INTERSPECIES CONSERVATION OF RETINAL GUANOSINE 5' TRIPHOSPHATASE.

by

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I. INTRODUCTION

The vertebrate retinal rod is a modified sensory receptor specially adapted for photon detection and is responsible for black-and-white vision. [1] The rod can be divided into three morophologically distinct components, each with a separate and unique function. The outer segment develops from a modified sensory cilium and is responsible for light detection; the inner segment is connected to the outer segment by a cytoplasmic bridge and carries out the cell's metabolic and synthetic functions. The synaptic region transmits information from the retinal rod to the rest of the brain.

Structurally the rod outer segment (hereafter designated simply as ROS) is composed of several hundred to several thousand (varying from species to species) flattened, closed vesicles called discs, which are surrounded by a plasma membrane. [2] The lumen of the discs does not communicate with the extra-cellular space. [3] In the vertebrate retina, new discs are constantly being formed by transverse invagination and endocytosis of the plasma membrane at the base of the ROS. Loss of old discs occurs by a pinching off within a plasma membrane envelope at the tip of the ROS. [4,5,6] This rate of turnover varies within species, (i.e.,

one to two weeks in mammals and one to two months in amphibia) and the rate can be accelerated upon illumination. [1,4] The plasma membrane constitutes only 0.5% of the total ROS membrane while the disc membrane makes up the other 99.5%. Methods for obtaining highly purified intact disc preparations are well established. [7,8,9,10,11] The purified ROS membranes were found to be comprised of 50% protein and 50% lipid by weight. [12] Sodium dodecyl sulfate (SDS) polyacrylamide gel analysis has shown that approximately 85% of the protein from this membrane is the visual pigment molecule, rhodopsin. [1,8,12,13,14,15] Because of the large percentage of rhodopsin in the membrane, its characteristics have been well studied. The other 15% of membrane protein consists of a complex mixture of components, many with specific enzymatic activities. These include a guanosine 5'-triphosphatase (GTPase), [16,17,18,19] a cyclic 3', 5' guanosine monophosphate phosphodiesterase (cGMP-PDE), [16,21,22,23] and a rhodopsin kinase. [16,21,22,23]

Visual pigments such as rhodopsin consist of a chromophore, 11-cis retinal, covalently bound to an apoprotein, opsin. Detection of light depends on the absorption of a photon by the 11-cis retinal chromophore group of rhodopsin which then yields the all-trans form of retinal, also called metarhodopsin II [15], and the release of free opsin. [24] The absorption of the photon by the chromophore also triggers a sequence of events leading to the eventual release of a transmitter substance (probably Ca^{++} ions) from

the photoreceptor membrane and a decrease in the permeability of the plasma membrane to Na^+ ions resulting in hyperpolarization of the membrane. The evidence for direct communication between the plasma membrane and the ROS disc membrane is now being studied using ^{45}Ca-exchange and light-scattering experiments [25], but the exact mechanism linking the cis-trans isomerization of rhodopsin with the change in Na^+ permeability is not yet known.

Neutron diffraction experiments show that over half of the rhodopsin is imbedded in the hydrophobic region of the lipid bilayer. [26] Digestion of rhodpsin with pronase or thermolysin [17,27,28,29] did not alter its spectral properties, suggesting that it is the chromophore part that is buried within the hydrophobic core. Further proof of the existence of a hydrophobic core region was obtained by using the photoactive agent (^3H)-azido napthalene, a hydrophobic molecule, which labels rhodopsin at a site protected from water soluble proteases. [30] The presence of hydrophilic regions would suggest exposure to the membrane surface, and indeed it has been found that rhodopsin binds ^{125}I-concanavalin A (Con A), succinyl-Con A, and wheat germ agglutin. [128] These results suggest that carbohydrate moiety is on the interior of the discs. Further orientation studies using the membrane-impermeable reagents ^{35}S-N-(4-azido-2-nitrophenyl)-2-aminoethane sulfonate[31], and sodium isethionyl acetimedate hydrochloride [9] now suggest that rhodopsin is a transmembrane protein oriented with its carboxy terminus on

the exterior and its amino terminus on the interior of the ROS membrane.

Neither the chemistry nor the physiology of the enzymes which are part of the remaining 15% of the membrane protein is fully understood, but many advances have been made in the last few years. It is suggested that a GTPase and a cGMP-phosphodiesterase, both present in the ROS membrane, are both activated upon bleaching of rhodopsin [32,33,34,35] since their action spectrum matches its absorption spectrum. [36] This further suggests that the bleaching of rhodopsin may be the primary step in enzyme activation.

It is well established that the PDE elutes from the membrane under low ionic strength and thereby loses its light-activation properties [37], indicating that the interaction of the PDE with other membrane-bound molecules is essential to the light-activation process. Wheeler and Bitensky [16] reported that GTP is also required for this light-dependent PDE activity suggesting that GTPase may also play an important role in the activation sequence. The activation of PDE occurs very rapidly and is of sufficient magnitude to play a significant role in the visual response. [19,35,38] Yee and Liebman [39], using continuously monitored proton release, found that the PDE was half maximally activated at 1/80,000 bleached rhodopsins, and had a velocity of 4×10^5 cGMP hydrolyzed/second/bleached rhodopsin. [17] ROS PDE was first isolated from the frog [37] and found to possess subunits of 120,000 and 110,000 daltons. It was later isolated from the

cow [40] and was only found to possess a single component of 105,000 daltons. The PDE constitutes 0.5% to 1% of the ROS membrane protein and is present in a ratio of 1:900 (PDE:rhodopsin). Other investigators [21,41,42] have reported a doublet of 88,000 and 84,000 daltons from cow ROS PDE as opposed to the single component, but the disparity can be explained by use of different gel systems which have also given different molecular weights for rhodopsin. [8] The PDE's from different species have been found to be highly related. [42]

A peripherial light-activated GTPase has also been isolated from ROS disc membranes [21,41,43,44,45,46] and found to have a molecular weight of 37,000 and 39,000. It is not active in its eluted form but only when reconstituted with bleached ROS membranes (or purified rhodopsin). [21,36,41, 43,46] This enzyme constitutes about 7% of the membrane protein [43] and becomes tightly bound upon illumination under moderate ionic strength. [21] Some have reported the presence of a separate 'H' factor [47,48] which is required for GTP hydrolysis but not PDE activation. It is generally accepted that the presence of the GTPase is required for PDE activation and for the subsequent reversal of PDE activation. It shows a remarkable similarity to PDE in both sensitivity to light and action spectrum. [17] A sawtooth pattern of PDE activity is observed which corresponds exactly to the P_i liberated by GTPase activity. [17] This suggests that the allosteric site for PDE activation corresponds to the GTPase catalytic

site. The concentration of GTP required for half-maximal activation of the PDE (0.07 - 0.08 μM) and the K_m of the GTPase (<.1 μM) are comparable. [49]

Another enzyme present in ROS is rhodopsin kinase. This enzyme has a molecular weight of 68,000 and is a peripheral component that phosphorylates rhodopsin in a cyclic nucleotide independent manner. [50] The possibility that this enzyme is involved in the visual process seems remote as the reaction time ($T_{1/2}$ = 1 - 2 minutes) is too slow. [43,50]

Extensive evidence strongly suggests that these enzymes (excluding rhodopsin kinase) play an important role in the retinal visual response. Perhaps the most convincing studies involve animals with retinal degenerations [51,52] such as the rd mouse phenotype. These animals show a deficient photoreceptor cGMP-PDE activity and a ten-fold increase in retinal cGMP. [42,53,54,55] The GTPase could represent a new class of proteins that physiologically fluctuate between membrane-bound and free states providing communication between membrane-bound receptors and other cellular elements.

Since the possible importance of this enzyme in the visual response has been documented, a comparison of different evolutionary species may be valuable in characterizing those parts of the molecule important in the mechanism of that response. In this work I have studied the GTPase from four evolutionary divergent species; the pig, cow, chick, and frog. After establishing the fact that what I had isolated was indeed a GTPase, I proceeded to evaluate to what extent, if

any, this enzyme had been conserved throughout evolution. As a means of ascertaining and characterizing relatedness, tryptic peptide mapping and photoaffinity labeling were used. The presence of a higher molecular weight species, proposed to be a dimer of GTPase, in some of the preparations was observed and also characterized. If the enzyme turned out to be highly conserved this would suggest that the major portion of the protein is somehow involved in the mechanism of response and such knowledge could benefit future studies trying to characterize the molecular basis of the visual excitation process.

