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TRANSIENT PHOSPHORYLATION BY ATP OF A 160 000 DALTON PROTEIN IN ROD OUTER SEGMENTS OF *BUFO MARINUS*

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Radioactive phosphate was incorporated from [γ-^{32}P]ATP into a 160 000 dalton protein from preparations of highly purified toad retinal rod outer segment membranes. Maximal incorporation occurred at 1 μM ATP, and turnover in the presence of nonradioactive substrate was rapid, showing that the 160 kdalton protein catalyzes ATP hydrolysis. The 160 kdalton intermediate was sensitive to hydroxylamine, suggesting an acyl linkage between the protein and phosphate. Ionic requirements for phosphorylation showed the ATPase is different from other membrane-bound ionic pumps. The phosphorylated intermediate was almost completely suppressed by 20 μM vanadate, and partial suppression occurred at lower concentrations. About one 160 kdalton protein was labelled per 30 000 molecules of rhodopsin. Although [γ-^{32}P]GTP labeled the protein, the ATPase was far more specific for adenine than guanine nucleotides. The specificity for ATP and sensitivity to vanadate of the intermediate suggest a relation to an ATP-dependent structural change which occurs in stacks of outer segment discs (Thacher, S.M., (1980) Fed. Proc. 39, 2066).

Introduction

Many ATPase activities have been described in retinal rod outer segment preparations of the toad and other vertebrate species [1–4]. One of them, a Mg^{2+}-ATPase, is stimulated as much as 2-fold by bleaching the visual pigment rhodopsin [4], but its function is not known. ATP also has an effect on rod outer segment structure which is measured as a change in light-scattering from a suspension of stacks of outer segment discs [5–7]. The light-scattering change is inhibited by vanadate with a K_I of about 0.3 μM, and this and other evidence suggest that an ATPase is necessary for the change. The light-stimulated ATPase is not inhibited by micromolar vanadate, and apparently it is a distinct entity [7].

Two well-characterized membrane-bound ATPases which perform ion transport in other systems, the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-ATPase of sarcoplasmic reticulum, have phosphorylated intermediates which can be trapped by denaturation in trichloroacetic acid or SDS. The major polypeptides of each can be identified by SDS-gel electrophoresis when [γ-^{32}P]ATP is the reaction substrate. The phosphate on each band turns over rapidly and is sensitive to the presence or absence of ions which the ATPase pumps [8,9]. A similar phosphoprotein to these two in gastric mucosa probably corresponds to an ion pump purified from this tissue which transports both protons and potassium [10,11].

A phosphorylated protein from rod outer segments having similar properties to the ion transport ATPases is described here. It is clearly distinguished

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Abbreviations: SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

from the other known ATPases and is sensitive to vanadate. Its possible relation to the ATP-dependent structural change is discussed.

Materials and Methods

Rod outer segments were broken from the retinas of two or three well-fed *Bufo marinus* [4] by vigorous shaking in 2 ml 42% (w/w) sucrose containing 10 mM KCl, 0.5 mM $MgCl_2$, 6 μM $CaCl_2$, 10 mM sodium-Hepes, pH 7.3 (a buffer used for all sucrose solutions). The material was passed through 30 μm pore size nylon mesh in a Millipore Swinnex-25 filter holder, thus removing fragments of the retina. A wash of 4–5 ml 36% sucrose followed, and the filtrate was divided into three Beckman SW-50 centrifuge tubes. Sucrose solutions of 33% (w/w) and 26% (w/w), followed by water, were layered above. After a 10-min spin at 10000 × *g*, two bands appeared: outer segments on top of the 33% sucrose and unidentified material called 'debris' below. The outer segment fraction was diluted in 1 vol. of distilled water and layered over a gradient of 26–36% sucrose for isopycnic centrifugation (10 min at 10000 × *g*). Two closely spaced bands in the middle of the gradient were each removed by Pasteur pipette, the upper called 'ROS' (for 'rod outer segment') and the lower, containing less material, 'dirty ROS'. Intact outer segments do not appear in either fraction. Both fractions and the 'debris' were diluted with water and recovered by centrifugation for 10 min at 15000 × *g*. All pellets were taken up in 40 mM Tris-Hepes, pH 7.3/50 μM Tris-EGTA/30 μM $MgCl_2$, the buffer used, with various additions, for the phosphorylation reactions. This is procedure A. In procedure B, the pellets were resuspended instead in 0.3 mM EGTA/0.5 mM EDTA, pH 7.4 after the first spin in order to break up the disc membranes more thoroughly, spun down again, and dispersed in phosphorylation buffer. All solutions were bubbled with argon gas, and membrane pellets were resuspended under argon. Membranes were assayed immediately or stored at −70°C. The yield was usually about 1 mg rhodopsin, as determined spectroscopically [4].

