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Light-Stimulated, Magnesium-Dependent ATPase in Toad Retinal Rod Outer Segments†

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ABSTRACT: Bleaching of rhodopsin increases adenosine and guanosine triphosphatase (ATPase and GTPase) activities present in the retinal rod outer segments (ROS) of the toad *Bufo marinus*. The light-stimulated activity travels with either broken or intact ROS widely separated on a metrizamide density gradient. The effect is primarily linked to the bleaching of rhodopsin in the red rods since the enzyme is half-stimulated by bleaching about 10% of the rhodopsin in either orange or blue light. The K_ms for ATP and GTP are 30 μM and 0.21 mM, respectively. Both activities appear to be catalyzed by the same enzyme since adenylyl imidodiphosphate (AMP-PNP) is a more effective inhibitor of both the ATPase and the GTPase than its guanyl analogue, GMP-PNP. The stimulation by bleaching is unaffected by the monovalent cations Na^+ or K^+ or by three different inhibitors of the mitochondrial ATPase. The light stimulation requires Mg^{2+}, and it is not seen when Ca^{2+} is the only divalent cation added. The chelating agent 2,2′-ethylenedioxybis(ethyliminodiacetic acid) increased the light stimulation still further, while adding Ca^{2+} lessened it. The activity was 0.9 ± 0.25 μmol of P_i released (mg of rhodopsin)$^{-1}$ h^{-1} and increased by a factor of 2.1 ± 0.3 in the light under optimal conditions. Low concentrations of Triton X-100 do not remove the light effect so it is not due to a membrane permeability change. The light stimulation is not minicked by the addition of *all-trans*-retinal. The fractional light activation which occurs when bleached and native membranes are mixed together is linear with the proportion of bleached membranes present.

The rod outer segments (ROS)[1] of the vertebrate retina can generate an electrical signal upon absorbing a single quantum of light (Yau et al., 1977). A sodium current flows into the outer segment of the rod cell in the dark and is inhibited in the light (Tomita, 1970; Hagins et al., 1970), changing the electrical potential across the plasma membrane. For dark-adapted vertebrate retinas half-maximal response of the rod requires about 30 quanta absorbed per rod (Fain & Dowling, 1973) or, in the case of the animal used in this study, the toad *Bufo marinus,* the bleaching of about one rhodopsin in 10^8 (Fain, 1975). Two possible mechanisms for amplifying the light signal to an electrical one have been advanced from the data published so far. One is that a phosphodiesterase is activated by the bleaching of rhodopsin and that the change in levels of 3′,5′-c-GMP in the ROS leads to a change in the sodium permeability of the outer segment plasma membrane (Woodruff et al., 1977). Another is that Ca^{2+} ions, sequestered in the flat discs whose membranes contain rhodopsin and which fill the ROS, are released into the outer segment cytoplasm when light bleaches rhodopsin and cause the sodium current to drop (Hagins and Yoshikami, 1974).

The theory that an ion might be released from the discs upon bleaching prompted an investigation to see what role an adenosine triphosphatase (ATPase) activity of the ROS might play in transporting ions across the disc membrane. The ionic gradients across ROS disc membranes in a living cell are not known (as opposed to those across the plasma membrane), although there is some evidence for a gradient of free Ca^{2+}. The Ca^{2+} content of freshly isolated ROS discs has been measured by Szuts & Cone (1977) and found to be about 0.1–0.2 Ca^{2+} ion per rhodopsin molecule or about 5 mM inside the disc, although most of this Ca^{2+} may be bound. In addition, the Ca^{2+} concentration in dark-adapted outer segment cytoplasm is about 1 μM since somewhat higher Ca^{2+} concentrations can affect the light response in the presence of the ionophore X537A (Hagins & Yoshikami, 1974). An ATP-dependent uptake of Ca^{2+} into bovine discs has also been reported (Schnetkamp et al., 1977) and may represent transport against a concentration gradient. None of the ATPase activities which are found in ROS preparations has been correlated with this process. In fact, it has been difficult to prove that any ATPase does come from the ROS. A Mg^{2+}-ATPase has been measured in bovine ROS preparations relatively free from mitochondrial contamination (Hendriks, 1975; Berman et al., 1977) but no activation by Ca^{2+} of this ATPase has been shown.

This work describes an ATPase in the ROS of the toad *Bufo marinus* which is activated as much as 2.5-fold by light (and

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[1] Abbreviations used: ROS, rod outer segment; EGTA, 2,2′-ethylenedioxybis(ethyliminodiacetic acid); EDTA, ethylenediaminetetraacetic acid; AMP-PNP, adenylylimidodiphosphate; GMP-PNP, guanylylimidodiphosphate; PK, pyruvate kinase; LDH, lactic dehydrogenase; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate.

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which may be the same as that reported by McConnell & Scarpelli (1963)). The light activation is used to demonstrate that the ATPase is present in the ROS. In addition, some properties of this light-stimulated ATPase, including its specificity for GTP and its dependence on ionic conditions and the fraction of rhodopsin bleached, are reported. A light-activated guanosine triphosphatase (GTPase), which is half activated when one part in 2000 of the rhodopsin is bleached, has recently been described for frog ROS (Robinson & Hagins, 1977; Wheeler & Bitensky, 1977), but the ATPase and GTPase activity described here is shown to have very different properties.

