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Interspecies conservation of retinal guanosine 5′-triphosphatase

Characterization by photoaffinity labelling and tryptic-peptide mapping

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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 M_r), which is a GTP-binding component, together with a β-(37000 M_r) and a γ-subunit (10000 M_r). 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-M_r components, that of the frog consists of a single 75000-M_r component. Labelling of the GTP-binding components with the photoaffinity label 8-azidoguanosine [γ-^{32}P]triphosphate demonstrated that the 37000-M_r components of the cow, pig and chick and the 75000-M_r component of the frog were major GTP-binding components. In addition, peptide maps of radioiodinated tryptic peptides indicate that the frog 75000-M_r protein is highly related to the pig transducin. These results demonstrate evolutionary conservation of retinal transducin and the presence of a higher-M_r, but nonetheless highly conserved form, of transducin in the frog. The relationship of this component to the recently reported rod-outer-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 M_r values corresponding to approx. 37000, 39000 and 10000 (Fung *et al.*, 1981; Takemoto *et al.*, 1981*a*). It has been reported that the purified 39000-M_r subunit contains a guanine-nucleotide-binding site (Fung *et al.*, 1981*a*). 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

Abbreviations used: ROS, rod outer segment(s); GTPase, guanosine 5-triphosphatase; 8-N_3 GTP, 8-azidoguanosine triphosphate; p[NH]ppG, guanosine 5′-[β,γ]imidotriphosphate; Mops, 4-morpholinepropanesulphonic acid; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate; PEI, poly(ethyleneimine); DPCC, diphenylcarbamoyl chloride.

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GTPase activity of the retinal GTPase and to ADP-ribosylate the 39000-M_r 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-M_r subunits of this bovine transducin have demonstrated that these subunits are highly related, but not identical with each other (Takemoto *et al.*, 1981*a*, *b*). Furthermore, 8-N_3 [γ-^{32}P]GTP-labelling studies have indicated that the 37000-M_r subunit is a GTP-binding component (Takemoto *et al.*, 1981*a*, *b*), and studies with p[NH]ppG have shown that the 39000-M_r 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 M_r of 60000 on Sephadex G-100. Those authors considered that this 60000-M_r 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 40μM-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 0.05μg of dialysed hexyl-agarose-purified enzyme and 5μg 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-$MgCl_2$, 0.1mM-PMSF and 1mM-dithiothreitol, pH7.4, the total volume of the incubation mixture being 200μl. The reaction was initiated by the addition of 5μM-[γ-^{32}P]GTP substrate (227mCi/mmol; New England Nuclear Corp.). The reaction was terminated after 8min at 37°C (Fung *et al.*, 1981).

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

Fractions were collected immediately after the extracted material had been loaded on to the column. The fraction volumes were 4ml each and 10μl of each fraction was assayed for GTPase activity. Activities are expressed as pmol of P_i liberated/min and are the averaged results from two different samples. Samples containing salt were not dialysed before assay; thus an equal molar amount of salt was added to the buffer and controls in these cases.

Fraction	Reconstituted activity
1	0.069
2	0.071
3	0.076
4 (After 80mM-NaCl)	0.098
5 (After 300mM-NaCl)	0.230

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 ^{125}I (Amersham International; 100mCi/ml). The reaction was initiated by the addition of chloramine-T (0.25μg/μl final concn.) for 1min. 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

film as described by Takemoto *et al.* (1981*b*). Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as standard. Human erythrocyte membrane proteins were used as M_r markers for SDS/polyacrylamide-gel electrophoresis (Fairbanks *et al.*, 1971).

Preparation of 8-N_3 GTP

The 8-N_3 [γ-^{32}P]GTP was prepared from unlabelled 8-N_3 GTP. Approx. 200μCi of [^{32}P]PO_4^{3-} (Amersham) were added to a plastic test tube under dim light. The reaction mixture contained 125μl of 0.1M-Tris/HCl, pH7.5, 100μl of 0.1M-sucrose, 5μl of 0.05M-$MgCl_2$, 5μl of 0.02M-cysteine hydrochloride, 20μl of 0.05M-glyceraldehyde 3-phosphate, 5μl of 1mM-NAD and 500μl of 0.1mM-8-N_3 GTP.

