Light-activated hydrolysis of cyclic GMP is achieved through the photoexcitation of rhodopsin, a process which then triggers the replacement of GDP for GTP by a retinal guanosine 5'-triphosphatase referred to as 'transducin'. The transducin–GTP complex then switches on the phosphodiesterase [Fung, Hurley & Stryer (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 152–156]. The bovine transducin consists of an α-subunit (39000 Mr), which is a GTP-binding component, together with a β-(37000 Mr) and a γ-subunit (10000 Mr). We have purified retinal transducin from cow, pig, chick and frog. The enzyme specific activities and sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic profiles indicate that this enzyme is similar in all species except the frog. Whereas the bovine, pig and chick transducins consist of major 37000- and 39000-Mr components, that of the frog consists of a single 75000-Mr component. Labelling of the GTP-binding components with the photoaffinity label 8-azidoguanosine [γ-32P]triphosphate demonstrated that the 37000-Mr components of the cow, pig and chick and the 75000-Mr component of the frog were major GTP-binding components. In addition, peptide maps of radiiodinated tryptic peptides indicate that the frog 75000-Mr protein is highly related to the pig transducin. These results demonstrate evolutionary conservation of retinal transducin and the presence of a higher-Mr, but nonetheless highly conserved form, of transducin in the frog. The relationship of this component to the recently reported rod-outter-segment inhibitor protein [Yamazaki, Stein, Chernoff & Bitensky (1983) J. Biol. Chem. 258, 8188–8194] is discussed.

The retinal ROS is the light-activated system in which rhodopsin is photoisomerized from the 11-cis-retinal to the all-trans form. The outer segment contains a light-activated GTPase [also referred to as 'transducin' (Stryer et al., 1981)] and a cyclic GMP phosphodiesterase (Wheeler & Bitensky, 1977). The bleaching of rhodopsin activates the GTPase, which then activates the phosphodiesterase (Fung et al., 1981). The GTPase therefore acts as an amplified signal carrier in this light-activation phenomenon (Stryer et al., 1981).

Efforts to purify the GTPase have demonstrated that the activity is associated with peripherally bound components that are selectively eluted from bleached ROS membranes with micromolar concentrations of GTP (Hurley, 1980; Kühn, 1980). The purified enzyme consists of three subunits with Mr values corresponding to approx. 37000, 39000 and 10000 (Fung et al., 1981; Takemoto et al., 1981a). It has been reported that the purified 39000-Mr subunit contains a guanine-nucleotide-binding site (Fung et al., 1981a). All three subunits are apparently required for the photoexcited exchange of GTP for GDP (Fung, 1983). Recently, cholera toxin has been reported to inactivate the
GTPase activity of the retinal GTPase and to ADP-ribosylate the 39000- Mr subunit (Abood et al., 1982). This possible functional homology between the retinal GTPase and N protein (also called G- or G/F protein) of adenylate cyclase has been verified by peptide mapping of the purified polypeptides (Manning & Gilman, 1983).

Since the retinal GTPase is involved in the visual-amplification process, unambiguous identification of the catalytic and GTP-binding moieties is a necessary prerequisite for understanding the role that this enzyme plays. Tryptic-peptide maps of the radioiodinated 37000- and 39000- Mr subunits of this bovine transducin have demonstrated that these subunits are highly related, but not identical with each other (Takemoto et al., 1981a, b). Furthermore, 8-N 3 [γ-32P]GTP-labelling studies have indicated that the 37000- Mr subunit is a GTP-binding component (Takemoto et al., 1981a, b), and studies with p[NH]ppG have shown that the 39000- Mr subunit binds GTP (Fung et al., 1981). In contrast with bovine ROS, Yamazaki et al. (1983) have reported that elution of frog ROS membranes with GTP released a phosphodiesterase inhibitor, an occurrence that was accompanied by the release of a GTP-binding component. The inhibitor had an apparent Mr of 60000 on Sephadex G-100. Those authors considered that this 60000- Mr component was a complex of inhibitor and GTP-binding subunits. In an effort to determine whether this protein is related to ROS transducin from other species we have purified, photoaffinity-labelled, and tryptic-peptide-mapped these ROS proteins isolated from the frog, cow, pig and chick.