II. MATERIALS

A. Autoradiography

KLX - Developer and Fixer - Eastman
Intensifying Screens - Dupont
XRP - 1 - film - Kodak

B. Chromatography

20 x 20 cm 13255 Cellulose - Eastman
20 x 20 PEI Cellulose - Eastman
Hexyl - Agarose - Miles - Yeda Ltd.
AG 1 - X - 100 Resin - BioRad

C. Enzymes

Glyceraldehyde - 3 - Phosphate - Dehydrogenase - Sigma
3 - Phophoglycerate Kinase - Sigma
Trypsin - Sigma

D. Filters

Dialysis Tubing (12,000-14,000 cut-off) - Fisher
Glass Micro-Fibre Filter (2.4 cm) - Whatman

E. Reagents

Acrylamide - Electrophoresis Pure - Bio Rad
Ammonium Bicarbonate - Fisher Scientific
Ammonium Molybdate - Fisher Scientific
Ammonium Persulfate - Fisher Scientific
BSA - Sigma
8-Azido-GTP - Boyd Haley, Univ. of Wyoming
Chloramine T - Sigma
Coomassie Brilliant Blue R Dye - Sigma
Cysteine HCl - Sigma
Dimethyl POPOP - r.p.i. Scintillator
Dithiothriotol - Sigma

EDTA - Fisher
Glacial Acetic Acid - Fisher
Glycine - Fisher
GTP - Sigma
[γ - ^{32}P] GTP - New England Nuclear
Hydrochloric Acid - Fisher
^{125}Iodide (carrier free) - New England Nuclear
Isopropanol - Fisher
Lithium Diiodosalicylate (LIS) - Eastman
Magnesium Chloride - Baker
Manganese Chloride - Baker
β - Mercaptoethanol - Sigma
Methanol - Fisher
N, N' - Methylene Bis Acrylamide - Eastman
Perchloric Acid - Mallinckrodt
3-P-Glyceraldehyde - Sigma
Phosphoric Acid - Fisher
Phenylmethylsulfonylfloride (PMSF) - Sigma
Pyridine - Fisher
Pyronin Y - Eastman
Sodium Azide - Sigma
Sodium Chloride - Fisher
Sodium Dodecyl Sulfate - Sigma
Sodium Hydroxide - Fischer
Sodium Monophosphate - Mallinckrodt
Sucrose - Fisher
Tetraethylamine (TEA) - Mallinckrodt
Toluene - Fisher
Tris - Fisher
Triton X-100 - r.p.i. Scintillator

E. Tissues

Chicken Heads - Saline Processing Plant, Salina, Ks.
Cow Eyes - Pfantesil Packing Company, Emporia, Ks.
Frogs - Carolina Biologicals
Pig Eyes - Kaw Valley Processing Plant, Wamego, Ks.

III. METHODS

Isolation of ROS Membranes from Retina

Preparation of ROS disc membranes followed the procedure of Baehr et. al. [20] with the exception that dissection of retinas and subsequent purification steps were conducted under normal light rather than dim red light.

The retinas were removed from approximately 25 cow eyes, 50 pig eyes, 100 frog eyes, and 600 chicken eyes, following two to four hours of dark adaptation. All of the steps were done at 4°C unless otherwise stated. Subsequent to removal, the retinas were placed in 1 ml of buffer A per retina containing 30% sucrose w/w, 65 mM Sodium Chloride, 2 mM Magnesium Chloride, 5mM Tris, 1 mM Dithiothriotol (DTT), and 0.5 mM phenylmethylsulfonylfloride (PMSF) at a pH of 7.4. The mixture was shaken vigorously for a few minutes in a stoppered erlenmeyer flask and then transferred to a 30 ml plastic centrifuge tube. After four minutes of centrifugation at 4000 rpm in a Sorvall SS-34 rotor the resulting supernatant (called S1) was decanted into another flask and the pellet (P1) was resuspended in 1 ml of buffer A per 10 ml of original mixture. P1 was then homogenized for two minutes with a loose teflon homogenizer and then re-centrifuged for four minutes at 4000 rpm. The second supernatant was added to S1 and diluted 2:1

with buffer B containing 10 mM Tris, 1 mM $MgCl_2$, 1mM DTT, and 5 mM PMSF, pH 7.4 This diluted mixture was centrifuged for four minutes at 7000 rpm and the pellet, P2, was saved. P2 was then resuspended in ten ml of buffer C containing 26.3% Sucrose w/v, 1 mM DTT, 10 mM Tris, and 0.5 mM PMSF, pH 7.4 (with a density of 1.10 g/ml) and homogenized. Approximately 2 ml of this homogenate was overlayed onto a discontinuous sucrose gradient of 1.1, 1.3, and 1.5 g/ml sucrose in buffer B. The resulting tubes were placed in a 50.1 swinging bucket rotor and centrifuged for 45 minutes at 24,000 rpm on a Beckman L3-50 Ultracentrifuge. The partially purified membranes were removed from the 1.1/1.3 interface and diluted with 2 ml of buffer E containing 10 mM Tris, 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, and 1mM DTT, pH 7.4. The diluted membranes were then pelleted three to four times in buffer E for 15 minutes at 14,000 rpm in an SS-34 rotor to remove any residual sucrose.

The washed membranes were placed, on ice, in a DA-10 Aristo light box and illuminated for 30 minutes with occasional stirring. After bleaching, the membranes are washed three to four more times in buffer B with the Tris concentration altered to 100 mM, for 15 minutes at 14,000 rpm. The membranes are now ready for GTPase extraction.

Enzyme Extraction and Purification

The extraction of the enzyme was according to Kuhn [45] and the subsequent purification was according to Fung et al. [56] Once the membranes were isolated, bleached, and washed, 10 ml of buffer F containing 10 mM Tris, 0.5 mM $MgCl_2$ and 80 μM GTP was added. The mixture was shaken vigorously, then spun down for 15 minutes at 14,000 rpm in an SS-34 rotor. Buffers were kept at 4° C and made fresh at least every two weeks. A 2 mM stock solution of GTP was kept frozen at -70°C so that buffer F could be made fresh every time (400 μl of stock GTP/10 ml of buffer F). At this step, with some preparations, a pellet was no longer visible but extraction was still carried out the same. Other methods of extracting peripheral membrane proteins was also tried, including 40 mM Lithium Diiodosalicylate (LIS), 1 M NaCl, 0.01% Sodium dodecyl sulfate (SDS), and 5 mM EDTA, instead of GTP.

The resulting supernatant containing the extracted enzyme was then loaded on a hexyl-agarose column which had been washed with buffer G containing 10 mM MOPS, 2mM $MgCl_2$, 0.1mM PMSF, and 1mM DTT, pH 7.4. The remaining pellet which consisted of depleted ROS membranes, was extracted two to three more times to ensure nearly complete removal of the GTPase, then stored in buffer B at -70°C to be used later in the GTPase assay.

The hexyl agarose column (0.5 x 5.0 cm) had a bed volume of 3 ml and was stored in 0.2% sodium azide (NaN_3) between usage to prevent bacterial growth. A separate column was

packed for each species to eliminate any cross-species contamination.

After the sample was loaded onto the column, it was washed with five to six bed volumes of buffer G and then one bed volume of buffer G containing 80 mM NaCl. Finally three to four volumes of buffer G containing 300 mM NaCl were passed through and 2 ml factions collected. 100 µl of each fraction was then assayed for GTPase activity in the presence of bleached ROS membranes (described in later section) and the peak fractions were then pooled and dialyzed.

Dialysis

Dialysis tubing (16 mm dry diameter, molecular weight cut-off 12,000-14,000) was prepared by boiling for five minutes in sodium bicarbonate ($NaHCO_3$) and EDTA solution to remove any contaminants. After the tubing had cooled the pooled fractions were placed in the tubing and dialyzed against 800 ml of 1 mM sodium monophosphate solution, pH 7.4. Dialysis continued for 24 hours with two to three changes during that time. The dialyzed material was then stored at -70°C in aliquots of 500 µl to be used for the GTPase assay, SDS - polyacrylamide gel electrophoresis, and photoaffinity labeling.

Purification of [γ - ^{32}P] Guanosine 5' - Triphosphate

[γ - ^{32}P]GTP was purified according to Methods in Enzymology. [57] A Pasteur pipette was filled with four cm of AG 1-

X4 resin (200-400) mesh and 100 µl of the sample was loaded on. The column was then washed with 30 ml of 30 mM HCl to remove any inorganic phosphate, GDP, or GMP present. The [γ - ^{32}P] GTP was then eluted with 250 mM HCl and 1 ml fractions were collected. Ten µl of each fraction was taken and counted in scintillation cocktail to determine peak activity. The fractions with the most activity, consistently tubes four through six, were pooled and neutralized using 6N NaOH. The pH of the material was determined using pH 1-11 litmus paper. The neutralized and purified material was then stored in 200 µl aliquots at 0°C in plastic vials.

Coomassie Blue Protein Assay

The Coomassie blue binding assay of Bradford [58] was used to determine the protein concentrations of the hexyl-agarose purified material. Since concentrations were so small, 5 ml of the dialyzed material was lyopholyzed, taken up in 100 µl of de-ionized water, and then used in the assay.

The dye reagent was prepared using 200 mg of Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid. This was then diluted to one liter with de-ionized water. Two ml of the dye-reagent was added to 100 µl of the hexyl-agarose material, allowed to incubate for ten minutes, then read at 560 nm on a Perkin-Elmer 5513 spectrophotometer. BSA was used to generate a standard curve.