The reaction was initiated by the addition of glyceraldehyde-3-phosphate dehydrogenase (70 units/mg; Sigma) and 3-phosphoglycerate kinase (2500units/mg; Sigma) in 10mM-Tris/HCl, pH7.4. The reaction was for 30min at 30°C. The 8-N_3 [γ-^{32}P]GTP was separated from free [γ-^{32}P]PO_4^{3-} on t.l.c. plates (PEI-cellulose, Eastman) by using a 1M-formic acid/0.5M-LiCl solvent system. Incorporation of ^{32}P into 8-N_3 GTP was 80–90%.

Photoaffinity labelling

8-N_3 [γ-^{32}P]GTP (0.5nmol, 2×10^6c.p.m.) was dissolved in methanol and dried on to the walls of 12mm × 75mm disposable test tubes. To these tubes was added 100μl each of hexyl-agarose-purified material in 300mM-NaCl/4mM-$MnCl_2$. Tubes were vortex-mixed vigorously and incubated at 0°C for 1min. The contents were transferred to the bottom half of 35mm × 10mm plastic dishes (Falcon Plastics) and irradiated at a distance of 1cm for 2min with the long-wavelength mode of a Mineral Lite (Ultra Violet Products, model UVSL-25) (Takemoto *et al.*, 1981*a*).

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.*, 1981*b*). After autoradiography the band were excised from the dried gel and digested with DPCC-treated trypsin (0.05mg/ml; Sigma, type XI) at 37°C for 14h in 12mm × 10mm glass tubes. After digestion, samples were freeze-dried, then dissolved in 5μl of distilled water. Approx. 30000c.p.m. was spotted on to an Eastman cellulose plate (10cm × 20cm; 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 phosphatidylcholine and purified rhodopsin (Takemoto *et al.*, 1981*a*) 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 5μg 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 0.05μg of GTPase with 5μg of depleted ROS membranes assayed for 3–8min 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 6nmol of P_i 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-agarose-purified GTPases from the pig, cow, chick and frog. Two major bands

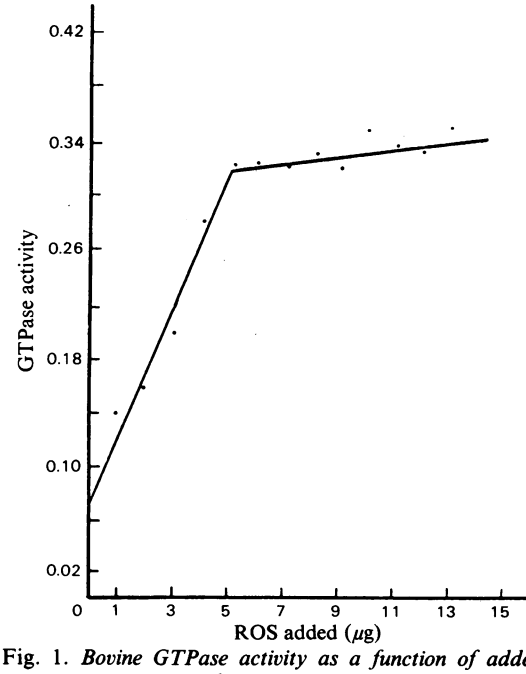

Fig. 1. *Bovine GTPase activity as a function of added bovine ROS*

Activity is expressed as pmol of P_i liberated/min per pg of GTPase. See the Materials and methods section for further details of the assay.

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.