Materials and Methods

ATP, GTP, PEP[1], β-NADH, LDH, pyruvate kinase (PK), Hepes, AMP-PNP, and metrizamide were purchased from the Sigma Chemical Co. Carrier-free [^{32}P]phosphate was from New England Nuclear. [^{3}H]ATP and [^{3}H]GTP were from ICN as was GMP-PNP. Efrapeptin (A23871) was obtained from Robert Hamill (Eli Lilly) and aurovertin was the kind gift of Harvey Penefsky (Public Health Research Institute, New York). *all-trans*-Retinal was prepared by Paul K. Brown (Harvard University). Triton X-100 was from Rohm and Haas and deoxycholic acid (Sigma) repurified (McClennan, 1970) by J. Anderson (Harvard).

Care of Toads and Preparation of ROS. The toad *Bufo marinus* (Mogul-Ed, Oshkosh, Wis.) was fed live crickets (Fluker's Cricket Farm, Baton Rouge, La.) for at least 10 days after arrival before use and maintained above 75 °F in a moist environment (Nace et al., 1974). The importance of good care became clear when, on two separate occasions in November and December 1976, toads were sacrificed within a few days of arrival. No light-stimulated ATPase could be measured in the ROS and in both shipments the activity returned to other animals sacrificed after 2 weeks of laboratory care.

The cornea and lens were cut from the front of the dissected eye of dark-adapted animals, and the retina was loosened and lifted out of the eyecup. This and all other operations were performed under indirect red light (General Electric bulb BCJ). All solutions (except those used in assaying the ATPase) were bubbled with nitrogen before use, and all preparations were kept on ice, under nitrogen, prior to assay (Dratz & Farnsworth, 1976).

Purification of ROS membranes was accomplished in two ways: (1) 4–6 retinas were shaken vigorously by hand in 1–2 mL of 33% sucrose (w/w) with a buffer solution used for most sucrose solutions (10 mM Hepes, 10 mM KCl, 1 mM dithiothreitol (DTT), pH 7.2). In experiments where the effect of Ca^{2+} was examined, the KCl/Hepes solution was passed over Chelex-100 (Bio-Rad) to remove 0.2 millimoles Ca^{2+} per mol of Hepes detected by atomic absorption. The ROS were diluted with 26% (w/w) sucrose and layered over 33% (w/w) sucrose in three SW-50 (Beckman) nitrocellulose tubes and spun for 10 min at 10 000 rpm. The crude ROS were diluted into a lysis solution, 0.5 mM Hepes, 0.5 mM KCl, pH 7.2. The ROS fragments were layered into three SW-50 tubes onto a 30.5% (w/w) sucrose cushion whose density was checked by refractive index at room temperature (Raubach et al., 1974). After 15 min at 15 000 rpm both membranes on top of the sucrose cushion and at the bottom of the tube were collected and washed separately once or twice in lysis solution at 20 000*g* for 15 min. The resulting pellets, taken up in lysis solution, were stored at −70 °C and retained light-stimulated ATPase activity for as long as 2 weeks. Loss of the light-stimulated activity usually occurred after storage on ice for 24 h. Early experiments used ROS taken directly from the first sucrose step (or from a 26–36% (w/w) sucrose gradient) without a wash in lysis solution, and their use is noted in the text. Some lysed ROS preparations were pelleted onto a 5% dextran T110 (Pharmacia) barrier in SW-50 tubes (35 000 rpm for 2 h) similarly to the method of Smith et al. (1975).

(2) Retinas were shaken in a solution of 104 mM NaCl, 10 mM metrizamide, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Hepes titrated to pH 7.2 with KOH, and layered on a gradient of 5–24% (w/v) metrizamide where the metrizamide replaced the NaCl to maintain an isoosmotic or slightly hyperosmotic medium (Robinson & Hagins, 1976). After 10 min at 8000*g* in an SW-50 tube, two bands of ROS were eluted by puncturing the bottom of the tube: intact (at about 8% metrizamide) and broken (at about 18% metrizamide) as judged by phase microscopy. This separation has also been observed on isoosmotic gradients of bovine serum albumin (Falk & Fatt, 1973). The metrizamide enters the broken ROS and makes them more dense than the intact ones.

[γ-^{32}P]ATP and GTP were synthesized by a modification of the method of Glynn & Chappell (1964). The enzymatic exchange reaction with [^{32}P]phosphate was run for 5 h to label GTP as opposed to 2 h for ATP.