Guanosine 5'-Triphosphatase Assay

GTPase activity was measured by the method of Fung et al. [56] with some modifications according to Kuhn. [45] The reaction mixture, kept on ice, contained 0.05 µg of dialyzed, hexyl-agarose material and 5 µg of depleted ROS membranes in assay buffer consisting of 10 mM MOPS, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT, pH 7.4. The reaction was initiated by the addition of the substrate, 5 µ M [γ - ^{32}P] GTP ($\sim$ 3 x 10^4 cpm/assay, specific activity 22.7 mCi/mmole) in a 20 µl volume resulting in a final total volume of 200 µl. Two tubes, one containing no enzyme or membranes and the other devoid of membranes only, were prepared to determine background and basal activities respectively. After eight minutes incubation at 37°C the reaction was stopped by the addition of 1 ml of solution A, containing 150 mM perchloric acid and 0.125 mM KPO_4, and one ml of solution B, containing 10 mM ammonium molybdate and 20 mM tetraethylamine in HCl (TEA-HCl). A dense precipitate formed immediately upon addition of solution B. The tubes were vortexed and allowed to sit on ice for ten minutes. The precipitate was then collected on glass filters using a suction filter apparatus. The tubes were washed with one ml of solution C (10 mM TEA-HCl, 200 mM perchloric acid, and 2.5 mM KPO_4), which was poured through the filter. The filters were then washed with at least 50 ml of solution C. The filters were finally placed in plastic scintillation vials with 10 ml of scintillation cocktail and counted. 20 µl of the substrate was directly placed in a vial and counted also

to determine the maximum number of counts added to the assay. Values were computed as a percentage of the maximum. The amount of P_i liberated was calculated from the specific activity of the substrate.

Sample Calculation:

1. Specific activity of substrate = 22.7 mC_i/mmole
2. 1 C_i = 2.20 x 10^{12}dpm = 1.10 x 10^{11}cpm (assuming 50% counting efficiency)
3. 2.27 x 10^9 cpm = 1 mmole of substrate.
4. cpm in maximum x 1 mmole / 2.27 x 10^9cpm = total number of mmoles added.
5. cpm in sample x 1 mmole / 2.27 x 10^9cpm = total number of mmoles unhydrolyzed.
6. number of mmoles left ÷ 10^{-6} = nmoles of P_i liberated.
7. Specific activity of sample = nmoles P_i liberated ÷ eight minutes assay time ÷ µg of protein in sample.

The retinal GTPase is an unusual enzyme since to be fully activated it requires bleached ROS membranes. [21] The GTPase itself has minimal hydrolyzing activity, but when reconstituted the activity observed is increased to a much greater extent. The ROS membranes alone show some hydrolyzing activity due to residual bound GTPase and are therefore extracted three to four additional times to ensure maximum depletion of the bound enzyme. This minimizes the error when calculating the specific activity of the reconstituted enzyme (that is, none or very little of the P_i liberated is due to the membrane addition itself but only to the effect it exerts on the GTPase).

Two prior experiments were conducted to determine the effect the amount of ROS membrane added back had on activity and also to determine that the amount of enzyme being used in the assay was optimal with the amount of substrate being used, (namely that the enzyme was saturated). In the first experiment all factors were kept constant, using the assay procedure previously described, except for the amount of ROS membrane added, varying from 0 to 10 µg. The activity was then determined for each sample, and the amount of membrane which gave maximal activity was used for all succeeding assays. The second experiment was conducted with all factors being kept constant except for the amount of hexyl-agarose material added from 0 to 0.10 µg. The activity was measured and an amount chosen for all following experiments for which the activity was on the linear region of the graph. These two experiments ensured that all factors in the assay used were conducive to optimal specific activity of the enzyme being studied. Two controls were also run using 10 µl directly off the column. The first was to determine whether GTPase was being enriched with 300mM salt and the second to see whether another protein, BSA was tried, would stimulate the enzyme's activity. The same protocol was used for every species but due to different concentrations of each hexyl-agarose preparation, the volumes used were varied as needed so that the amounts of protein per assay were approximately equal.

Polyacrylamide Gel Electrophoresis

Samples were prepared for electrophoresis by taking 100 µl of the dialyzed, hexyl-agarose material and radioiodinating them according to Takemoto et al. [59] To each sample was added 10 µl of de-ionized water, 5 µl of 20 mM Tris, 5 µl of 20% SDS, 5 μC_i of ^{125}Iodide, and 25 µl of a 1 mg/ml solution of chloramine T. After one minute incubation the reaction was quenched with an equal volume of sample buffer containing 2% β - mercaptoethanol, 20mM Tris, 20% SDS, 10 mM sucrose and bromothymol blue.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed the procedure of Laemmli [60] with a 4.0% stacking gel and 7.5% resolving gel. Fifty µl of sample was applied per well and the gels were then electrophoresed at 12 mA for one and a half hours at 4°C. Amperage was increased to 30 mA for the remaining two hours. Human erythrocyte membranes and BSA were used as markers. [61]

After electrophoreses was completed, the gels were placed in a 25% isopropanol and 10% acetic acid solution for several hours with two to three changes to remove any free iodine or SDS. The acetic acid was present to fix the protein band and minimize diffusion. The gels were then stained in a solution consisting of Coomassie Brilliant Blue R dye dissolved in 25% isopropanol and 10% acetic acid for two to three hours and destained for the same time in 10% methanol. The gels were then dried and exposed for 15 to 30 minutes (depending on the concentration of protein and extent of iodination) to Kodak

XRP-1 film and DuPont Cronex intensifying screens. [59] After developing the autoradiograph to locate the position of the bands, the bands were excised from the gel and prepared for tryptic peptide mapping.

Tryptic Peptide Mapping

Two dimensional tryptic peptide mapping was carried out according to the procedure of Takemoto et al. [59] After the desired protein band was exised from the gel it was placed in 2 ml of the digesting solution containing 0.05 mg per ml of trypsin in 0.05 M ammonium bicarbonate (NH_4HCO_3). The sample was then incubated at 37°C for twenty-four hours. After digestion was complete, the solution was transferred with a Pasteur pipette to another tube (being careful not to transfer any of the gel) and lyopholyzed. However, before lyopholization took place the decanted liquid was counted to ensure sufficient counts were present to continue with the mapping. Approximately 50,000-100,000 cpm are needed to ensure good results.

If sufficients counts were present the lyopholyzed material was taken up in 10 µl of de-ionized water and spotted 1.5 cm up and over from the bottom corner of a 20 x 20 cm cellulose thin-layer chromatography (TLC) plate. A spot of pyronin Y dye was also placed next to the sample spot to monitor the migration. The TLC plate was lightly sprayed with electrophoresis buffer containing acetic acid, formic acid and water in a ratio of 3:1:16 before it was placed on the

apparatus. Two plates were then electrophoresed for 50 minutes (or until the dye had migrated 12.5 cm) at 30 mA, or at approximately 1000 volts at room temperature. The plates were next dried and placed in a chromatography tank with just enough chromatography solvent to cover the bottom. The chromatography solvent consisted of a mixture of butanol, pyridine, acetic acid and water in a ratio of 7:5:1:4. The plates remain in the tank at room temperature until the solvent reaches the top of the plate, approximately one hour, and are then removed, dried and wrapped in plastic (as the cellulose easily chips off). The plates are then exposed to Kodak XRP-1 film and DuPont Cronex intensifying screens [59] for at least 24 hours at -70°C (longer exposure time may be needed depending on how many counts were initially spotted on the plate.)

To obtain a mixed pattern, an equal number of counts from each species were added together in a tube, lypholyzed, and then spotted. The procedure was then exactly as previously described.

[^{32}P] - PO_4 Exchange with 8-Azido-Guanosine 5'-Triphosphate

Cold 8 - N_3 - GTP was provided by B.J. Haley and then exchanged with [^{32}P] - PO_4 to give [γ -^{32}P] 8 - N_3 - GTP according to the procedure of Haley. [63]

Approximately 200 μC_i of [^{32}P] - PO_4 (HCl free) was added to a disposable plastic tube and the following solutions were added to it, on ice and under dim red light; 12.5 µl of .1 M

Tris (pH 7.5); 100 µl, .1 M sucrose; 5 µl, .05 M $MgCl_2$; 5 µl, .02 M cysteine HCl; 20 µl, .05 M 3-P-glyceraldehyde; 5 µl, 1 mM NAD and 500 µl, .1 mM 8-N_3-GTP dissolved in 10 mM Tris (pH 7.4). The reaction was imitated by the addition of a diluted enzyme mix. The mix consisted of 10 µl of glyceralderhyde -3-P-dehydrogerase (70 units/mg) and 1.5 µl of 3-phosphoglycerate kinase (2500 units/mg) diluted 1:10 with 10 mM Tris (pH 7.4) and 15 µl was used to start the reaction. The reaction was then allowed to proceed for 30 minutes at 30°C. At the end of this time the reaction mixure was placed on ice to retard the reaction and the amount of conversion was monitored on a PEI cellulose TLC plate using a 1 M formic acid: .5 M lithium chloride solvent system. [^{32}P] - PO_4 was used as a standard and 1 µl was spotted on the plate next to 1 µl of the reaction mixture. Approximately 80-90% of the [^{32}P] - PO_4 was incorporated into the 8-N_3 GTP spot after the 30 minute reaction time. This material was kept at 4°C, protected from exposure to light and next used for the photoaffinity labeling experiment.