Species	Specific activity (nmol of P_i liberated/min per μg of enzyme added)		Increase (fold)
	Reconstituted	Basal	
Pig	29.0	6.4	4.5
Cow	29.0	4.6	6.4
Chick	31.0	3.6	8.6
Frog	30.0	4.0	7.5

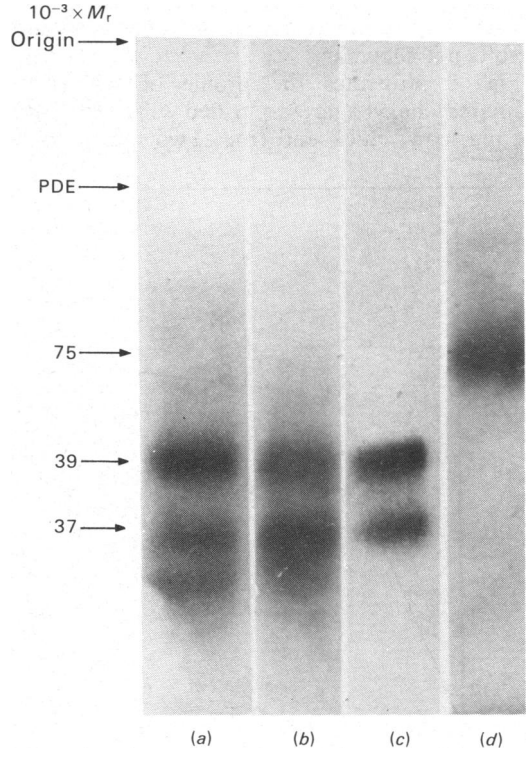

Fig. 2. *Protein profiles of purified and radioiodinated GTPases from ROS of different species*
After purification by hexyl-agarose chromatography, the GTPase preparations were radioiodinated and resolved in 7.5% (w/v) polyacrylamide gels. The gels were dried and exposed to Kodak XRP-1 film for 6–16 h at −70°C. The Figure shows GTPases from: (*a*) pig; (*b*) cow; (*c*) chick; (*d*) frog. Abbreviation used: PDE, phosphodiesterase.

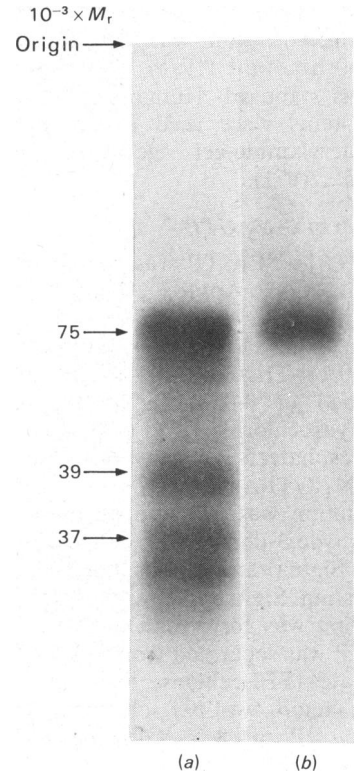

Fig. 3. *Effect of freeze–thawing on the apparent M_r of purified GTPase*
After purification by hexyl-agarose chromatography, the purified GTPase preparation was freeze–thawed five times at temperatures of −70 and 22°C respectively. The preparation was then radioiodinated, resolved on 7.5% (w/v) polyacrylamide 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.

corresponding to M_r 37000 and 39000 were present in the pig, cow and chick (Figs. 2*a*, 2*b* and 2*c*). This is similar to previous results reported for the cow GTPase (Kühn (1980). However, the frog exhibited only one band of approx. M_r 75000 (Fig. 2*d*).

Fig. 3 shows that, after freeze–thawing of the purified GTPase from the pig, a similar 75000-M_r component appears in the gels. This was also found in the chick and, to a much smaller extent, in the cow (results not shown). The appearance of this higher-M_r species always accompanied a decrease in the intensities of the two lower bands. Freeze–thawing of the frog 75000-M_r species, however, did not cause the reappearance of 39000-M_r or 37000-M_r components, and treatment with 2-mercaptoethanol or boiling also had no effect (results not shown). Likewise the 75000-M_r com-