[γ-^{32}P]ATPase and GTPase Assay. Equal volumes of ROS in lysis solution, containing from 0.2 to 0.5 mg/mL rhodopsin, threefold concentrated reaction buffer (containing salts, 3 mM DTT), were held together on ice for 10 min, at least, and 80 μL was added to new, distilled water-washed tubes (10 × 75 mm). The reaction was started with the addition of 40 μL of radiolabeled nucleotide and took place at 37 ± 2 °C in a water bath. The reaction was quenched with 0.5 mL of 5% trichloroacetic acid, and the tube was placed on ice. The mix was added to a 0.1 g/mL slurry of acid-activated charcoal in 5% trichloroacetic acid, spun down on a tabletop centrifuge, and 0.5 mL of the supernatant was counted for radioactivity. Free [^{32}P]phosphate in unreacted controls gave a background of 1–2% of the total counts. Most assays were done in triplicate and the median value used. Graphed error bars show the range of values. ATPase activities were calculated in units of micromoles of phosphate released per mg of rhodopsin per hour.

ATPase or GTPase activity was linear with time when an ATP or GTP regenerating system was used (PEP and PK), and this was coupled to NADH oxidation (Barnett, 1970) to measure the production of ADP or GDP directly. NADH and LDH were added at the end of the assay, along with 10 mM EDTA to stop the ATPase, and the amount of pyruvate formed during the assay was measured by NADH oxidation at 340 nm after 300 μL of 0.3 N NaOH had stopped the reaction (Lowry & Passonneau, 1972). Controls where added ADP exceeded NADH were run in the presence of ROS and were used as a baseline to compare activities with and without bleaching. With a regenerating system the actual ATP or GTP hydrolysis will be greater than the apparent hydrolysis measured by the release of [γ-^{32}P]phosphate due to the dilution of the specific activity of the ATP or GTP by the regenerating system. Actual phosphate release (ATP or GTP hydrolysis) depends on the fraction of $^{32}P_i$, $f[^{32}P_i]$, released as follows: f(P_i released as a fraction of total ATP) = $-\ln(1 - f[^{32}P_i])$.

Chromatography of ^{3}H-Labeled Nucleotides. ATPase or GTPase reactions were quenched by the addition of one-third volume of 1.2 N perchloric acid, and the ClO_4^- anion precipitated with the addition of an equal volume of 1.1 N $KHCO_3$, 0.5 N KOH. Chromatography was done in either 1 M LiCl (Randerath & Randerath, 1967) on PEI plates (Brinkmann) or on cellulose thin-layer plates (Eastman 13254) in isobutyric acid:1 N ammonia (100:60 v/v) (Cantley & Hammes, 1973).

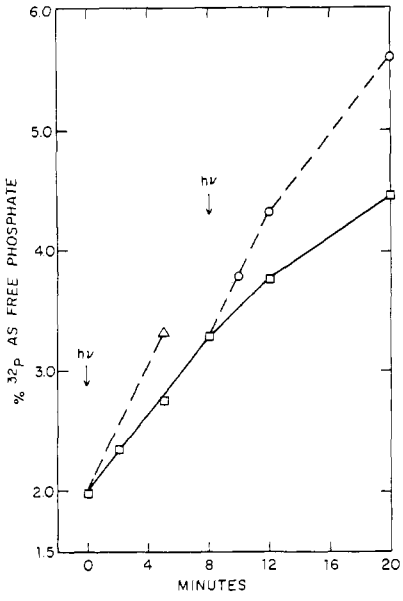

FIGURE 1: Effect of light stimulation on ATPase activity. ROS taken directly from sucrose gradient were diluted into a reaction solution of 15 mM KCl, 1 mM DTT, 1.5 mM $MgCl_2$, 1.5 mM ATP, 15 mM Tris, pH 7.2 (plus efrapeptin and strophanthidin), on ice. Tubes were exposed to white light at the times indicated. Percent ATP hydrolyzed during incubation in the dark (□); after bleaching at zero time (△); after bleaching at 8 min (○).

TABLE I: Light-Stimulated ATPase of Fractions from Metrizamide Gradient after One Wash in Lysis Buffer.[a]

	activity in μmol of P_i (mg rhodopsin^{-1} h^{-1})		
fraction	dark (no inhibitors)	dark (plus efrapeptin & strophanthidin)	bleached (plus inhibitors)
upper (intact ROS)	4.9	0.81	1.55
mid gradient		1.6	2.64
lower (broken ROS)	2.3	0.85	1.57

[a] Conditions: 40 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.33 mM ATP, 10 mM Hepes, pH 7.2; 30 min at 37 °C.

The latter method was used to separate free guanosine or adenosine from the di- and triphospho nucleotides.

Light Dependence of the ATPase Activity. ROS in a quartz cuvette were bleached by light from a slide projector filtered by a $CuSO_4$ solution as a heat filter and either a Kodak Wratten no. 45 filter (blue light) or a Schott-Jena OG2 filter (orange light, cutoff at 570 nm). Aliquots of ROS were removed from the cuvette, which was kept on ice when not being irradiated, and assayed for ATPase activity after anywhere from two to 200 s accumulated bleaching. The logarithm of the fraction of rhodopsin unbleached depends linearly on the time of bleaching in theory, and this relation held. Rhodopsin concentrations were calculated from the light minus dark difference at 500 nm of samples in 1.5% Triton X-100, 20 mM NH_2OH, using the approximate molecular weight and extinction coefficient of 40 000 for rhodopsin (Daemen, 1973). Small amounts of bleaching were calculated from the time of irradiation. One hundred percent bleaching was accomplished by moving the sample directly in front of the projector and was done with orange light for almost all experiments.