Materials and methods

Materials

Adult cow, chicken and pig eyes were obtained within 24h of death, from local slaughterhouses. Frog (Rana pipiens) eyes were obtained from adult frogs received live from Carolina Biological Supply Co., Burlington, NC, U.S.A.

Purification of retinal ROS GTPase

Retinas were dark-adapted for 2–4h before dissection under dim red light. ROS were prepared from these retinas as described by Baehr et al. (1979). The GTPase was eluted from bleached ROS membranes with 40 μM-GTP as described by Kühn (1980), then further purified by elution from hexyl-agarose with 300mM-NaCl (Fung et al., 1981). Table 1 illustrates a purification scheme for cow GTPase.

GTPase assay

GTPase was assayed by the method of Fung et al. (1981), as modified by Kühn (1980). The reaction mixture contained 0.05 μg of dialysed hexyl-agarose-purified enzyme and 5 μg of ROS membranes that had been depleted of GTPase activity (Fung et al., 1981). This reaction took place in an assay buffer consisting of 10mM-Mops, 2mM-MgCl2, 0.1mM-PMFS and 1mM-dithiothreitol, pH7.4, the total volume of the incubation mixture being 200μl. The reaction was initiated by the addition of 5 μM-[γ-32P]GTP substrate (227mCi/mmol; New England Nuclear Corp.). The reaction was terminated after 8 min at 37°C (Fung et al., 1981).

Preparation of depleted ROS membranes

ROS membranes were prepared and depleted of GTPase activity as previously described (Baehr et al., 1979). After the extraction of GTPase with 50μM-GTP, the membranes were re-extracted three to four additional times to ensure maximum depletion of bound enzyme.

SDS/polyacrylamide-gel electrophoresis and radioiodination of GTPase preparations

Samples (100μg) of the hexyl-agarose-purified GTPase were dialysed overnight against distilled water. Iodination was in 2% SDS/0.05M-Tris/HCl, pH7.5, using 500μCi of 125I (Amersham International; 100μCi/ml). The reaction was initiated by the addition of chloramine-T (0.25μg/μl final concn.) for 1 min. This reaction was quenched by the addition of an equal volume of sample buffer [1% (w/v) sucrose/2% (v/v) 2-mercaptoethanol/4% (w/v) SDS/0.063M-Tris/HCl, pH6.8].

Samples were electrophoresed by the method of Laemmli (1970). Electrophoresis was at 4°C for 2–4h. After the run the gel was stained in 0.1% Coomassie Brilliant Blue R in propan-2-ol/acetic acid/water (5:2:13, by vol.) for 7–8h. Gels were destained, dried, and exposed to Kodak XRP-1

Table 1. Hexyl-agarose column elution profile of cow GTPase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Reconstituted activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.069</td>
</tr>
<tr>
<td>2</td>
<td>0.071</td>
</tr>
<tr>
<td>3</td>
<td>0.076</td>
</tr>
<tr>
<td>4 (After 80mM-NaCl)</td>
<td>0.098</td>
</tr>
<tr>
<td>5 (After 300mM-NaCl)</td>
<td>0.230</td>
</tr>
</tbody>
</table>
film as described by Takemoto et al. (1981b). Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as standard. Human erythrocyte membrane proteins were used as M₀ markers for SDS/polyacrylamide-gel electrophoresis (Fairbanks et al., 1971).