Radioactive nucleotides. [γ-^{32}P]ATP and [γ-^{32}P]-GTP were enzymatically prepared at specific activities of 50–100 Ci/mmol [12]. The crude reaction mixture was diluted with 3 ml H_2O and labeled nucleotides were separated from free $^{32}P_i$ on Dowex AG 1-X2 resin [13]. To confirm that the [γ-^{32}P]GTP was not contaminated with [γ-^{32}P]ATP, the two nucleotides were separated in 1.0–4.0 M sodium formate, pH 3.4, on polyethyleneimine cellulose plates (Brinkmann) which had been water-washed [14].

Phosphorylation of membranes. Reaction buffer, membrane preparations, and labelled nucleotides (0.5–1 μM ATP or 5 μM GTP) were pre-equilibrated to the temperature of the assay. Phosphorylations lasted from 4 s to 1 min in a volume of 100 μl containing 20–50 μg of rhodopsin. The conditions were closely similar to those used by Avruch and Fairbanks [15]; The low $MgCl_2$ used by them is important because high $MgCl_2$ causes excess background phosphorylation. To measure turnover of phosphoproteins, nonradioactive ATP or GTP was added in excess several seconds after the labeled nucleotides. The addition of 0.2 ml 10% trichloroacetic acid quenched all reactions, and was followed by 0.1 ml of 0.5 mg/ml bovine serum albumin, 5 mM ATP, 5 mM P_i, and then 0.6 ml 5% trichloroacetic acid. Except as noted, assays were carried out in dim red light on unbleached ROS membranes, prepared under dim red light also [4].

Precipitates were water-washed once more for gel electrophoresis, or washed twice more in 5% acid containing ATP and P_i for analysis of total $^{32}P_i$ incorporation.

Samples for SDS-polyacrylamide gel electrophoresis were solubilized in SDS, sucrose, sample buffer and 15 mM dithiothreitol, without boiling to prevent the formation of rhodopsin multimers. The two gel buffer systems used were pH 7.4 Tris-acetate [15] or pH 2.4 phosphate [16]. Figures show only the pH 7.4 gels. The acrylamide concentration was 5.6%. Radioactivity trapped at the top of gels was often less if gels were stored one to three days before use. Molecular weight markers were β-galactosidase (116000) [17] and myosin (200000); a [^{35}S]methionine labeled lysate of *Escherichia coli* K12 (a gift of H. Shuman, Harvard University) provided markers at 155 and 165 kdaltons, the β and β′ subunits of RNA polymerase [18]. Gels were dried on filter paper and exposed to Kodak XR-5 film with a DuPont Cronex Lightning Plus intensifying screen at −70°C [19]. Exposure densities were analyzed by

an Ortec 4310 densitometer on films that were not overexposed.

Phosphorylated intermediate: hydroxylamine treatment. An acid precipitate of labeled membranes was taken up in 0.08 M sodium acetate, pH 5.8, with or without 0.2 M NH_2OH (HCl), which was prepared fresh and unbuffered for each experiment. After 30 min incubation on ice, the membranes were precipitated again in 5% trichloroacetic acid. Additional bovine serum albumin was added, and the precipitated material was washed twice before electrophoresis [20].

Results

When purified rod outer segment membranes were incubated briefly with [γ-^{32}P]ATP, a protein of molecular weight of about 160 000 was labeled. This phosphoprotein turned over rapidly with the addition of nonradioactive ATP, and bleaching of rhodopsin had no effect on either its incorporation of $^{32}P_i$ or its turnover (Fig. 1). Maximum incorporation into the 160 kdalton band took place in less than 5 s, showing that the 160 kdalton protein catalyzes significant ATP hydrolysis. Rhodopsin is the other major protein seen on the autoradiogram, but it incorporated significant radioactivity only after bleaching. Its time course of phosphorylation is slow [21] and, as shown in Fig. 1, the radioactivity incorporated does not turn over during a short incubation.