Phosphorylation of rhodopsin (Miller et al., 1977) was measured over times of 2 to 6 min to obtain the initial rates of phosphorylation.

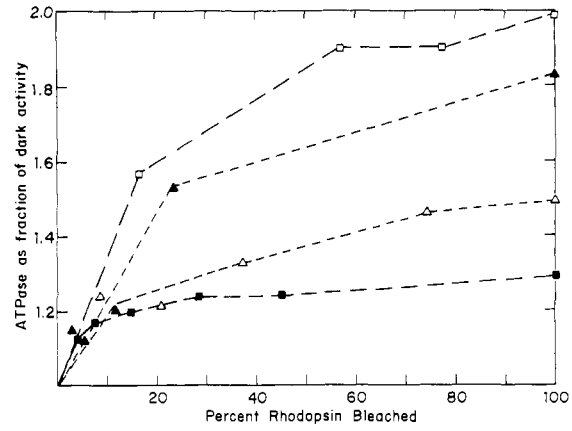

FIGURE 2: ATPase as a function of percent bleaching (measured at 500 nm) by either orange or blue light. The ATPase activity in the dark has been set at one for comparison between experiments. Bleaching in orange light, dark activity was 0.43 μmol mg^{-1} h^{-1} (□) or 0.8 (■); bleaching in blue light, dark activity was 0.95 (△) or 0.5 (▲). Membranes for sample (▲) were washed and assayed in 0.5 mM EGTA (see text and Figure 4 for EGTA wash procedure.).

Atomic absorption analysis of Ca^{2+} content in retinal ROS was done on a Perkin-Elmer 303 with the help of Robert Quirk (Dept. of Geological Sciences, Harvard University). Known amounts of Ca^{2+} added to ROS samples were detected with an efficiency of better than 80%.

Results

Bleaching and ATPase Activity. A light-stimulated ATPase or GTPase activity (see Figure 1) was seen in a variety of crude and highly purified ROS preparations described above. The light-stimulated activity did not decrease with time and appeared whether the rhodopsin was bleached before or after the addition of ATP. Bleached membranes were usually assayed in the dark, but continuous illumination in white light did not alter the results. The light-stimulated ATPase travelled with both intact and broken retinal ROS on a metrizamide gradient, yielding the same specific activity in both fractions (Table I), and this also held for the light-stimulated GTPase. By contrast, the specific activity of the mitochondrial ATPase, measured independently of (Na + K)-ATPase activity, was not the same in each fraction, being higher in the top one. The light activity was observed in partially intact ROS but no semblance of intact ROS structure was required for this effect. The most commonly used preparation—ROS washed twice in lysis solution—appeared as dispersed refractile bodies only a few nanometers in diameter under a phase microscope.

The relationship between the stimulation of ATPase activity and rhodopsin bleaching at 500 nm is shown in Figure 2. Orange light and blue light give a half-maximal response for roughly the same amount of rhodopsin bleached, and this varies between 5 and 15% bleaching. *Bufo marinus* has both red rods (λ_{max} = 502 nm) and green rods (λ_{max} = 433 nm) of similar dimensions (Hárosi, 1975), but the red rods outnumber the green seven to one (Fain, 1976). The data are sufficiently accurate to show that the red rod pigment (rhodopsin) and not the green rod pigment is responsible for the major part of the light activation. The rate of bleaching of the green rod pigment with respect to rhodopsin will be much faster in blue light than in orange. If the green rod pigment was entirely responsible for the light effect, the blue light and orange light activation curves would be separated by a factor of about ten along the horizontal axis of Figure 2.

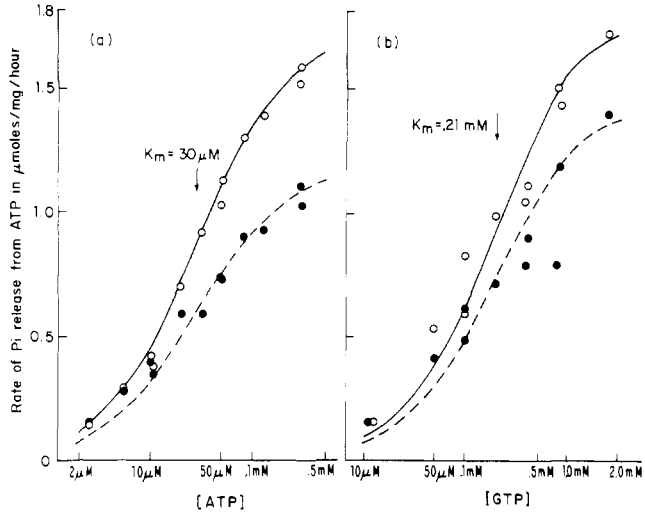

FIGURE 3: Activity as a function of nucleotide concentration. Assay conditions: 37 °C; in 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1.5 mM PEP, 10 mM Hepes (pH 7.2), PK, efrapaptin, and strophanthidin. Rhodopsin and nucleotide concentrations were varied to obtain 4–20% hydrolysis, and hydrolysis was linear with rhodopsin concentration and time. K_m and V_{max} were obtained by eye from Eadie–Hofstee plots of the data. (a) ATP: K_m = 30 μM; V_{max} = 1.2 in native membranes (●), 1.75 in bleached (○). (b) GTP: K_m = 210 μM; V_{max} = 1.5 in native (●), 1.9 in bleached (○).