**Preparation of 8-N₃ GTP**

The 8-N₃ [γ-³²P]GTP was prepared from unlabelled 8-N₃ GTP. Approx. 200 μCi of [³²P]PO₄ (Amersham) were added to a plastic test tube under dim light. The reaction mixture contained 125 μl of 0.1M-Tris/HCl, pH 7.5, 100 μl of 0.1M sucrose, 5 μl of 0.05M-MgCl₂, 5 μl of 0.02M cysteine hydrochloride, 20 μl of 0.05M-glyceraldehyde 3-phosphate, 5 μl of 1mM-NAD and 500 μl of 0.1M-8-N₃ GTP.

The reaction was initiated by the addition of glyceraldehyde-3-phosphate dehydrogenase (70 units/mg; Sigma) and 3-phosphoglycerate kinase (2500 units/mg; Sigma) in 10mM-Tris/HCl, pH 7.4. The reaction was for 30 min at 30°C. The 8-N₃ [γ-³²P]GTP was separated from free [γ-³²P]PO₄ in t.l.c. plates (PEI-cellulose, Eastman) by using a 1M-formic acid/0.5M-LiCl solvent system. Incorporation of ³²P into 8-N₃ GTP was 80–90%.

**Photoaffinity labelling**

8-N₃ [γ-³²P]GTP (0.5 nmol, 2 × 10⁶ c.p.m.) was dissolved in methanol and dried on to the walls of 12 mm × 75 mm disposable test tubes. To these tubes was added 100 μl of each hexyl-agaroce-purified material in 300 mM-NaCl/4 mM-MnCl₂. Tubes were vortex-mixed vigorously and incubated at 0°C for 1 min. The contents were transferred to the bottom half of 35 mm × 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) (Takemoto et al., 1981a).

**Tryptic-peptide mapping**

Proteins were solubilized, radioiodinated, and resolved by gel electrophoresis as described above. Preparation of gels and autoradiography were as previously described, Kodak XRP-1 film and Dupont Cronex intensifying screens being employed (Takemoto et al., 1981b). After autoradiography the band were excised from the dried gel and digested with DPCC-treated trypsin (0.05 mg/ml; Sigma, type XI) at 37°C for 14 h in 12 mm × 10 mm glass tubes. After digestion, samples were freeze-dried, then dissolved in 5 μl of distilled water. Approx. 30 000 c.p.m. was spotted on to an Eastman cellulose plate (10 cm × 20 cm; Eastman 13255) and resolved by using the two-dimensional system of Elder et al. (1977).

### Results

Retinal ROS GTPase activity is measurable only when reconstituted with bleached vesicles of phosphatidylincholine and purified rhodopsin (Takemoto et al., 1981a) or with GTPase-depleted ROS membranes (Kühn, 1980). Fig. 1 illustrates that the bovine ROS GTPase exhibits a linear increase in activity with increasing concentration of depleted ROS membranes to 5 μg of protein. Similar results were obtained using GTPase purified from the pig, chick and frog (results not shown). Ideal reaction conditions for each enzyme were found to be 0.05 μg of GTPase with 5 μg of depleted ROS membranes assayed for 3–8 min at 37°C.

Table 2 illustrates that the specific activities of the GTPases are similar from all four species. Basal activity (without added ROS membranes) varied from 4 to 6 nmol of P₁ liberated/min per μg of enzyme. Reconstituted activity increased by approx. 4–8-fold, depleted ROS membranes showed no GTPase activity, and bovine serum albumin did not substitute for the ROS membranes (results not shown).