Only Mg^{2+} was required for phosphorylation, which was inhibited by excess EDTA. Varied calcium ion concentrations present during phosphorylation, from less than 10^{-8} M to 20 μM using EGTA as a Ca^{2+} buffer, did not affect radioactivity in the 160 kdalton band in outer segments prepared by procedure B or cause new bands to appear. The cations Na^+ and K^+ also had little influence on the 160 kdalton phosphorylated band.

Fig. 2 demonstrates that the 160 kdalton band originates in the outer segment membranes by comparing the three fractions obtained from retinal homogenates on sucrose density gradients. The content of the 160 kdalton band is the same in 'ROS' and 'dirty ROS', as measured by densitometry in Fig. 2. Samples of both fractions had equal rhodopsin content. The other bands in the 'dirty ROS' appear to be contaminants from the 'debris', which had no

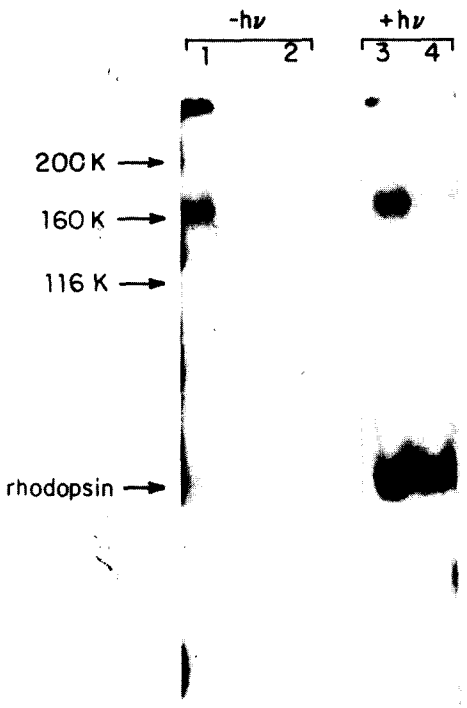

Fig. 1. A pH 7.4 gel of the phosphorylated proteins of purified rod outer segment membranes, using a 5 s incubation with 0.5 μM [γ-^{32}P]ATP at room temperature in phosphorylation buffer containing 20 mM KCl. In lanes 1 and 2, dark-adapted ROS membranes are phosphorylated; in lanes 3 and 4 partially bleached membranes, having received a few seconds' exposure to bright white light, are used. A 5-s chase with 1 mM nonradioactive ATP followed the phosphorylation in lanes 2 and 4.

spectroscopically detectable rhodopsin. The presence of a contaminating membrane species which gives rise to the 160 kdalton phosphorylated band is unlikely; such a membrane contaminant would have to appear in the 'ROS' and 'dirty ROS' fractions in proportion to their rhodopsin content, but nowhere else.

One of the contaminant bands in the 'dirty ROS' is the (Na^+ + K^+)-ATPase, having an apparent molecular weight of about 125 000 and arising most likely from the presence of rod inner segment plasma membranes [1,22]. It appears in lanes 3 and 7, where it is stabilized by ouabain, but not in lane 5, where, in the absence of ouabain, K^+ destabilizes the phosphorylated form of the enzyme [8,23]. The (Na^+ + K^+)-ATPase intermediate is relatively labile in the pH