TABLE II: Effect of Efrapeptin on a Relatively Impure Preparation of ROS.

additions[a]	native	bleached	bleached–native
none	2.5	2.24	−0.26
2 μg/mL efrapeptin	0.97	1.25	+0.28

[a] 20 min at 37 °C, 10 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 0.3 mM ATP, 10 mM Hepes, pH 7.2. ROS pellet from base of sucrose cushion.

Properties of the ATPase Reaction. The ATPase and GTPase activities, in native or bleached preparations, were due to the release of the γ-phosphate only from the nucleoside triphosphates. The coupled assay, which measured the production of ADP or GDP directly, showed almost without exception that a light-stimulated production of the diphosphate occurred. Due to the relative insensitivity of this method, it was not certain whether the ATPase or GTPase measured by the release of [^{32}P]phosphate was entirely accounted for by the production of ADP or GDP. Other breakdown products of ATP or GTP were detected only at very low levels by thin-layer chromatography using [^{3}H]ATP or GTP as a substrate, however. The ATP regenerating system was present in these assays so that processes which break down ADP or GDP even further would not be able to take place. The overall rate of production of other nucleotides, in the dark or as a result of bleaching, was less than 5% of the ATPase or GTPase activity measured by the release of [^{32}P]phosphate in parallel assays (50 μM ATP or 100 μM GTP were the substrate concentrations). A light-stimulated cycle of the phosphorylation and dephosphorylation of rhodopsin did not explain these results. The increased levels of phosphorylation by ATP or GTP upon bleaching were low and accounted for less than 10% of the increase in ATPase or GTPase activity. Most likely the kinase was washed off the membrane in the lysis solution. Conditions similar to those under which ROS enzymes, such as the kinase for rhodopsin, are known to come off the membrane—washing

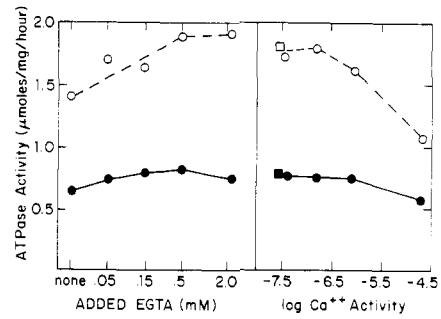

FIGURE 4: Effect of EGTA and Ca^{2+} addition on ATPase activity. ROS were washed twice in lysis solution containing 0.3 mM EGTA and taken up in EGTA-free lysis buffer. Reaction conditions: 20 min at 37 °C; in 10 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 0.7 mM PEP, 0.33 mM ATP, 10 mM Hepes, pH 7.2, PK, efrapeptin, and strophanthidin. Native (●) and bleached (○) activities with added EGTA or EGTA-buffered Ca^{2+} (with 0.5 mM EGTA). Native (■) and bleached (□) activities in EGTA-buffered Ca^{2+} (with 2 mM EGTA). Ca^{2+} activities were calculated using a Ca–EGTA binding constant of $10^{6.8}$ M at pH 7.2 (Sillén & Martell, 1964).

the membranes in 1 mM EDTA, pH 7.6 (Kühn et al., 1973; Miki et al., 1975; Wheeler et al., 1977)—did not diminish the light effect either.

The ATPase and GTPase activities appear to be related. The rates of hydrolysis of ATP or GTP were about the same in a single preparation, although they ranged over a factor of three in different preparations. The approximate K_m for GTP (210 μM) is higher than that of ATP (30 μM), and the dependence of the ATPase and GTPase on substrate is shown in Figure 3. The ATPase and GTPase are both inhibited by the nucleotide analogue AMP-PNP much more effectively (60% inhibition of ATPase and GTPase activities after bleaching by 0.1 mM Mg^{2+}-AMP-PNP and 80% inhibition by 0.3 mM Mg^{2+}-AMP-PNP with 0.5 mM Mg^{2+}-ATP or 0.5 mM Mg^{2+}-GTP as substrate) than by GMP-PNP (5% inhibition of both activities by 0.1 mM Mg^{2+}-GMP-PNP, 35% inhibition by 0.3 mM Mg^{2+}-GMP-PNP). These data can be understood if both the ATPase and the GTPase are catalyzed by the same enzyme. In that case, the greater inhibition by AMP-PNP would reflect the fact that the enzyme active site binds adenine nucleotides more tightly than guanine nucleotides. This surmise is supported by the lower K_m for ATP.