Photoaffinity Labeling of Enzyme with [γ - ^{32}P] 8 -N_3 GTP

The labeling of the hexyl-agarose material with the photoaffinity reagent [γ - ^{32}P] 8-N_3GTP [62], provided by B.J. Haley, was carried out according to Takemoto et al. [35] Five µl of 400 µM [γ - ^{32}P] 8 - N_3 - GTP dissolved in methanol was dried onto the walls of a 12 x 75 mm disposable test tubes under dim red light. To this tube was added 50 µl of the

hexyl-agarose material that contained 4m$\underline{M}$ manganous chloride ($MnCl_2$). In the absense of Mn^{++} ion no binding was observed. [35] The tube was quickly vortexed and the contents transferred to the bottom half of a 35 x 10 mm plastic petri dish. The sample was then irradiated at a distance of 1 cm for two minutes with the long wavelength mode of a UVSL-25 Mineral Lite. After irradiation the sample was dissolved in an equal volume of sample buffer containing β-mercaptoethanol. The samples were subsequently resolved on 7.5% polyarcylamide gels according to Laemmli. [60] Human erythrocyte membranes [61], BSA and lysozymes were used as molecular weight markers. The gels were run at 4°C for one hour at 12 mA and the remaining time at 30 mA, then stained, destained, dried, and exposed to Kodak XRP-1 film and DuPont intensifying screens. [59] Controls were also run consisting of hexyl-agarose material plus label not irradiated and hexyl-agarose material plus excess cold GTP which was then labeled and irradiated. These controls were run on the same gels with the labeled samples.

IV. RESULTS

Isolation of ROS Membranes

To obtain a sufficient amount of enzyme from this preparative procedure, a large number of eyes was required. I found that at least 50 pig eyes were required for a band to be observed on the sucrose gradient, but even if no band was observed the solution at the interface was still removed and the subsequent purification steps undertaken. When this was the case, bands were still visible on the autoradiogram but high amounts of ^{125}iodide was required and long exposure time (hours as compared to minutes). I also found that 25 cow eyes yielded sufficient membranes to show a sharp band on the sucrose gradient, as did 100 frog eyes and 600 chicken eyes.

The eyes were all kept at 4°C in the dark for a few hours prior to dissections of the retina since this allowed for much easier removal. The rest of the preparation was done under normal light, as opposed to the reported dim red light, and it had no effect except that the band on the sucrose gradient was slighlty more diffuse than when the preparations were done under dim light. The band is highly distinguishable from other components as it was bright orange in color, though the color diminishes somewhat upon bleaching. The main purpose of the bleaching step is to tightly bind the enzyme to the

membrane, which requires both light and moderate ionic strength. Since the preparation was done under normal light the bleaching time was usually shortened.

Guanosine 5' - Triphosphatase Extraction and Purification

After the bleaching step, the membranes were thoroughly washed to remove any unbound material and were then extracted with 80 mM GTP. Godchaux and Zimmerman [19] found that if the enzyme was bound tightly to membranes in the absence of GTP and at moderate ionic strength, it could then be specifically eluted by solutions containing low concentrations of GTP. GDP and ATP do not work as eluting agents nor does GTP work if the binding is done at low ionic strength. The other traditional methods of extraction tried, LIS and EDTA for example, appeared to work nearly as well as the GTP method only they rendered the enzyme inactive and the specific activity could not be determined.

After extraction, the material was applied to a hexyl-agarose column. Table 1 shows the elution profile of a cow enzyme preparation. A slight amount of activity was seen coming off at 80 mM NaCl, but the majority of it was seen after the 300 mM salt addition. Approximately 10 ml was collected off the column after the 300 mM addition and then dialyzed. Half of this was lyopholyzed, taken up in 10 µl of

TABLE 1

HEXYL-AGAROSE COLUMN ELUTION PROFILE OF COW GTPASE

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

NOTE: Fractions were collected immediately after the extracted material had been loaded onto the column. The fraction volumes were 4 ml each and 10 µl of each fraction was assayed for GTPase activity. Activities are expressed as picomoles of P_i liberated/minute and are the results of averaging two different samples. Samples containg salt were not dialyzed prior to assaying thus an equal molar amount of salt was added to the buffer and controls in these cases.

de-ionized, and a protein measurement taken because the concentrations of the samples coming off the column were very minute. The other half of the column effluent was frozen at -70°C to be assayed later. If the enzyme wasn't kept at this temperature the activity was quickly lost.

Guanosine 5' - Triphosphatase Assay

After the hexyl-agarose material was dialyzed to remove the salt and a protein measurement obtained, a sufficient volume was then used in the assay to ensure approximately equal amounts of protein added for all species. The amount of enzyme added was varied with all other parameters being kept constant to generate a curve illustrating the optimum amount of enzyme one could add and still be on the linear portion of the graph (that is there was sufficient substrate in the reaction mixture to saturate the enzyme.) Figure 1 shows the results of this experiment. It appeared that there was sufficient substrate up to at least .07 µg of enzyme. Another experiment was also conducted where the amount of ROS membrane added was varied with all the parameters being kept constant to ensure maximal fold increase in activity. Figure 2 illustrates the results of this experiment. This experiment showed a linear dependence of GTPase activity on the amount of ROS membrane protein added up to 5 µg was added the dependence seemed to level off. This verified the findings of Kuhn also. [44]

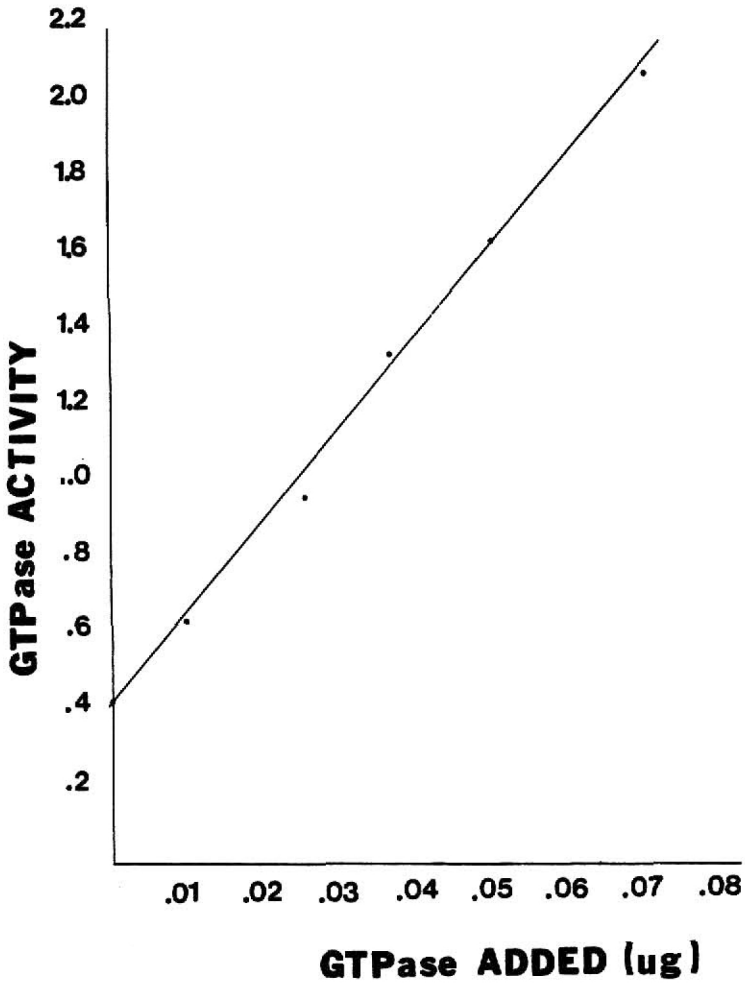

Fig.1. Graph illustrating that the specific activity is linearly dependent on the amount of enzyme added to the assay mixture, indicating that there is sufficient substrate in the mixture to accomodate up to at least 0.07 µg of GTPase. Activity expressed as picomoles of P_i liberated/minute. The enzyme used to generate this curve was from a cow preparation and .5 µg of ROS membranes was used to reconstitute the enzyme.