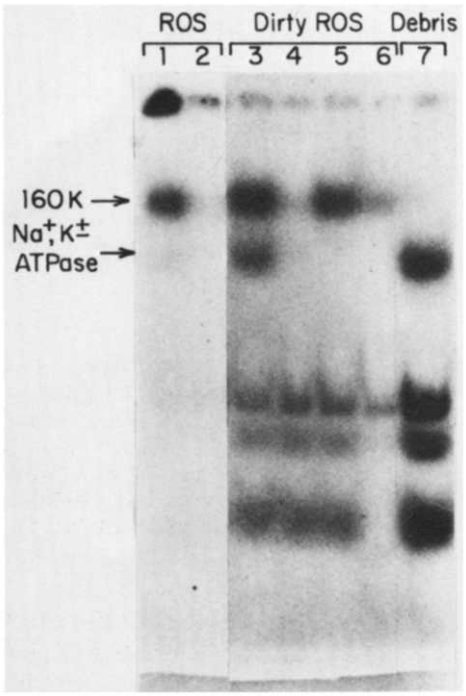

Fig. 2. Outer segment and contaminating retinal membrane fractions washed in buffer B and phosphorylated for 10 s at 29°C in 1 μM [γ-^{32}P]ATP in the dark. 'ROS' fraction: in lane 1, the phosphorylation buffer contains 30 μM ouabain; in lane 2, 30 μM ouabain and 20 μM vanadate. 'Dirty ROS' fraction: lane 3, phosphorylation buffer plus 30 μM ouabain; lane 4, 30 μM ouabain plus 20 μM vanadate; lane 5, no ouabain is present and 5 mM KCl is added; lane 6, same as lane 5, except for a 10-s chase with 30 μM ATP. 'Debris': in lane 7, phosphorylation buffer contains 30 μM ouabain. Radioactivity trapped at the top of the gel (as in lane 1) was usually insensitive to vanadate, indicating its independence of the 160 kdalton intermediate. Samples in lanes 1–6 had equal rhodopsin content.

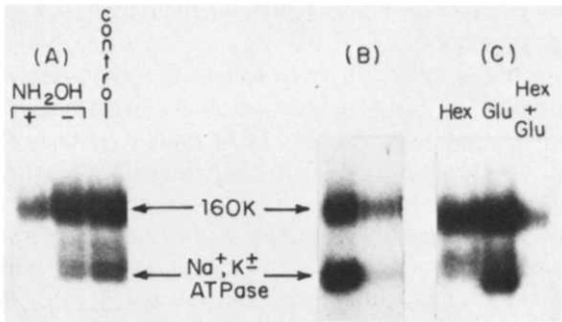

Fig. 3. Panel A. Hydroxylamine sensitivity of the 160 kdalton intermediate. In the two lanes labeled 'NH$_2$OH', phosphorylated membranes were incubated with or without 0.2 M hydroxylamine (see Materials and Methods). The 'control' membranes received no incubation. Turnover of the intermediate: in panel B, lane 1, 0.5 μM [γ-^{32}P]ATP was incubated for 5 s with ROS at 27°C; in lane 2, this was followed by a chase with 3 μM nonradioactive ATP for 5 s. In panel B, after 5 s in 0.5 μM [γ-^{32}P]ATP, the following were added for 15 s to each 65 μl ROS incubation mixture: 10 μl of a 1 : 20 dilution of hexokinase in a 3.2 M ammonium sulfate suspension (lane 1), 10 μl 20 mM glucose (lane 2) or 10 μl of each simultaneously (lane 3). In both panels B and C, sodium-Hepes, pH 7.4, replaced the Tris-Hepes buffer. The ammonium ion destabilizes the ($Na^+ + K^+$)-ATPase in panel C, lane 1.

7.4 gels shown here; when examined in pH 2.4 gels, it is more stable [15,24] and is seen in all preparations, including the most pure, when the assay includes Na^+, but not K^+. Whether the ($Na^+ + K^+$)-ATPase is in fact an outer segment component is not certain, however. The 160 kdalton intermediate was better preserved and sharper after electrophoresis at pH 7.4, so the latter gel system was used most often. The phosphate on both the 160 kdalton band and the ($Na^+ + K^+$)-ATPase was sensitive to hydroxylamine, suggesting that there is an acyl-phosphate bond on the protein (Fig. 3A).

It was difficult to demonstrate complete turnover of the 160 kdalton intermediate at low ATP concentrations. In Fig. 3B the ($Na^+ + K^+$)-ATPase turns over comparatively faster than the 160 kdalton intermediate during a 3 μM ATP chase following phosphorylation by 0.5 μM [γ-^{32}P]ATP. In other preparations, only a 50% loss of radioactivity at 160 kdalton could be observed after a similar 5-s chase. The reasons for partial turnover in these cases are not known. Also, exhaustion of ATP by hexokinase-catalyzed phosphorylation of glucose produced near complete dephosphorylation on a short time scale (Fig. 3C).