Fig. 2 illustrates the profile of the radioiodinated, hexyl-agaroce-purified GTPases from the pig, cow, chick and frog. Two major bands...
Table 2. Specific activity of GTPase

ROS protein (5 μg) and enzyme (0.05 μg) were used in each assay for reconstituted activity. The increase (fold) is a relative term indicating the enhancement produced by the addition of the ROS. The 0.05 μg of enzyme was prepared by dilution from a 10 μg/ml stock solution in all cases.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reconstituted</th>
<th>Basal</th>
<th>Increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>29.0</td>
<td>6.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Cow</td>
<td>29.0</td>
<td>4.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Chick</td>
<td>31.0</td>
<td>3.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Frog</td>
<td>30.0</td>
<td>4.0</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Fig. 2. Protein profiles of purified and radioiodinated GTPases from ROS of different species

After purification by hexyl-agarose chromatography, the GTPase preparations were radioiodinated and resolved in 7.5% (w/v) polyacrylamide gels. The gels were dried and exposed to Kodak XRP-1 film for 6–16 h at −70°C. The Figure shows GTPases from: (a) pig; (b) cow; (c) chick; (d) frog. Abbreviation used: PDE, phosphodiesterase.

Fig. 3. Effect of freeze–thawing on the apparent Mr of purified GTPase

After purification by hexyl-agarose chromatography, the purified GTPase preparation was freeze–thawed five times at temperatures of −70 and 22°C respectively. The preparation was then radioiodinated, resolved on 7.5% (w/v) polyacrylamide gels, dried, and exposed to X-ray film for 16 h at −70°C. (a) pig GTPase after freeze–thawing; (b) frog GTPase before freeze–thawing.

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Fig. 4. Photoaffinity labelling of purified GTPase preparations from different species

(a), (c) and (e) are the autoradiographs of radioiodinated preparations; (b), (d) and (f) are autoradiographs of each preparation after treatment with the photoaffinity label, [γ-32P]8-N3 GTP. (a) and (b), pig GTPase; (c) and (d), bovine GTPase; (e) and (f), frog GTPase.

ponent of the pig cannot be converted to the smaller subunits.

The results of photoaffinity-labelling the purified GTPase are shown in Fig. 4. The 37000-Mr subunit of the pig and cow enzyme were labelled with the 8-N3[γ-32P]GTP in a competitive manner (Figs. 4b and 4d). Only the 75000-Mr, GTPase purified from the frog retinal ROS was labelled (Fig. 4f), indicating that it is a major GTP-binding component. When the pig and chick GTPase were subjected to freeze-thawing, the appearance of a 75000-Mr aggregate paralleled labelling of this component with [γ-32P]8-N3 GTP (results not shown). In all cases the specificity of binding was verified by competition with excess of unlabelled GTP.

On the basis of the results of SDS/polyacrylamide-gel electrophoresis, freeze-thawing and photoaffinity of GTPase from different species, it is possible that the 75000-Mr, GTPase from frog ROS may indeed be related to the 39000- and 37000-Mr, GTPase components from other species. Tryptic maps of the radioiodinated peptides of these polypeptides demonstrate extensive homologies (Fig. 5). At least six peptides (numbered 1–6) are found in both the 75000-Mr component of the frog and the 39000-Mr, component of the pig. In addition, peptide mapping also demonstrated that the 39000-Mr and the 37000-Mr, components from all species were homologous (results not shown).

Fig. 5. Maps of radioiodinated tryptic peptides from the GTPases of chick and frog

After radioiodination and resolution by SDS/polyacrylamide-gel electrophoresis, the 39000-Mr band of chick GTPase and the 75000-Mr band of frog GTPase were treated with trypsin, and the resulting peptides were resolved by electrophoresis in the first dimension (left to right) and chromatography in the second dimension (bottom to top). Peptides were revealed by autoradiography for 48 h at −70°C. Numbered arrows indicate some peptides that are common to both species. Bold arrows indicate the sample origin. A, 75000-Mr, component of frog GTPase; B, 39000-Mr, component of chick GTPase.

Taken together with the previously published homologies of the bovine 39000-Mr, and 37000-Mr, components (Takemoto et al., 1981a), the results demonstrate that the GTPases from all species studied comprise a family of highly conserved polypeptides of M, 75000 (frog dimer) and 39000 + 37000 (bovine, pig, chick).