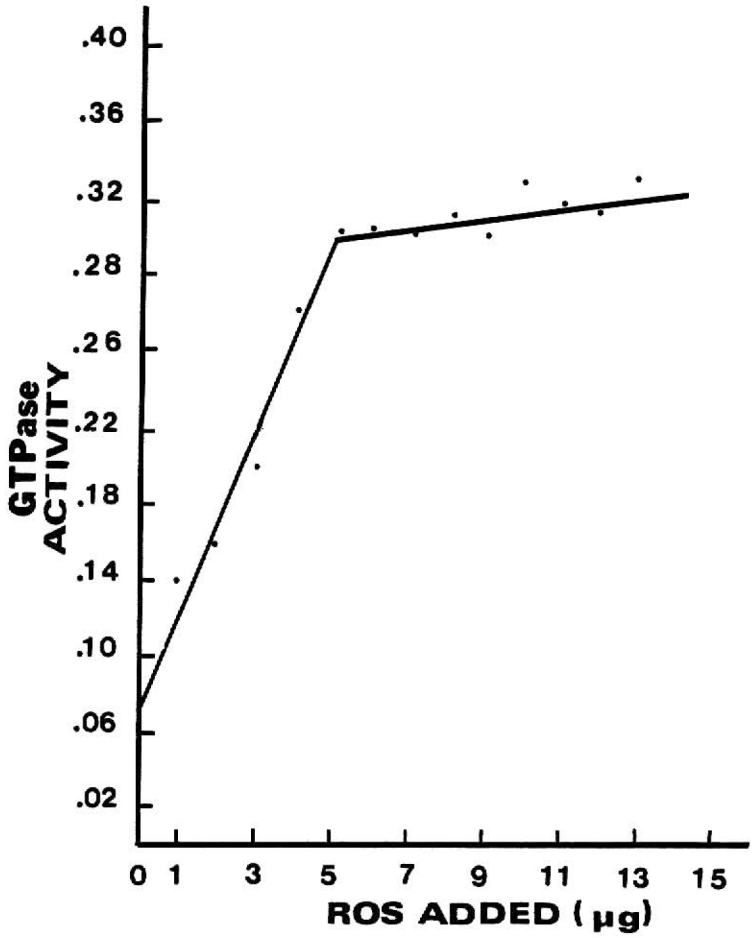

Fig.2. Graph illustrating that the specific activity of the GTPase is dependent on the ROS protein concentration up to 5 µg. Activity is expressed as picomoles of P_i liberated/minute/picogram of GTPase.

Table 2 illustrates the specific activity of the enzyme alone verses reconstituted and verifies what Kuhn [45] already stated, that the enzyme does require the addition of bleached ROS membranes (or purified rhodopsin) for it to be in its fully active form. The activities were very similar for all four species, showing activity increases of about five to nine fold, with the pig enzyme showing the smallest increase. This smallest activity increase seems to be due to the pig enzyme's basal activity being higher than the other species, because the reconstituted activity is still comparable to the others. The chick and frog possess the lowest basal activities and therefore show the largest increases in specific activity. These values are very comparable with those obtained by Godchaux and Zimmerman using cow GTPase. [44] Table 3 shows that any activity left unextracted on the membranes plus the basal activity on the enzyme does not add up to the reconstituted activity expressed. This demonstrates that there is a definite enhancement in the enzyme's hydrolyzing activity in the presence of bleached ROS membranes. Table 4 shows control samples run with the assay. Control set #1 verifies that it is indeed a GTPase being enriched with the 300 mM salt addition and contol set #2 shows that only the specific ROS protein stimulates the enzyme, not BSA. An additional experiment was tried using the ROS membranes from one species added to the enzyme from a different species and the enhancement was approximately the same.

TABLE 2

**SPECIFIC ACTIVITY OF GTPASE:
BASAL VS. RECONSTITUTED FOR ALL SPECIES**

Species	Reconstituted Specific Activity	Basal Specific Activity	Fold Increase
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

NOTE: 5 µg of ROS protein and 0.05 µg of enzyme were used per assay for reconstituted activity. The fold increase is a relative term indicating the enhancement produced by the addition of the ROS. Activities are expressed as nanomoles P_i liberated/minute/microgram of enzyme added.

TABLE 3

BASAL ACTIVITY OF EXTRACTED GTPASE VS. DEPLETED ROS MEMBRANES

Species	Extracted GTPase	Depleted ROS	GTPase + ROS
PIG	0.19	0.21	0.90
COW	0.20	0.45	1.58
CHICK	0.20	0.23	1.70
FROG	0.20	0.21	1.44

NOTE: 5 µg of GTPase and 0.05 µg of ROS were used to determine activities. Activities are expressed as picomoles of P_i liberated/minute.

TABLE 4

ACTIVITIES FOR GTPase CONTROL EXPERIMENT

Control Set #1	GTPase Activity
Buffer wash	0.063
Buffer wash + ROS	0.069
300 mM eluant	0.076
300 mM eluant + ROS	0.230

Control Set #2	GTPase Activity
eluted GTPase	0.076
eluted GTPase + BSA	0.076
eluted GTPase + ROS	0.230

NOTE: The first set was done to show that it is a GTPase being isolated and it is specifically eluted with 300 mM salt. 10 µl fractions were collected and assayed with or without 5 µg of ROS as indicated. The second set shows that only ROS protein will stimulate the GTPase. Ten µl fractions were used again and 5 µg of both proteins. Activities are expressed as picomoles of P_i liberated/minute.

SDS Polyacrylamide Gel Electrophoresis

Figure 3 illustrates the profile of the hexyl-agarose purified enzyme preparations for all four species under observation run on 7.5% SDS-PAGE. Two bands corresponding to 37,000 and 39,000 daltons were present in three of the four species, namely, the pig, cow, and chick. Previous works by Kuhn [45], Bitensky [43], and Godchaux and Zimmerman [44], have also noted the appearance of a 39,000 and 37,000 doublet but these were all done using cow enzyme. No prior identification has been done on any other species up till now. Slight discrepancies have been reported on the molecular weights but this can easily be explained by the use of slightly different gel systems. The one exception is the frog which exhibited only one band of approximately 75,000 daltons. This band was also present in the chick and pig preparations after the enzyme had been subjected to repeated freeze/thaw treatment, see Figure 4 (chick not shown). In the pig and chick, the appearance of the higher molecular weight species always accompanied a decrease in the intensitites of the two lower bands in the preparation. The band rarely appeared in the cow preparation, and then only to a very small extent, no matter how many freeze/thaws it was subjected to. The first indication seemed to be that the band reflected a dimer formation since the frog exhibited only this band and still had the same activity as the other species. Treatment by boiling or 2-mercaptathanol did not diminish the intensity of the band so it apparently is of a very stable nature and is

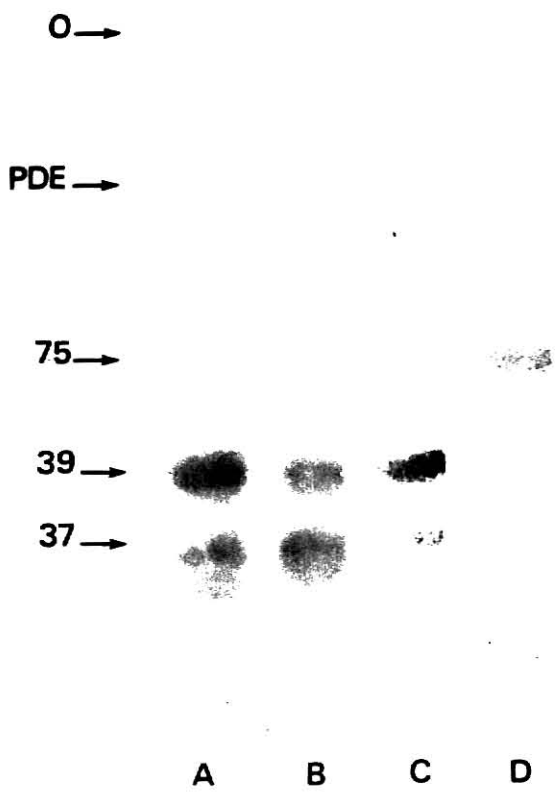

Fig.3. 7.5% SDS-PAGE profile of radioiondated, hexyl-agarose purified material. (100 µl of material was loaded on the gel.) Lane A, pig; lane B, the cow; lane C, chick; and lane D, frog. Molecular weights are approximations using human erythrocyte membrane proteins as markers. The above is a composite of four separate autoradiographs, each exposed for approximately 15 minutes to one hour.

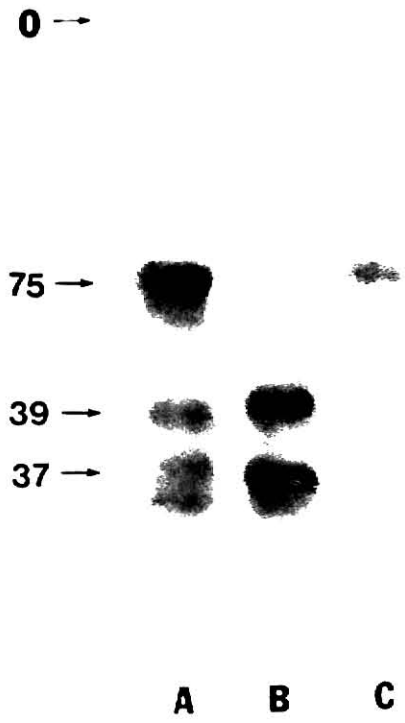

Fig.4. 7.5% SDS-PAGE profile of radioiondated, hexyl-agarose purified material after repeated subjection to freeze/thaw treatment. (100 µl of material was loaded on the gel.) Lane A, pig; lane B, cow; and lane C, frog.

not linked by disulfide bonds. It is unknown whether the formation was an experimental artifact in the frog, or actually exists in vivo, but it was present in each preparation. The higher molecular weight component never appeared in the cow preparation in sufficient quantity to characterize, and this being the species chosen for almost all previous studies, no reference could therefore be found of a dimer's existence.