The 160 kdalton band in both 'ROS' and 'dirty ROS' disappeared when 20 μM vanadate was present during phosphorylation and the effect was very specific for that band (see Fig. 2, lanes 2 and 4). All other phosphorylated bands in the 'dirty ROS', except for the ($Na^+ + K^+$)-ATPase [26], were unaffected even though they turn over rapidly (cf. lanes 4 and 6). Vanadate suppression of $^{32}P_i$ incorporation in purified outer segment membranes, in the presence

of K^+, was used to quantitate the 160 kdalton protein; counting of gel slices was not done because of the lability of the phosphoprotein at pH 7.0 (data not shown). A minimum estimate of the ATPase of about one copy per 30 000 rhodopsin molecules is deduced from the vanadate-suppressed radioactivity incorporated into membranes of known rhodopsin (M_r = 38 000 [25]) content, and from the specific activity of [γ-^{32}P]ATP. This estimate assumes that each 160 kdalton ATPase is labeled once and that the only vanadate-sensitive phosphorylated material is the 160 kdalton band seen on gels. The EDTA-wash procedure B did not reduce vanadate-sensitive $^{32}P_i$ incorporation. Total radioactivity chased by ATP was about twice as great as the vanadate-sensitive portion in the 'ROS' fraction, indicating that other rapidly exchangeable material was present.

Vanadate probably inhibits the turnover of the 160 kdalton ATPase; it is unlikely that steady state phosphorylation is reduced because vanadate makes the enzyme turn over faster. Fig. 4 shows a slower incorporation of $^{32}P_i$ between 7 and 30 s in the presence of 20 μM vanadate than in a control lacking vanadate, implying that vanadate inhibits the approach to steady state. Inhibition of phosphorylation at lower vanadate concentrations was variable, although 50% inhibition could be observed at 1 μM vanadate. With 1 μM ATP as substrate, the ATPase activity of ROS sensitive to 10–30 μM vanadate was $(1.2 \pm 0.2) \cdot 10^{-2}$ μmol ATP/mg rhodopsin when assayed for periods of 2 min or less.

The 160 kdalton band can also be phosphorylated with 5 μM [γ-^{32}P]GTP, although at only about one-quarter the intensity of 1 μM [γ-^{32}P]ATP of the same specific activity (Fig. 5A). The incorporation of phosphate reached a plateau more slowly, in less than 30 s (cf. Fig. 5A and B), but otherwise the intermediate behaved like the one labeled by [γ-^{32}P]ATP: it had an identical migration on SDS gels, was inhibited by vanadate, and turned over in the presence of excess ATP or GTP. In addition, as Fig. 5B shows, when a small amount of nonradioactive ATP was also present during the phosphorylation, at one-tenth of the concentration of the [γ-^{32}P]GTP, no radioactivity was incorporated at 160 kdaltons. In the converse experiment, [γ-^{32}P]ATP phosphorylation was reduced no more than 50%, as determined by scans of an autoradiogram, when a 10–50-fold excess of GTP was present simultaneously, confirming that the enzyme has a much higher affinity for ATP than GTP.

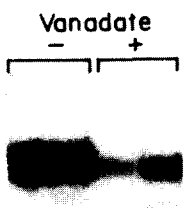

Fig. 4. Phosphorylation of 'ROS' at 29°C in the presence of 1 μM [γ-^{32}P]ATP. Phosphorylation times were 7 s in both lanes 1 and 3, 30 s in lanes 2 and 4. 20 μM vanadate was included in lanes 3 and 4.

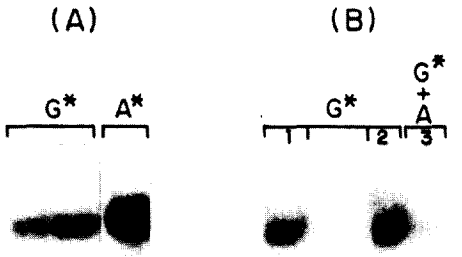

Fig. 5. Phosphorylation of ROS by GTP. Panel A, phosphorylation at 29°C with 5 μM [γ-^{32}P]GTP for 7 and 30 s (lanes 1 and 2) or 1 μM [γ-^{32}P]ATP for 7 s (lane 3). Both nucleotides had specific activities of 30 Ci/mmol. Panel B, phosphorylation with [γ-^{32}P]GTP 30 and 60 s (G*, lanes 1 and 2). Nonradioactive ATP (0.5 μM) was present in addition to 5 μM [γ-^{32}P]GTP for a 30-s incubation (G* + A, lane 3).

