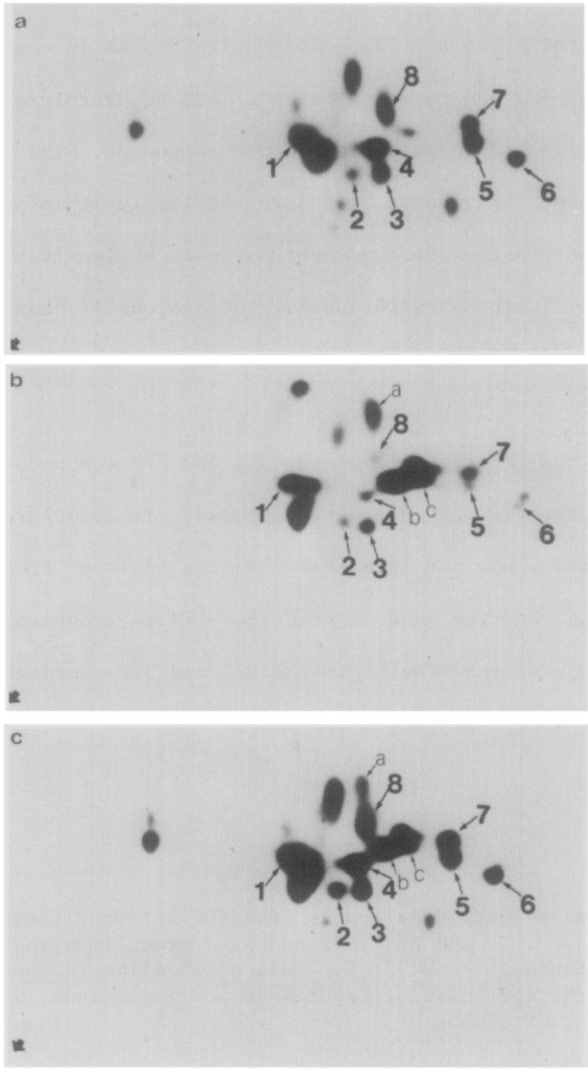

Figure 2: Mapping of radioiodinated tryptic peptides from the 37K and 39K components. Peptides containing 50,000 cpm were resolved in two dimensions as described by Takemoto et al. (14), followed by autoradiography using Kodak XRP-1 film and Cronex intensifying screens. Exposure was for 16 hours. a, 39K; b, 37K; c, mixture of equal counts of peptides from 39K and 37K.

37,000 and 39,000 daltons (7-9). To ascertain that these are the actual molecular components that bind GTP in the catalytic process, we have used [γ-^{32}P]8-N_3GTP, a radioactive photoaffinity analogue of GTP. This analogue has been previously used to characterize GTP-binding sites on the tubulin molecule (11,16).

Our results indicate that the major GTP-binding component of a highly purified GTPase preparation is the 37K component. This is in contrast to the results of Fung et al. (9) who report binding of the nonhydrolyzable analogue guanosine 5'-[β- γ-imido]triphosphate to the 39K component. The reasons for this discrepancy are not fully known, although sterically it is possible that [γ-^{32}P]8-N_3GTP may bind to the 39K component but not covalently attach to it following photolysis. In this regard, we have occassionally observed a small amount of binding of this photoaffinity analogue to the 39K component (Takemoto, D.J., unpublished).

To help clarify the relationship of the 39K and 37K components, maps of their radioiodinated tryptic peptides were compared. It is apparent that these two proteins are similar, but not identical. On the basis of these maps, the photoaffinity labeling, and the guanosine 5'-[β, γ-imido]triphosphate binding data of Fung et al. (9), we feel that both the 37K and 39K components probably possess binding specificity for GTP.

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