Another band usually appeared on the pig and cow gels which corresponded to retinal PDE (see arrows on Fig.'s 3 and 4), but it was very minor. It has been reported [64] that this phenomenon (PDE:GTPase complex) is dependent upon the EDTA and 2-mercaptathanol concentrations in the sample preparation. The unresolved PDE and GTPase proteins act as a single complex on native gels, analytical ultracentrifigation, sucrose gradient sedimentation, and gel filtration, explaining their co-purification throughout the entire procedure. This previous work was done using cow retinal preparations only, but it also seems to apply to the pig system also. The band never appeared on chick or frog gels.

Radiolabeling of 8-Azido-Guanosine 5'- Triphosphate.

It has always been difficult to synthesize a useful affinity analog because, previously, the active site had to contain an amino acid that had the potential to form a covalent bond with the reactive group on the nucleotide analog. [62] This problem has now been alleviated by the use

of photoaffinity analogs which generate nitrenes or carbenes that when activated with light [65,66,67,68] that react with any C-H group. When these anaolgues are also labeled with ^{32}P, so that the approximate molecular weight of the analogue-binding polypeptide can be determined.

The actual exchange reaction used to gamma-label the 8-N_3 GTP was quite simple and straightforward, the only disadvantage being that everything had to be done under dim red light. The final purification step of running the reaction mixture through a Sephacyl column was omitted because any unreacted material (i.e., unlabeled analogue, P_i, or $^{32}P_i$) was assumed to run with the dye front when electrophoresed on SDS gels. The reaction mixture itself was run on a SDS gel and did indeed migrate with the dye front. The thin-layer chromatography on PEI-cellulose showed almost total incorporation of the $[^{32}P_s]$ - PO_4 into the 8 - N_3 - GTP, with a specific activity of approximately 1 mC_i/mmole.

Specific Labeling of Guanosine 5' - Triphosphatase with 8-Azido -Guanosine Guanosine 5 - Triphosphate.

To determine which component of the GTPase actually binds GTP, the hexyl-agarose material was incubated with the photoaffinity analogue according to Takemoto. [35] The experiment was tried using both preparations of analogue; the one provided by B. J. Haley, already labeled (specific activity 5 mC_i/mmole) and the one labeled immediately before the experiment (1mC_i/mmole). The results were the same for both

preparations. The only component which covalently bound the photoaffinity reagent was the 37,000 molecular weight species in the pig, see Figure 5, and the cow, see Figure 6. These results agree with previous published findings using cow GTPase. Photoaffinity labeling with any species other than the cow has never been reported. [35] Fig. 7 shows that using the frog preparation, the reagent was covalently bound to the 75,000 dalton component, substantiating the previous hypothesis that this 75K component is a GTPase and possibly a dimer. This further suggests that perhaps the dimer is of a 37-37 or 37-39 nature, but eliminates the 39-39 possibility assuming that the frog acts similar to the other species (that is, the 37,000 component is responsible for the binding). The molecular weight markers run alongside also suggest a lower weight for the dimer than a 39-39 would give. Another possibility is that the 39,000 component never exists in the frog at all, but only a 37,000 one (either monomer or dimer). One observation that would tend to support this theory is the appearance of a 37,000 band on gels using a frog enzyme preparation that had been stored for an extremely long time and subjected to many freeze/thaw treatments. The band could only be seen if the sample was radiolabeled with a large amount (at least 6 μC_i) of $^{125}I^-$ and the gel exposed for at least 36 hours. Even under these extreme treatments a 39,000 band never appeared. Interestingly, in the pig, repeated freeze/thaw treatments seemed to promote this higher molecular weight band rather than diminish it, but it was capable of

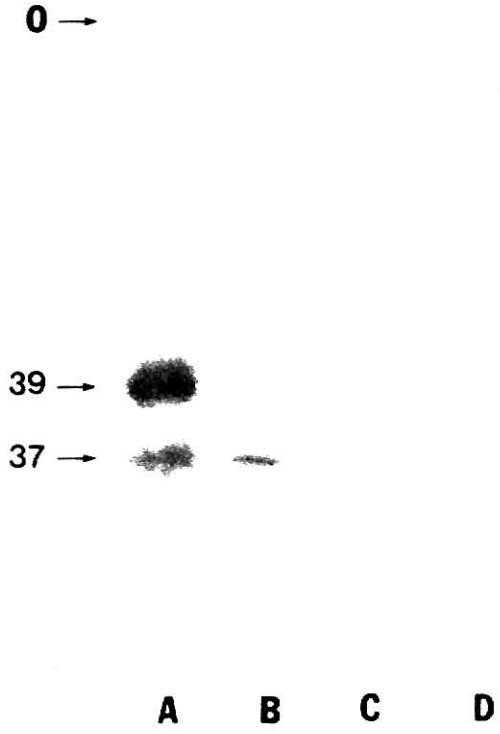

Fig.5. 7.5% SDS-PAGE profile of A) 100 µl radioiondated hexyl-agarose purified pig GTPase B) cold enzyme incubated with 50 µl of the photoaffinity reagent 8-N_3 [γ - ^{32}P] GTP, and photolyzed C) cold enzyme + reagent with no photolysis and D) cold enzyme + 100X excess cold GTP + ^{32}P reagent and photolyzed. The above autoradiograph was exposed for 24 hours.

Fig.6. 7.5% SDS-PAGE profile of A) 100 µl radioiondated hexyl-agarose purified cow GTPase B) cold enzyme incubated with 50 µl of the photoaffinity reagent 8-N_3 [γ - ^{32}P] GTP, and photolyzed C) cold enzyme + reagent with no photolysis and D) cold enzyme + 100X excess cold GTP + ^{32}P reagent and photolyzed. The above autoradiograph was exposed for 24 hours.

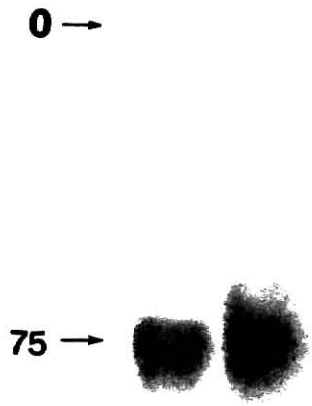

Fig.7. 7.5% SDS-PAGE profile of A) 100 µl radioiondated hexyl-agarose purified frog GTPase B) cold enzyme incubated with 50 µl of the photoaffinity reagent $8\text{-}N_3$ [γ - ^{32}P] GTP, and photolyzed C) cold enzyme + reagent with no photolysis and D) cold enzyme + 100X excess cold GTP + ^{32}P reagent and photolyzed. The above autoradiograph was exposed for 24 hours.

binding the photoaffinity reagent also, see Figure 8. This verifies that the 37,000 dalton component is involved in the higher molecular weight species. The reagent was also observed binding to the 75,000 band in the chick preparation. This binding was dependent in all cases upon photolyzing light (Figures 5,6,7, and 8 - lane C) and was prevented when the enzyme preparation was preincubated with a 100X molar excess of cold GTP prior to addition of the photoaffinity reagent and subsequent photolysis (Figures 5,6,7, and 8 - lane D). Based on these results it seems likely that at least the 37,000 component binds the GTP and therefore takes part in the catalytic hydrolysis.

Tryptic Peptide Mapping

To better characterize the relationship between the 37,000, 39,000 and 75,000 dalton components in the same species, the radioiodination bands were excised from the gels, digested with trypsin, and then submitted to two-dimensional electrophoesis and chromatography. Figure 9 shows the results of the 39,000 and 37,000 maps of the radioiodinated tryptic peptides of the pig GTPase. The spots identified as A through H are common to the 39,000 maps of the pig, cow, chick, and partially of the frog. The spots identified with the numbers 1 through 3 are spots common to all 39,000 maps and also all 37,000 maps also. This is true for every species. Spots vary in intensity due to the inate differences in iodination caused by conformational aspects of the various peptides. This is discussed in more detail later with Table 5.

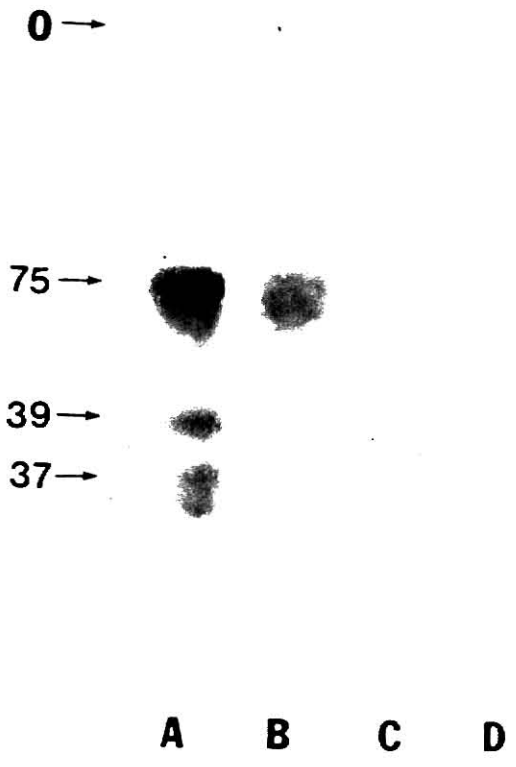

Fig.8. 7.5% SDS-PAGE profile of A) 100 µl radioiondated hexyl-agarose purified pig GTPase subjected to freeze/thawing B) cold enzyme incubated with 50 µl of the photoaffinity reagent 8-N_3 [γ - ^{32}P] GTP, and photolyzed C) cold enzyme + reagent with no photolysis and D) cold enzyme + 100X excess cold GTP + ^{32}P reagent and photolyzed. The above autoradiograph was exposed for 24 hours.