Discussion

The function of the ATPase corresponding to the 160 kdalton phosphorylated intermediate is speculative. Three properties of the 160 kdalton intermediate suggest that it is similar to known membrane-bound enzymes which catalyze ion transport, such as the $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase of the sarcoplasmic reticulum. These properties are: its close association with ROS membranes (from which it

could not be extracted), its rapid turnover, and its sensitivity to hydroxylamine when denatured. Sensitivity to hydroxylamine rules out an ester or phosphohistidine bond in the 160 kdalton intermediate, and suggests that an acyl-phosphate bond occurs from either aspartic or glutamic acid [27]. In the cases of the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-ATPase, hydroxylamine sensitivity is due to the presence of an aspartyl-phosphate bond [9,28]. The $(H^+ + K^+)$-ATPase of gastric mucosa also has an hydroxylamine-sensitive phosphorylated intermediate [10,11].

By its ionic requirements, the 160 kdalton intermediate is distinct from all three ion pumps: it is not suppressed by K^+ (see Fig. 2, lane 5) as two of them are. In membranes washed and assayed in EGTA, which removes free calcium, 160 kdalton phosphorylation can still take place. This rules out any identity with the Ca^{2+}-ATPase. No new phosphoprotein appears in outer segment membranes upon Ca^{2+} stimulation, making the presence of significant Ca^{2+}-ATPase in the outer segment unlikely. The Mg^{2+}-ATPase of outer segments which is enhanced by bleaching rhodopsin is unlikely to produce the 160 kdalton intermediate because it is not inhibited by vanadate below 5 μM [7,29]. Ion transport by the 160 kdalton ATPase is obviously not ruled out. There is only one reliable experiment which shows active ion transport by any component of the outer segment, however: in an intact retina, Ca^{2+} is extruded from the outer segment upon stimulation by light [30,31]. The enzymatic basis of this is unknown;

The 160 kdalton ATPase is almost certain to be part of the outer segment, as Fig. 2 indicates. Other known sources of contamination appear on a sucrose gradient at predominantly higher densities than the outer segment membranes: the mitochondria [1,4], and the inner segment plasma membrane, using the $(Na^+ + K^+)$-ATPase as a marker [1,22]; according to Fig. 2, these contaminants cannot account for the 160 kdalton ATPase. Flotation of osmotically shocked outer segment membranes on 5% Ficoll is thought, but not proven, to leave behind a pellet of plasma membrane fragments [32]. The 160 kdalton ATPase appears in the floated material, however, suggesting that it may be a disc membrane component (Kalish, D. and Thacher, S., unpublished data). In the toad disc, which contains about 10^6 rhodopsin molecules, about 40 copies of the ATPase would be present [25]. The presence of the ATPase in rod cilia was not ascertained [33].

The 160 kdalton ATPase may be involved in the outer segment structural change observed in vitro. Upon ATP addition, a 10–30% drop in turbidity of a suspension of ROS disc stacks occurs within minutes [5–7]. Ionic transport or a change of volume of the disc internal space has been ruled out as the explanation [7,29]. In bovine ROS, an ATPase which is transiently active with the time course of the turbidity change, and is inhibited by vanadate, had also been found [5]. The relation of the 160 kdalton ATPase to the structural change is suggested by two observations. One is the vanadate inhibition of both processes. Submicromolar vanadate can cause half-maximal inhibition of the structural change, and 1 μM vanadate also suppresses the 160 kdalton phosphorylation significantly. The nucleotide affinity of the phosphoprotein, which is much greater for ATP than GTP, also closely parallels that of the structural change, where GTP is ineffective as an energy source or even in interfering with the effect of ATP. The correlation would imply that a new kind of membrane-bound ATPase has been described, which is involved in structural change or movement of membranes, not ionic transport. The 160 kdalton ATPase is similar to the dynein ATPase of motile cilia in its vanadate inhibition and specificity for ATP, but a phosphorylated intermediate for this enzyme has not been described [34–36]. Latent-activated dynein of *Tetrahymena* [34] (a gift of Mary Porter, Woods Hole, MA), which has substantial vanadate-sensitive activity after extraction from flagellae, showed no phosphorylated intermediate in the 160 kdalton region (data not shown) [36]. Because purification of the ATPase from outer segments is likely to be very difficult, identication of a similar protein in other systems by its intermediate would be valuable for studying its function.

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