Differential Control of Protein Kinase Activities of the Retinal Photoreceptor. Cation Effects on Phosphorylation by Adenosine and Guanosine 5′-Triphosphates†

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ABSTRACT: ATP-kinase activity in photoreceptor membranes is maximal at equimolar amounts of ATP and Mg^{2+}. GTP-kinase activity is maximal with high concentrations of Mn^{2+}. Under these conditions, calcium ion markedly inhibits phosphorylation with ATP but not with GTP. GTP-kinase activity is maximal at pH 6.5 and decreases at higher pH; little change is seen in ATP-kinase activity over the pH range of 6.0–7.5. GTP-kinase activity is inhibited by ATP and other adenine compounds; ATP-kinase activity is less sensitive, although 1 mM adenosine decreases activity by about threefold. No marked differences in the protein moieties phosphorylated by ATP or GTP were seen in bovine or frog outer segment membranes. Fluxes in cation and metabolite concentrations as well as availability of ATP and GTP could thus exert a major influence on protein kinase activity in the photoreceptor unit, affording the possibility of differential phosphorylation under various physiological conditions.

Retinal photoreceptor outer segments exhibit a light-dependent ATP-protein kinase activity with low phosphorylation in dark-adapted photoreceptor units but markedly higher phosphorylation upon light adaptation (Kuhn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Weller et al., 1975). We have previously compared phosphorylation in isolated bovine rod outer segment membranes utilizing ATP and GTP and found substantial differences with the two phosphate donors (Chader et al., 1975, 1976).

Photoreceptor outer segments also exhibit extraordinary high guanylate cyclase activity (Pannbacker, 1973; Goridis et al., 1973; Bensinger et al., 1974), an activity that shows distinct cation requirements (e.g., Mn^{2+} and Mg^{2+}) and effects of several nucleoside triphosphates, e.g., ATP (Krishnan et al., 1978). Differential cation requirements are seen in other guanylate cyclase preparations as well (Frey et al., 1977). In light of these striking cation effects and the apparently unique dependence of photoreceptor units on guanine nucleotides, we have further investigated the differential phosphorylation of outer segment membranes by ATP and GTP, especially with reference to possible control of kinase activity by cations and other metabolites that might naturally modulate protein kinase activity in the photoreceptor unit.

Materials and Methods

Dark-adapted bovine and frog rod outer segments were prepared by differential sucrose gradient centrifugation as previously described (Krishna et al., 1976) and bleached under normal room lighting when appropriate prior to incubation. The final buffer used was 40 mM Tris-HCl, pH 7.0. "Soluble" protein kinase was prepared essentially as first reported by Kuhn et al. (1973) using 10 mM Tris buffer, pH 7.6, containing 5 mM Na_2EDTA. The extracted outer segment membranes were then washed in distilled water and treated with 4% potassium aluminum sulfate ("alum") to remove residual kinase activity as described by Frank & Buzney (1975).

Protein Kinase Assay. Assay tubes contained [γ-^{32}P]ATP (26.6 Ci/mmol) or [γ-^{32}P]GTP (21.9 Ci/mmol) obtained from New England Nuclear Corp. (Boston, MA) and an appropriate concentration of divalent cation. The chloride salts of the cations were used in all cases. The specific activity of the radioactive nucleotide was 50–200 cpm/pmol, and the final concentration in each case was 0.1 mM. To start the reaction, 50–100 μg of outer segment protein was added for a final volume of 100 μL in 40 mM Tris buffer, pH 7.0. Incubation was at 37 °C for 60 s. The assay was linear with time and protein concentration under these conditions. Boiled membrane samples or membranes treated with 10% trichloroacetic acid prior to incubation were used as controls. In pH experiments, the outer segment membranes were centrifuged to obtain a firm pellet, the buffer was discarded, and new buffer (40 mM Tris-HCl) of appropriate pH was added. The pellet was resuspended and recentrifuged. The buffer was again discarded, and the membranes were finally resuspended in fresh buffer of appropriate pH at about 1 mg of protein/mL. In reconstitution experiments, "soluble" protein kinase was added back to kinase-depleted outer segment membranes brought to a protein concentration of about 1 mg/mL. Subsequent incubation was as described above. In all cases, the reaction was stopped by the addition of 0.5 mL of 10% trichloroacetic acid solution. The precipitated protein was collected on Metricel filters (0.45 μm; Gelman Instrument Co., Ann Arbor, MI) using a Yeda filtration apparatus (Yeda Scientific Instruments, Rehovot, Israel). Each sample was washed three times with 5 mL of 10% trichloroacetic acid solution and once with 5 mL of chloroform–methanol (2:1 v/v). Filters were dried and assayed for radioactivity after the addition of 1 mL of Cellosolve (Eastman Kodak, Rochester, NY) and 10 mL of Aquasol (New England Nuclear Corp.).