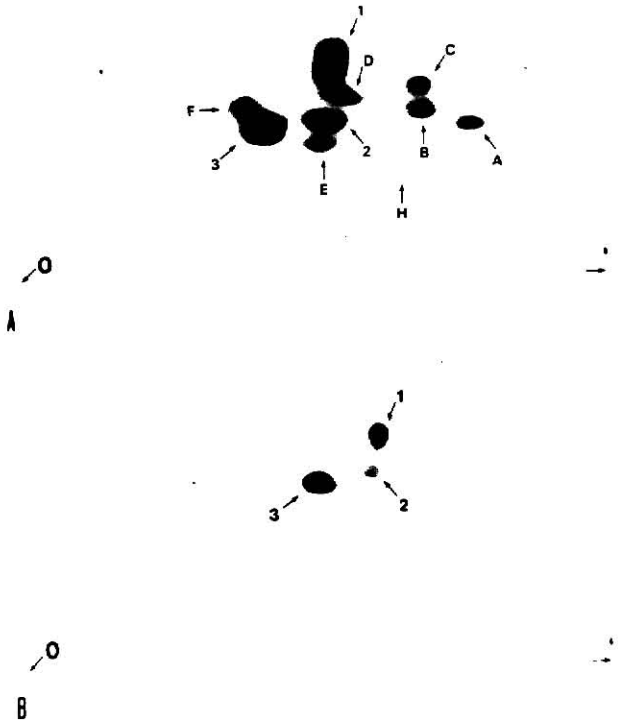

Fig.9. Results of mapping radioiodinated tryptic peptides of A) 39,000 component of pig GTPase and B) 37,000 component of pig GTPase. Horizontal arrows in lower right-hand corner show direction of 1st dimension electrophoresis and vertical arrows show 2nd dimension chromatography. The origin is indicated in the left bottom corner. The spots identified by capital letters are common to all 39,000 maps only while the spots identified with numbers are common to all 39,000 and 37,000 maps in all species. (There are some exceptions in the chick and frog maps.)

Figure 10 shows the results of the 39,000 and 37,000 maps of the radiodinated tryptic peptides of the cow GTPase. This is the only species previously mapped. [35] As in the pig, spots A through H are present in the 39,000 map only while spots 1 through 3 are in both. The maps appear very similar indicating a high degree of relatedness between the two enzymes.

Figure 11 illustrates the maps of the radioiodinated peptides of the chick enzyme. Here, too, spots A thorough H and 1 through 3 are in the 39,000 map but the 37,000 map differs from the pig and cow. While the pig and cow showed only three peptides on the 37,000 map, 1 through 3, the chick had these plus B, E, and F. It appears that the 37,000 component of the chick must differ somewhat in composition from those of the pig and cow, perhaps being more similar in composition to the 39,000 component. Figure 12 shows a comparison of the 39,000 maps of the pig, cow, and chick and the 75,000 of the frog. It is obvious that they are all highly related, with the pig and cow being nearly identical, as at least nine major peptides (A through H and 1,2, and 3) are conserved. Figure 13 shows the same comparison with the 37,000 maps. It appears that the map of the frog more closely resembles those 37,000 maps of the other species as it only has peptides 1,2,3,B,E, and F. But one cannot say it is exclusively of 37,000 character because it does possess peptides common to 39,000 maps also, namely B,C,E, and F. These seem to suggest that the proposed dimer may be of a 37-39 type. It may be possible that the frog is just highly suseptible to dimer formation as

Fig.10. Results of mapping radioiodinated tryptic peptides of A) 39,000 component of cow GTPase and B) 37,000 component of pig GTPase. Horizontal arrows in lower right-hand corner show direction of 1st dimension electrophoresis and vertical arrows show 2nd dimension chromatography. The origin is indicated in the left bottom corner. The spots identified by capital letters are common to all 39,000 maps only while the spots identified with numbers are common to all 39,000 and 37,000 maps in all species. (There are some exceptions in the chick and frog maps.)

Fig.11. Results of mapping radioiodinated tryptic peptides of A) 39,000 component of chick GTPase and B) 37,000 component of pig GTPase. Horizontal arrows in lower right-hand corner show direction of 1st dimension electrophoresis and vertical arrows show 2nd dimension chromatography. The origin is indicated in the left bottom corner. The spots identified by capital letters are common to all 39,000 maps only while the spots identified with numbers are common to all 39,000 and 37,000 maps in all species. (There are some exceptions in the chick and frog maps.)

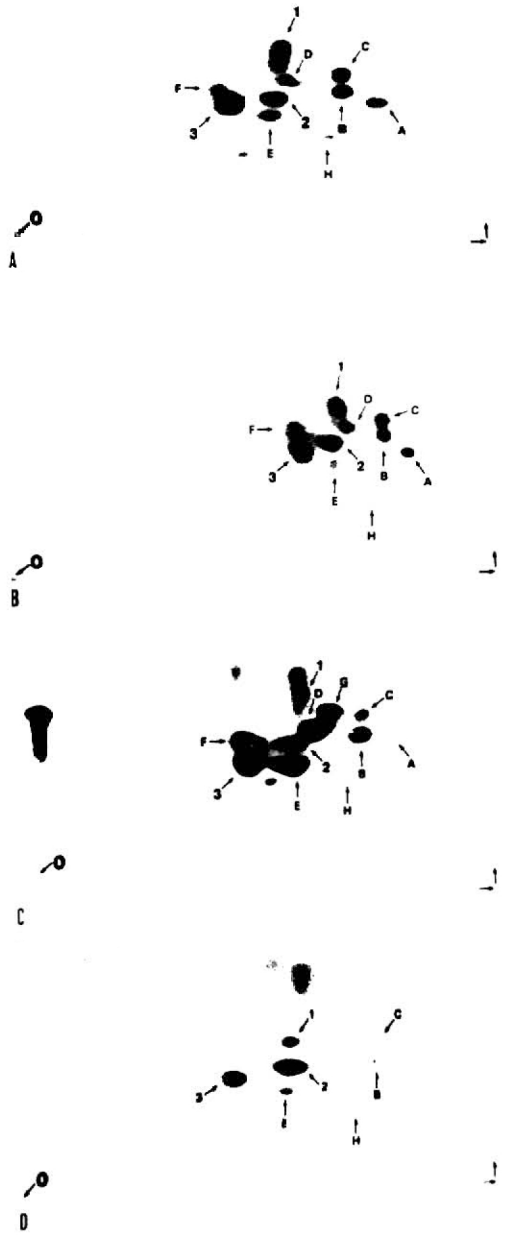

Fig.12. Interspecies comparison of the 39,000 maps. A) pig, B) cow, C) chick, and D) frog (75,000 map). Letters and numbers characterizing spots are the same as before. One can see the overall pattern is very similar among the various maps.

Fig.13. Interspecies comparison of the 37,000 maps. A) pig, B) cow, C) chick, and D) frog (75,000 map). Letters and numbers of spots are the same as before. The differences in iodination of peptides is apparent here if one looks at peptide 2 im maps A and B as compared to the same spot in maps C and D.

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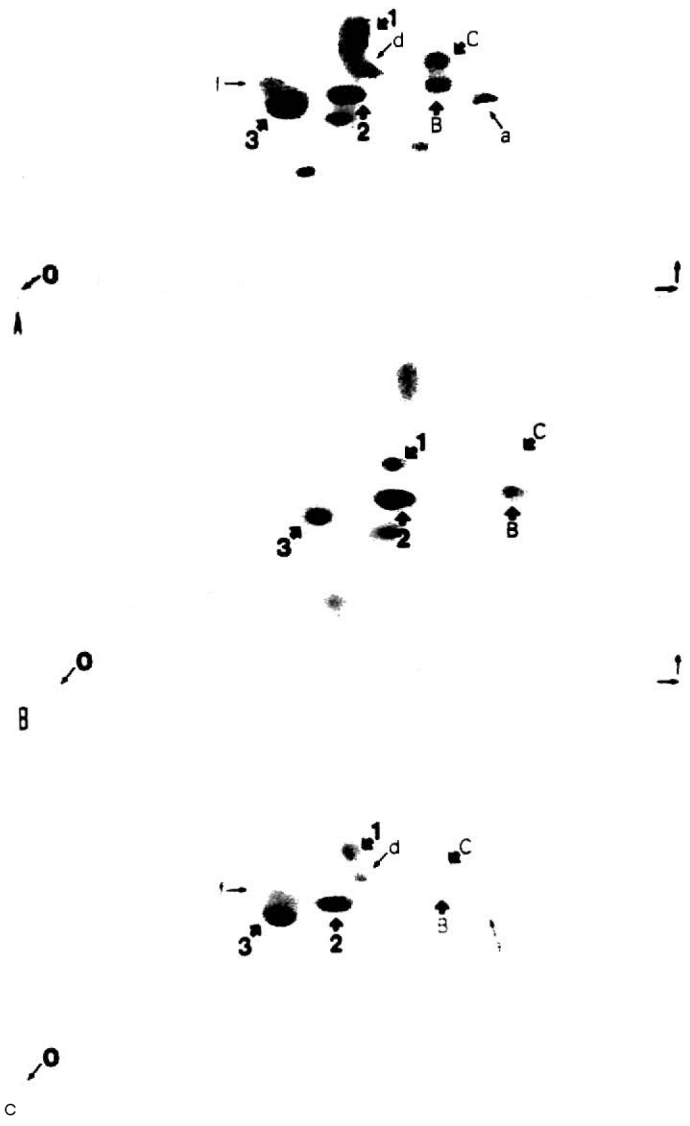