Discussion

Because of the importance of GTPase in the visual-excitation process of the retina, numerous attempts have been made to identify the molecular species associated with catalytic activity and with phosphodiesterase binding. We (Takemoto et al., 1981a, b) and others (Kühn, 1980; Fung et al., 1981) have obtained major components of Mr, approx. 37000 and 39000 from hexyl-agarose-purified retinal GTPase from bovine ROS. In addition, Fung et al. (1981) reported a smaller component of Mr, 10000. We have not observed this component in our purified preparations with numerous gel
systems either using radioiodinated samples or on stained gels.

To date no information is available on the GTPases from ROS of other species. In order to ascertain whether the 37000-\(M_r\) and 39000-\(M_r\) components are associated with GTPase activity in other species, we have purified the ROS GTPases from cow, pig, chick and frog. Our results indicate that, indeed, the 37000-\(M_r\) and 39000-\(M_r\) components are associated with GTPase activity in cow, pig and chick. However, the GTPase isolated from the frog migrated at 75000 \(M_r\) on SDS/polyacrylamide-gel electrophoresis. Although freeze–thawing of pig and chick GTPase may cause a 75000-\(M_r\) band to appear in these samples, numerous treatments of the frog 75000-\(M_r\) component failed to cause the appearance of 37000-\(M_r\) and 39000-\(M_r\) components. In very fresh preparations of frog, very faintly staining bands at 37000 \(M_r\) and 39000 \(M_r\) were observed.

As shown in the tryptic-peptide maps, it appears that the 75000-\(M_r\) component of the frog contains peptides similar to both the 37000 and 39000-\(M_r\) components. This is consistent with our previous observation that the 37000- and 39000-\(M_r\) components of bovine ROS are highly related (Takemoto et al., 1981a, b). Yamazaki et al. (1983) have recently reported that elution of frog ROS membranes with p[NH]ppG results in the appearance of a phosphodiesterase inhibitor with an apparent \(M_r\) of 60000. This was accompanied by the release of a GTP-binding protein. The authors hypothesized that the 60000-\(M_r\) component could represent a complex of the inhibitor and GTP-binding protein. Since our GTPase preparations are eluted in the presence of GTP, the frog 75000-\(M_r\) component that we observe may be similar to this complex; certainly it possesses GTP-binding capacity and GTPase activity. Furthermore, the peptide map of the 75000-\(M_r\) species from the frog and the 39000-\(M_r\) species of the pig suggest a high degree of relatedness. In all cases, however, the specific activities of the hexyl-agarose-purified GTPase and the requirement for exogenous depleted ROS membranes are very similar. It appears, therefore, that the frog GTPase may have a greater tendency to form a higher-\(M_r\) aggregate than the GTPase from other species.

In order to determine possible GTP-binding components of this enzyme, we labelled each purified GTPase with the photoaffinity GTP analogue 8-N\(_3\) \([\gamma^3P]\)GTP. This analogue has been previously reported to label the 37000-\(M_r\) component of the GTPase purified from bovine ROS (Takemoto et al., 1981a, b). Our results indicate that the 37000-\(M_r\) subunit is also a major GTP-binding component of the ROS GTPases purified from pig and chick retina. The 75000-\(M_r\) component of the frog was labelled with this analogue. Fung et al. (1981) reported binding of the analogue p[NH]ppG to the 39000-\(M_r\) component of bovine ROS GTPase. The reasons for this discrepancy are not known. However, since these analogues differ sterically, it is quite possible that the labelling reflects a difference in affinity for GTP. It has been suggested that the 39000-\(M_r\) component has a GTP-binding site, whereas the 37000-\(M_r\) component may be somehow involved in the exchange of GTP for GDP (Fung et al., 1981; Fung, 1983). It appears, therefore, that the 8-N\(_3\) \([\gamma^3P]\)GTP may label a catalytic moiety, whereas the p[NH]ppG may label a separate GTP-binding protein.

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