In control experiments, [8-^{3}H]GTP (10.6 Ci/mmol) or [2,8-^{3}H]ATP (32.5 Ci/mmol) purchased from New England Nuclear Corp. was added in the incubation mixture in place of the [γ-^{32}P]GTP or [γ-^{32}P]ATP. Incubation times were 30, 60, and 120 s at 37 °C; 0.5 mL of a 10% trichloroacetic acid solution was then added and samples were treated as described above.

Slab Gel Electrophoresis. Bovine or frog outer segment membranes were prepared and incubated for 2 min with [γ-^{32}P]ATP or [γ-^{32}P]GTP as described above. Membranes were centrifuged and dissolved in buffer containing 1% $NaDodSO_4$[1]

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Table I: Cation Effects on Phosphorylation of Photoreceptor Membranes[a]

cation	ATP-kinase act. (pmol per mg per min) at cation concn		GTP-kinase act. (pmol per mg per min) at cation concn	
	10^{-4} M	10^{-5} M	10^{-4} M	10^{-5} M
none	150 ± 20		*	
Mg^{2+}	2270 ± 130	570 ± 70	150 ± 70	*
Mn^{2+}	1490 ± 90	960 ± 60	1460 ± 130	70 ± 30
Co^{3+}	710 ± 40	480 ± 210	490 ± 80	*
Zn^{2+}	350 ± 60	220 ± 30	480 ± 40	*
Fe^{3+}	320 ± 20	110 ± 30	230 ± 90	*
Fe^{2+}	130 ± 50	*	200 ± 10	*
Sn^{4+}	50 ± 20	*	50 ± 10	*
Ba^{2+}	*	*	*	*
Ca^{2+}	*	*	*	*

[a] An asterisk denotes a value that was <10 pmol per mg of protein per min. Incubation was with 0.1 mM nucleoside triphosphate. Values given are averages of six samples from two experiments ± standard deviations.

and 1% mercaptoethanol. Each sample was then divided into two parts; one part was placed in a boiling water bath for 2 min and the other part was kept at 20 °C (Saari, 1974). Samples were then layered on a 10% polyacrylamide slab gel with an electrode buffer of 50 mM Tris, 385 mM glycine, and 0.1% $NaDodSO_4$. The electrophoresis was run at 75 V for 3.5 h. The slab gel was then fixed with 5% trichloroacetic acid and dried. The autoradiograph of the gel was developed after an exposure time of 20 h.

Results

Control Experiments. Protein phosphorylation values determined in the membrane preparations were not due to incorporation of ^{32}P into lipids, since washing with chloroform–methanol (2:1 v/v) did not markedly decrease the bound ^{32}P. The values determined also did not appear to be due to nonspecific binding of unreacted [^{32}P]ATP or [^{32}P]GTP to the membrane preparations or filters, since only small amounts of [8-^{3}H]GTP or [2,8-^{3}H]ATP were retained on the filter along with the outer segment membranes after washing with trichloroacetic acid and solvent solution (20–50 pmol per mg per min). This low level of nonspecific binding was identical in dark- and light-adapted membranes.