Ionic Requirements for Light-Stimulated Activity. The light-stimulated ATPase does not require any monovalent cation, in particular Na^+ and K^+, which can be replaced entirely by choline and Tris, nor is it altered by the addition of the (Na + K)-ATPase inhibitor strophanthidin. Replacement of Cl^- by SO_4^{2-} in every step of the ROS preparation and assay, as well as during the preparation of [γ-^{32}P]ATP, did not eliminate the light effect. Also, the light stimulation was not altered by Cl^- added back during the assay. The light minus dark activity was independent of any of three mitochondrial ATPase inhibitors, efrapeptin, aurovertin, and oligomycin, each of which is known to act at a different site on the mitochondrial ATPase (Lardy et al., 1975), and each was added at a concentration of 2 μg/mL. Mitochondrial ATPase and (Na + K)-ATPase activities accounted for about 20–30% each of the total dark ATPase activity in typical ROS preparations and thus inhibitors of these ATPases were routinely included in all assays.

Relatively impure ROS which pelleted through a 30.5% sucrose cushion in the last step of ROS preparation had high levels of mitochondrial ATPase contamination in them (about 60%) compared with the ROS membranes which floated on the sucrose. A light-inhibited ATPase activity was measured

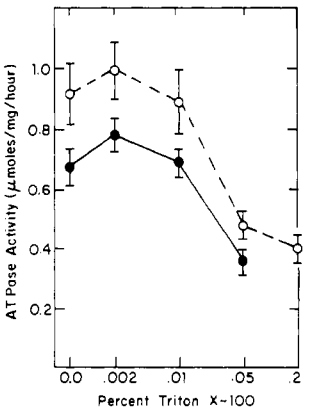

FIGURE 5: Effect of Triton X-100 on ATPase activity. One part 0.25 mg/mL rhodopsin in lysis solution kept on ice with one part 30 mM KCl, 6 mM $MgCl_2$, 6 mM DTT, 30 mM Hepes, pH 7.2, efrapeptin, and strophantidin for more than 10 min with detergent added at the concentrations shown. Bleached (○) and native (●) activities (30 min at 37 °C) in 0.33 mM ATP (after the addition of one part 1.0 mM ATP).

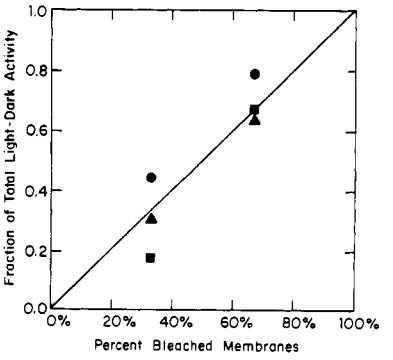

FIGURE 6: Mixing of native and bleached membranes. ATPase activity, above the level in native membranes, calculated as a fraction of the total light minus dark activity, was plotted with respect to the fraction of bleached membranes present in the mixture. Assay conditions: same as in Figure 4, plus 0.5 mM EGTA. (●) Dark activity, 0.9 μmol/mg^{-1} h^{-1} and light/dark ratio, 1.56. (■) Activity, 1.12, and ratio, 1.55. (■) Activity, 0.86, and ratio, 1.49.

in the impure material which was abolished or reversed by the addition of efrapeptin (Table II). Ostwald & Heller (1972), who did not use mitochondrial ATPase inhibitors in all their assays, may have described a similar light inhibition.

ATPase and GTPase activities, not sensitive to the mitochondrial inhibitors efrapeptin and oligomycin, but of similar magnitude to the Mg^{2+}-ATPase, could be stimulated by adding Ca^{2+} alone. Such an ATPase, requiring Ca^{2+} but not Mg^{2+}, has been reported in bovine and frog ROS preparations (Ostwald & Heller, 1972; Hendriks, 1975; Sack & Harris, 1977) but others have shown that this is a contaminant (Berman et al., 1977). Pigment bleaching had no effect on the Ca^{2+}-ATPase or GTPase of toad ROS preparations where a light-enhanced Mg^{2+}-ATPase was present. ATPase activity was completely abolished in 0.5 mM EDTA with no added divalent cations.

Mg^{2+} alone was sufficient for the light-stimulated ATPase to be observed. Neither the light-activated nor the dark Mg^{2+}-ATPase requires Ca^{2+} for activity, however. This is demonstrated in the left panel of Figure 4 which shows that as much as 2 mM EGTA did not reduce (and in fact enhanced) the light effect. A similar enhancement of the Mg^{2+}-ATPase of bovine ROS by adding EGTA has been noted (Berman et al., 1977). The total Ca^{2+} present in ROS preparations washed in 0.3 to 0.5 mM EGTA plus lysis solutions was checked by atomic absorption analysis and found to be about 2 Ca^{2+} per rhodopsin. In ATPase assays of these preparations the total Ca^{2+} concentration contributed by the ROS and the reagents was measured to be about 10 μM so that the free Ca^{2+} concentration was less than 10^{-9} M when 2 mM EGTA was present. To be sure that Ca^{2+} required for the ATPase activity was not sequestered inside the disc membranes and protected from the action of EGTA, a similar experiment was carried out in 0.01% Triton X-100 or 1 g of detergent/g of rhodopsin; adding 1 mM EGTA enhanced the light effect slightly.