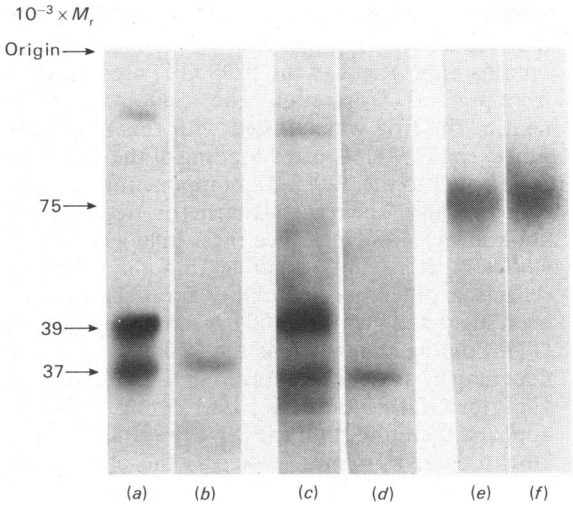

Fig. 4. *Photoaffinity labelling of purified GTPase preparations from different species*

(*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-^{32}P]8-N_3 GTP. (*a*) and (*b*), pig GTPase; (*c*) and (*d*), bovine GTPase; (*e*) and (*f*), frog GTPase.

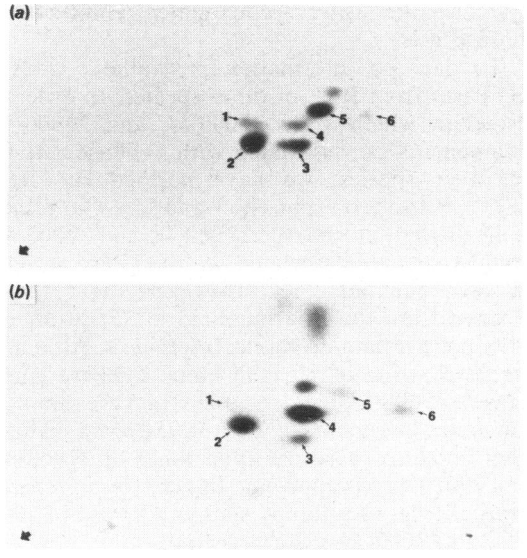

Fig. 5. *Maps of radioiodinated tryptic peptides from the GTPases of chick and frog*

After radioiodination and resolution by SDS/polyacrylamide-gel electrophoresis, the 39000-M_r band of chick GTPase and the 75000-M_r 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 48h at −70°C. Numbered arrows indicate some peptides that are common to both species. Bold arrows indicate the sample origin. A, 75000-M_r component of frog GTPase; B, 39000-M_r component of chick 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-M_r subunit of the pig and cow enzyme were labelled with the 8-N_3[γ-^{32}P]GTP in a competitive manner (Figs. 4*b* and 4*d*). Only the 75000-M_r GTPase purified from the frog retinal ROS was labelled (Fig. 4*f*), 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-M_r aggregate paralleled labelling of this component with [γ-^{32}P]8-N_3 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-M_r GTPase from frog ROS may indeed be related to the 39000- and 37000-M_r 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-M_r component of the frog and the 39000-M_r component of the pig. In addition, peptide mapping also demonstrated that the 39000-M_r and the 37000-M_r components from all species were homologous (results not shown). Taken together with the previously published homologies of the bovine 39000-M_r and 37000-M_r components (Takemoto *et al.*, 1981*a*), the results demonstrate that the GTPases from all species studied comprise a family of highly conserved polypeptides of M_r 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.*, 1981*a*, *b*) and others (Kühn, 1980; Fung *et al.*, 1981) have obtained major components of M_r approx. 37000 and 39000 from hexyl-agarose-purified retinal GTPase from bovine ROS. In addition, Fung *et al.* (1981) reported a smaller component of M_r 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 could 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.*, 1981*a*, *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 [γ-^{32}P]GTP. This analogue has been previously reported to label the 37000-M_r component of the GTPase purified from bovine ROS (Takemoto *et al.*, 1981*a*, *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 [γ-^{32}P]GTP may label a catalytic moiety, whereas the p[NH]ppG may label a separate GTP-binding protein.

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