Protein kinase activity can easily be extracted from ROS membranes into a "soluble" form (Kuhn et al., 1973; Weller et al., 1975; Farber et al., 1979). Soluble protein kinase activity averaged 327 ± 57 pmol of ^{32}P transferred per mg of ROS protein per min using [^{32}P]ATP and 214 ± 43 pmol of ^{32}P transferred per mg of protein per min using [^{32}P]GTP. Protein kinase activity was thus much lower when studied in the "soluble" form in reconstitution experiments than in the membrane-bound form used in the present study, as seen in Table I.

Cation Effects. The effects of 10^{-4} and 10^{-5} M concentrations of several cations on ATP- and GTP-kinase activities are shown in Table I. Using 0.1 mM [γ-^{32}P]ATP as phosphate donor, ATP-kinase activity was low (about 150 pmol of P_i transferred per mg of ROS protein per min) in the absence of added divalent cation (Table I). In comparison, no GTP-kinase activity was observed in the absence of exogenous cation. At 10^{-4} M, several cations stimulated ATP-kinase activity, with the highest activity seen with Co^{2+} and Mg^{2+}. Calcium and other cations such as Sn^{4+} and Ba^{2+} did not enhance the activity but rather inhibited basal activity. At 10^{-5} M, several cations (Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}) supported approximately the same level of ATP-kinase activity. GTP-kinase activity was stimulated best by Mn^{2+}, although Co^{2+} and Zn^{2+} showed substantial stimulation at 10^{-4} M. Little GTP-kinase activity was seen with any of the cations tested at 10^{-5} M (i.e., about 10 pmol per mg per min) except for Mn^{2+}.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; ROS, rod outer segment.

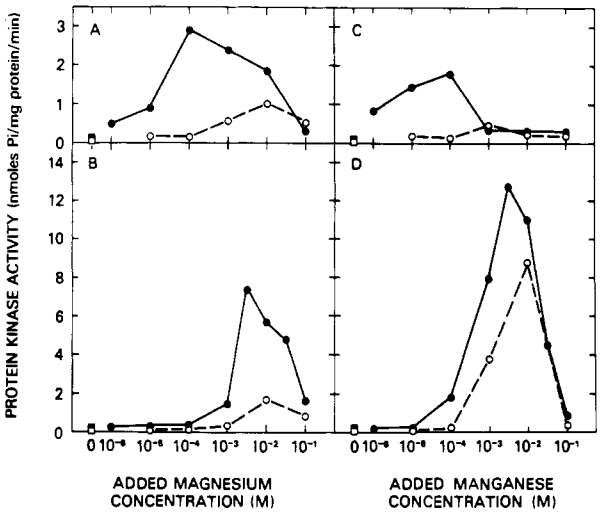

FIGURE 1: Effects of added magnesium (A, B) and manganese (C, D) on photoreceptor kinase activities. (A) ATP-kinase activity in the absence (●) or presence (○) of 10 mM calcium ion. (B) GTP-kinase activity in the absence (●) or presence (○) of 10 mM calcium ion. (C) ATP-kinase activity in the absence (●) or presence (○) of 10 mM calcium ion. (D) GTP-kinase activity in the absence (●) or presence (○) of 10 mM calcium ion. Activity with no added cation: absence (■) or presence (□) of 10 mM calcium ion. The radiolabeled nucleoside triphosphate concentration was 0.1 mM. Incubation was for 60 s at 37 °C. Values given are averages from two experiments with triplicate samples at each cation concentration examined.

The differential effects of magnesium (Mg^{2+}) and manganese (Mn^{2+}) cations on ATP- and GTP-kinase activities are shown in Figure 1. With Mg^{2+} (Figure 1A), increased ATP-kinase activity was observed even at 10^{-6} M cation, with maximal activity at 10^{-4} M cation. Activity was decreased at higher Mg^{2+} concentrations and was virtually completely inhibited at 100 mM cation. The addition of 10 mM calcium ion (Ca^{2+}) totally inhibited the effect of added Mg^{2+} up to 10^{-4} M. At 10^{-3} M Mg^{2+} and above, the inhibitory effect of Ca^{2+} was less pronounced.