As shown in Figure 4, added EGTA increased the light-stimulated activity as well as the ratio of light to dark activities, and this was observed in every preparation tested, although the magnitude of the EGTA effect was variable. Assays done in the absence of EGTA, before its effect was noticed, had an average ratio of light to dark activities of 1.45 ± 0.2 (20 preparations). Fresh samples washed twice in 0.3 mM − 0.5 mM EGTA plus lysis solution and assayed on the same day in the presence of 0.5 mM EGTA gave consistently better results: an average light to dark ratio of 2.1 ± 0.3 (six preparations) and a dark activity of 0.9 ± 0.25 μmol mg^{-1} h^{-1}. The maximum ratio observed was 2.5. Samples washed extensively in EGTA, followed by a wash in lysis solution lacking EGTA, gave inferior results.

TABLE III: The Effect of Added *all-trans*-Retinal.[a]

mol of all-trans/ mol of rhodopsin	native	bleached	bleached-native
0.0	0.80	1.07	0.27
0.22	0.93	1.17	0.24
0.97	0.89	1.08	0.19

[a] Conditions as in Table II, except 0.33 mM ATP, 1.5 mM PEP, PK, strophanthidin, efrapeptin, and 0.09 mg/mL rhodopsin were present.

Added EGTA may have improved the light stimulation by chelating the Ca^{2+} present in every assay (see above). The right-hand panel of Figure 5 shows how adding Ca^{2+} back could reduce both the light and dark ATPase activities. In 0.5 mM Ca^{2+} inhibition of the ATPase was no more than 50% as others have observed (Ostwald & Heller, 1972; Hendriks, 1975), and the light-stimulated and dark ATPases were reduced in activity approximately equally.

Effect of Retinal and Detergents. Titration of the ATPase activity with detergents sodium deoxycholate and Triton X-100 showed that both reduce light and dark activities simultaneously. At least a fivefold excess of detergent over rhodopsin was required before the light effect could not be measured. The results for Triton X-100 are shown in Figure 5.

The effect of *all-trans*-retinal, which is released from rhodopsin upon bleaching, and which might be activating the enzyme in some way, was examined. Up to 1 mol of all-trans/mol of rhodopsin added to an assay does not substitute for or replace the effect of light, reducing ATPase activity only slightly (Table III). The possibility of nonspecific all-trans activation can also be tested by mixing together bleached and native ROS membranes. The ATPase activity increased linearly with the proportion of bleached membranes which were present in an ATPase assay (see Figure 6). A comparison of Figures 2 and 6 shows that the ATPase is not activated by some freely diffusible substance produced stoichiometrically with the bleaching of rhodopsin.

Discussion

This work shows that a light-stimulated activity is present in the outer segments of the red rods of *Bufo marinus* which hydrolyzes both ATP and GTP. The evidence that this activity is not due to contamination in ROS preparations can be summarized as follows: First, the ATPase comigrates with broken and intact ROS on a metrizamide gradient. Also, the light activation depends similarly on bleaching red rhodopsin in orange or blue light. Taken together, these two facts show that bleaching red rods is required to observe the activation. The ROS structure can be completely destroyed by osmotic shock, without eliminating the light activity, and, further, no freely diffusible activator produced stoichiometrically with rhodopsin's bleaching, such as *all-trans*-retinal, is responsible for the effect. Thus one can say with some certainty that the enzyme activated by light is found in a membrane that contains rhodopsin and not in other material in an ROS preparation.

Since the light stimulation of the ATPase was considerably more variable than the dark activity, and could be lost during storage of the ROS at 0 °C for more than 24 h, it is worth pointing out that no experiment shows clearly that the two activities are due to a single enzyme. Lacking evidence to the contrary, the single enzyme hypothesis was assumed to be true during these experiments.

The ATPase activity can be enhanced more than twofold by bleaching the membranes and is highest in membranes washed and assayed in 0.5 mM EGTA. The effect of EGTA may have been due to the chelation of Ca^{2+} ions present in the sample, about 2 Ca^{2+} ions per rhodopsin. The levels of Ca^{2+} are much higher than in vivo levels if the data of Szuts & Cone (1977) showing only 0.1–0.2 Ca^{2+} per rhodopsin in freshly isolated frog ROS are correct. Others have recorded high levels of Ca^{2+} similar to those shown here (Hendricks et al., 1974), however, and the reasons for the discrepancies are not known. It is interesting to note that chelating agents protect ROS membrane lipids from iron-catalyzed oxidation (Dratz & Farnsworth, 1976), and this may partly explain the effect of EGTA on light activation.

A light-activated GTPase (Robinson & Hagins, 1977; Wheeler et al., 1977; Wheeler & Bitensky, 1977) not dealt with here is indeed a separate activity: its K_m for GTP is less than 1 μM, and bleaching of $^1/_{2000}$ of the rhodopsin, not $^1/_{10}$, is required for half-activation; also, it can be washed off the ROS membranes in 1 mM EDTA (Wheeler et al., 1977), whereas the ATPase described here cannot. A GTPase not sensitive to 0.05% bleaching from frog (Wheeler & Bitensky, 1977) has a similar K_m (90 μM) and V_{max} (0.75 μmol mg^{-1} h^{-1}) to the GTPase studied here (K_m = 210 μM, V_{max} = 1.5, in the dark). The GTPase and the ATPase described here are probably the same enzyme based on the relative inhibitions caused by AMP-PNP and GMP-PNP.