Fig.14. Results of mixing approximately 30,000 cpms of the A) pig 39,000 component and B) frog 75,000 to obtain the pattern in C). Heavy arrows indicate spots common to both maps and also the mix. Lighter arrows and small letters indicate spots common to the pig map only. The lettering and numbering system is the same as before. One can see that spots common to both (1,2,3,B, and C) are relatively dark in the mix pattern, while those unique to the pig (a,d, and f) are very light in the mix.

TABLE 5

IODINATION FACTORS OF MAJOR PEPTIDES PRESENT ON PEPTIDE MAPS OF ALL SPECIES

SPOT	PIG 39K	PIG 37K	COW 39K	COW 37K	CHICK 39K	CHICK 37K	FROG 75K
A	2	-	2	-	1	-	-
B	2	-	2	-	2	2	1
C	2	-	2	-	1	-	1
D	2	-	2	-	3	-	-
E	1	-	1	-	3	1	1
F	2	-	2	-	2	1	-
G	-	-	-	-	2	-	-
H	1	-	1	-	1	-	1
1	4	2	4	3	2	1	2
2	3	1	3	1	2	3	5
3	5	4	5	5	6	2	4

NOTE: Only major spots were counted; the ratios being rounded off to the nearest integer. In the 39K maps, spot H was used to normalize, in the pig and cow 37K maps spot 2 was used. In the chick 37K map and the frog 75K map spot 1 was used to normalize due an extreme difference in iodination.

Apparently there is something unique about this peptide in the chick GTPase.

For the 37,000 maps spot 2 was used to normalize the pig and cow maps but due to an extreme difference in iodination of this spot in the frog and chick maps, spot 1 was used for these. This suggests a somewhat different structure in the chick and frog enzyme. According to Table 5, it would appear that the peptide, referred to as spot 2, is much more accessible to iodination in the chick and frog enzyme, suggesting an altered conformation appearing somewhere during evolution. This eliminates the chance of also using a spot comparatively that is merely due to contamination (if the factor was less than one, it is assumed to be due to contamination.)

V. DISCUSSION

Due to the possible importance of GTPase in the visual excitation process in the retina, a biochemical characterization of it might elucidate the mechanism and role of this enzyme in the visual response. Recent studies have suggested that the binding of GTPase to bleached rhodopsin is necessary for maximum enzymatic activity. [17,41,45] Identification of the catalytic moiety of the enzyme is thus a necessary prerequisite to fully comprehend the excitation process on a molecular level. All previous purification studies [41,45,56] have obtained major components of approximately 37,000 and 39,000 daltons using cow GTPase. I also observed these two components in the pig and chick GTPase, neither of which have previously been studied. In the frog GTPase only one component of 75,000 daltons appeared, in contrast to the doublet. This enzyme has been used for kinetic studies but no prior reference to a 75K component was found.

To ensure that these were the actual molecular components of GTP ase, I first assayed them for specific GTP hydrolyzing activity and found it to be approximately the same, 30.0 nanomoles of p_i liberated/minute/picagram of protein, for all four

species. Godchaux and Zimmerman [44] found a specific activity of 26.0 mamomoles of P_i liberated/minute/microgram of protein using cow GTPase. No activity has been determined previously for pig, chick, or frog. I also found that this activity was highly dependent on the presence of bleached ROS membrane proteins, namely rhodopsin, which is in full argreement with all previous studies. [21] The frog GTPase whch had only the one component of around 75,000 daltons also exhibited the same specific activity. This component appeared later in other species after repeated freeze/thaw treatments and accompanied a relative decrease in the amount of the 37,000 and 39,000 components. I also used the [γ - ^{32}P] 8 -N_3 GTP, a radioactive, photoaffinity analoque of GTP to see which components bound GTP in the catalytic process. The results indicated that the major GTP - binding component of the GTPase was the 37,000 dalton protein in all species except when the higher molecular weight component was present. Photoaffinity labeling has been done previously using cow GTPase [53], but never pig, chick, or frog. They also found the 37,000 dalton component to bind the GTP-analogue. There has been no previous encounter with the higher molecular weight species in photoaffinity labeling experiments. In the latter case, I found that the 75,000 band strongly bound the analogue and I observed no binding in the 37,000 region. Other investigators [56] have reported binding of the nonhydrolyzable analogue, quanosine 5' - [β - γ - imido] triphosphate, to the 39,000 component of the cow GTPase, but the reason for the discrepan-

cy is not known. It is possible that both components possess some GTP - binding capabilities but due to some steric factors, certain analogues are able to bind and others aren't. This leads to the possibility of the higher molecular weight component being of a 37-37 or 37-39 dimer formation since the analogue did bind to it.

To help clarify the relationships between the 37,000, 39,000, and 75,000 components, I compared maps of each of their radioiodinated tryptic peptides. The 39K and 37K maps were highly related and extensively conserved from species to species and 75K map also appeared highly related to the 37K maps. On the basis of the maps and photoaffinity labeling it is possible that the 39,000 molecular component never exists in the frog at all. It is also possible that the higher molecular weight species is a precursor to the 39,000 and 37,000 components and is cleaved after translation. From the observed radioindated map patterns (Figure 9-11) it appears that the 37,000 component may be a smaller fragment of the 39,000 one due to the common spots. If this is so, cleavage may occur somewhere along the chain of one of the 37,000 components leaving the other fragment containing the full uncleaved chain resulting in a 39K and 37K component. This cleavage may not occur in the frog species and the higher molecular weight species therefore remains. This would also explain both components having GTP -binding capabilities and also how the binding site may be hidden in the tertiary structure of the 39,000 chain but fully exposed in the 37,000 fragment. An

interesting experiment that would help clarify these assumptions would be to determine whether the higher molecular weight species binds the imido analogue.

Since the tryptic peptide maps indicate at least nine major peptides are conserved over approximately 200 million years of evolution, it would seem likely that these peptides are necessary in the catalytic or regulatory functions of the enzyme and overall play an important role in the mechanism of visual response.

The large degree of homology maintained through evolution seems to indicate a very important physiological role has been assigned to the retinal GTPase. This is exemplified in other cases, namely, insulin and hemoglobin. Both proteins show large degrees of relatedness and homology among evolutionary divergent species also. Their physiological functions are well characterized as to their importance.

This is the first interspecies comparison ever done using retinal GTPase and it seems to suggest, as with insulin and hemoglobin, a high degree of homology and therefore a very important function. The peptides that are conserved may actually be located near or comprise the catalytic site for GTP hydrolysis. Additional studies with photoaffinity analogues may enable us to determine which peptides, in fact, do the actual binding, and further our knowledge of the visual process on a molecular level.

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INTERSPECIES CONSERVATION OF RETINAL GUANOSINE 5'-TRIPHOSPHATASE.

by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

The vertebrate retinal rod outer segment (ROS), responsible for light detection in the eye is comprised of thousands of flattened vesicles called discs. The membranes of these disc constitute 99.5% of the total ROS membrane. The membranes contain four major proteins; rhodopsin, and three others which have specific enzymatic activities. One, a GTPase comprises 7% of the membrane protein, and has previously shown in the cow to be comprised of two bands on SDS gels with molecular weights of 37,000 and 39,000. In the four species studied here (pig, cow, chick and frog) the 37 and 39K bands were present in all except for the frog where one band only appeared at approximately 75K. This 75K band also appeared in the chick and in the pig after numerous freeze/thaws, leading to the possibility of it being a dimer. Labeling with the photoaffinity analogue [γ -^{32}P] 8 -N_3 GTP shows that it is the 37K which binds the GTP. Further evidence of the 75K being a dimer was found as it also bound the analogue. Tryptic peptide mapping of the 32K, 37K, and 75K components of all species showed remarkable similarities between the two components of the same species and also among the four different species. The 37K appeared to be a smaller fragment of the 39K component. At least nine peptides in the 39K appeared conserved throughout evolution. Together, these

results demonstrate that the GTPase consists of two components of 37K and 39K daltons in the pig, cow, and chick and one component of 75K, possibly a dimer, in the frog; these components are highly conserved; and the function of this enzyme is most likely an important one in the mechanism of visual response.