In contrast to ATP-kinase activity, GTP kinase was not affected by Mg^{2+} until the 10^{-3} M level of cation was reached (Figure 1B). Maximal stimulation was observed at 5 mM, with an inhibitory effect observed at higher concentrations. As with ATP-kinase activity, GTP-kinase activity was strongly inhibited by addition of 10 mM Ca^{2+} when Mg^{2+} was the supporting cation.

Manganese (Mn^{2+}) ion was generally less effective in stimulating ATP-kinase activity than Mg^{2+}, although a positive effect was observed at lower (e.g., 10^{-6} M) Mn^{2+} concentration (Figure 1C). Inclusion of 10 mM Ca^{2+} virtually abolished the stimulatory effect of Mn^{2+}. The stimulatory effect of Mn^{2+} on GTP-kinase activity was great, with the highest activity observed at 5 mM (Figure 1D). At high Mn^{2+} concentrations (e.g., 100 mM), little GTP-kinase activity was observed. Ca^{2+} ion had a relatively small effect on GTP-kinase activity, with little inhibition observed if the Mn^{2+} concentration was 5 mM or above.

pH Effect. The effect of pH on ATP- and GTP-kinase activities in the presence of optimal concentrations of cation

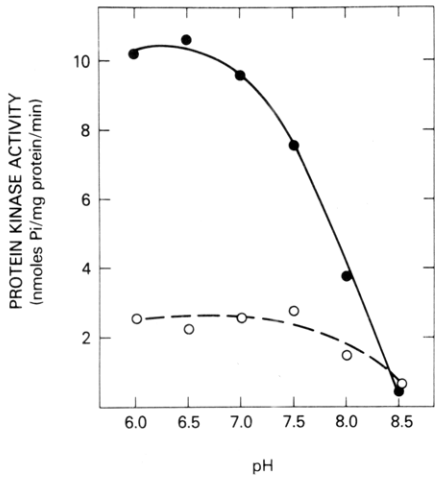

FIGURE 2: Effect of pH on ATP-kinase activity (○) and GTP-kinase activity (●). Cation concentration was 0.1 mM magnesium in assays with ATP and 5 mM manganese in assays with GTP. Nucleoside triphosphate concentration was 0.1 mM. Other conditions are as in Figure 1.

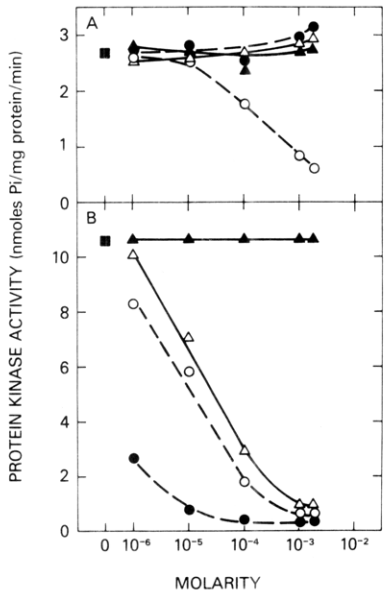

FIGURE 3: Inhibitor effects on ATP- and GTP-kinase activities. (A) ATP-kinase activity in the presence of no additive (■); GTP (●); adenosine (○); cyclic AMP (△); or cyclic GMP (▲). (B) GTP-kinase activity in the presence of no additive (■); ATP (●); adenosine (○); cyclic AMP (△); or cyclic GMP (▲). Radiolabeled nucleoside triphosphate concentration was 0.1 mM. Cation concentration was 0.1 mM magnesium in assays with ATP and 5 mM manganese in assays with GTP. Other conditions are as in Figure 1.

is shown in Figure 2. In these experiments, 0.1 mM Mg^{2+} was used with ATP and 5 mM Mn^{2+} was used with GTP. Little difference in ATP-kinase activity was observed from pH 6.0 to 7.5. Over pH 7.5, however, phosphorylation by ATP was decreased somewhat. GTP-kinase activity was significantly lower at pH 7.5 than between pH 6 and 7, and virtually abolished at pH 8.5.