The ATPase could be located in either the discs or the plasma membrane of the ROS. If the specific activity of the ATPase is the same as the (Na + K)-ATPase, 1200 μmol of phosphate (mg of protein)$^{-1}$ h^{-1} (Jørgensen & Skou, 1971), it would make up 0.1–0.2% of the ROS protein. The plasma membrane makes up 1% of the total membrane in amphibian ROS (Daemen, 1973) and could accommodate the ATPase easily.

The function of the ATPase is unknown. It cannot be washed away from the membrane in lysis buffer, and this suggests that it is an integral membrane protein. On the basis of its requirement for cations and its sensitivity to inhibitors it is unlike any well-described transport ATPase: the mitochondrial proton pump, the (Na + K) exchange pump of eukaryotic plasma membranes, the K^+-dependent proton pump of gastric mucosa (Sachs et al., 1976), or the Ca^{2+} pump of the sarcoplasmic reticulum (Ikemoto, 1974). An obvious explanation for the light effect might have been an increase in the permeability of the disc membrane after bleaching which would let a transport ATPase operate faster. The effect of detergent makes this unlikely. The possibility of this ATPase functioning in transport remains untested.

This enzyme should play a major part in the metabolism of ATP in rod outer segments given the evidence here. Data on the nucleotide metabolism of fresh, intact, frog ROS kept in the dark shows that the loss of ATP is slow (Robinson et al., 1975; Caretta & Cavaggioni, 1976) and is less than 5% of the rate which would be expected from the dark activity of the ATPase measured here. The discrepancy may be due to the presence of high levels of ADP in even these freshly isolated ROS (Robinson, W., & Hagins, W., personal communication) and its consequent inhibitory effect on the ATPase. Light and dark ATPases are reduced by a factor of about four when 1 mM Mg^{2+}–ADP is added to the substrate 1 mM Mg^{2+}–ATP (data not shown.)

The light stimulation of the ATPase may have a physiological role, but the extent of bleaching required for the stimulation, as well as the fact that it amounts to little more than a doubling of the ATPase activity at best, makes this question highly speculative. The stimulation is not as dramatic as the fivefold stimulation of a phosphodiesterase from frog ROS (Miki et al., 1975) or the GTPase described earlier. The significance of the light effect will become clearer when the function of the ATPase is known.

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Coupling of Aspartate and Serine Transport to the Transmembrane Electrochemical Gradient for Sodium Ions in *Halobacterium halobium.* Translocation Stoichiometries and Apparent Cooperativity†

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ABSTRACT: Cell envelope vesicles, prepared from *Halobacterium halobium,* accumulate aspartate and serine in response to both electrical potential across the membrane, $\Delta\psi$, and chemical potential difference for Na^+, $\Delta\mu_{Na^+} = RT/F \ln (Na_{out}/Na_{in})$. Since $\Delta\mu_{H^+}$ of either positive or negative sign fails to influence the accumulation of amino acids, it was concluded that H^+ is not cotranslocated and thus the transport must be energized solely by cotransport (symport) with Na^+. The electrical ($\Delta\psi$) and the chemical ($\Delta\mu_{Na^+}$) components of the Na^+ gradient could be imposed separately on the vesicles, and the accumulation of aspartate and serine in response to each of these was examined. The ratio of the electrochemical potential differences for the amino acids and for Na^+ in the stationary state gives the coupling ratio, which for efficient coupling corresponds to the translocation stoichiometry. Na^+/aspartate is approximately 2 and Na^+/serine is approximately 1 for transport driven by $\Delta\psi$, but the corresponding values are 4 and 2 for transport driven by $\Delta\mu_{Na^+}$. In the range examined, between 0 and 150 mV, the initial rates of transport are fourth power functions of $\Delta\psi$ for both amino acids tested, but first power (linear) and second power functions of $\Delta\mu_{Na^+}$ for aspartate and serine, respectively. In contrast, the transport rates are hyperbolic functions of the amino acid concentrations, and the K_m values are invariant with $\Delta\psi$ and $\Delta\mu_{Na^+}$. Thus, $\Delta\psi$ and $\Delta\mu_{Na^+}$ seem to have direct regulatory effects on the transport carriers, in addition to their energizing function. The variable stoichiometry and the apparent cooperativity suggest, although do not prove, that the transport carriers for aspartate and serine possess allosteric properties.

Na^+-linked membrane transport systems are widespread among eucaryotic cells (for a recent review, see Crane, 1977), and have been shown to exist also in bacteria (MacDonald & Lanyi, 1975, 1977; Tsuchiya et al., 1977a,b; Tokuda & Kaback, 1977; MacDonald et al., 1977b). It is now generally accepted that these systems accomplish the accumulation of amino acids and sugars by coupling the energetically uphill transmembrane movement of the substrates to the downhill

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