Nucleoside Effects. In the presence of 0.1 mM Mg^{2+}, adenosine is an effective inhibitor of ATP-kinase activity, although cyclic AMP is ineffective (Figure 3). As would be expected in an enzymatic reaction, nonradiolabeled ATP competed with the [γ-^{32}P]ATP in a stochiometric manner (data not shown). Neither GTP nor cyclic GMP had any stimulatory or inhibitory effect when tested to 2 mM. In contrast, GTP-kinase activity (with 5 mM Mn^{2+}) is extremely

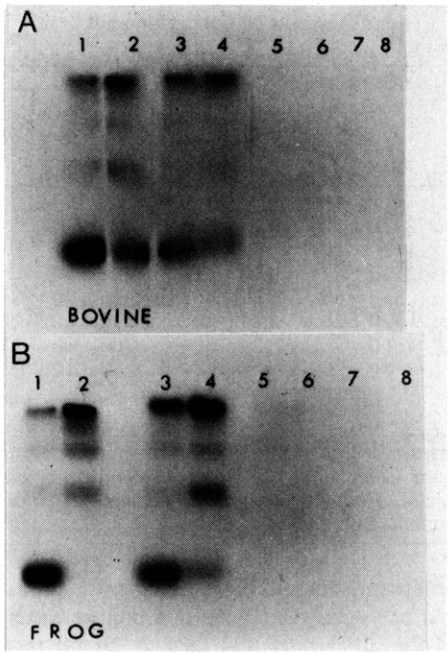

FIGURE 4: Autoradiographic pattern of ^{32}P incorporation into outer segment membrane proteins as separated by $NaDodSO_4$ gel electrophoresis. (A) Bovine outer segment membranes. (B) Frog outer segment membranes. Light-adapted (slots 1–4) or dark-adapted (slots 5–8) membrane samples were studied. Membranes were incubated with [γ-^{32}P]GTP (slots 1, 2 and 5, 6) or [γ-^{32}P]ATP (slots 3, 4 and 7, 8) as described in the text and dissolved in 1% $NaDodSO_4$ and 1% mercaptoethanol. Portions were then boiled for 2 min (slots 2, 4, 6, and 8) or remained at room temperature (slots 1, 3, 5, and 7) and were layered on 10% polyacrylamide slab gels containing 0.1% $NaDodSO_4$. Autoradiography was for 20 h.

sensitive to even low levels of ATP, with an approximately threefold inhibition seen with 10^{-6} M ATP. Nonradiolabeled GTP competed in a stochiometric manner with radiolabeled GTP as would be expected (data not shown). Adenosine and cyclic AMP were less effective than ATP but still significantly inhibited GTP-kinase activity even at 10^{-5} M.

Slab Gel Electrophoresis. Autoradiographic patterns of incorporation of ^{32}P from [^{32}P]ATP or [^{32}P]GTP into bovine and frog outer segment membranes are shown in Figure 4, A and B, respectively. Radiolabeled phosphorus bands were apparent only in the light-adapted samples (slots 1–4); little incorporation was observed in dark-adapted samples (slots 5–8). The major phosphorylated species in both cow and frog migrated in a manner similar to opsin whether incubation was with [^{32}P]GTP (slot 1) or [^{32}P]ATP (slot 3). If samples were boiled prior to application to the gel ([^{32}P]ATP, slot 2; [^{32}P]GTP, slot 4), significant aggregation was observed in both cow and frog membrane preparations, although higher molecular weight bands were evident even in the samples kept at room temperature prior to application (slots 1 and 3). In the boiled samples, a similar multimeric autoradiographic pattern was observed with [^{32}P]GTP (slot 2) compared to [^{32}P]ATP (slot 4), although somewhat less radiolabel was apparent in the most rapidly moving component (presumably opsin) with [^{32}P]GTP than with [^{32}P]ATP (slots 2 and 4, respectively).

Discussion

ATP-kinase activity can be easily extracted from outer segments under proper conditions using low ionic strength buffers or mild detergents (Kuhn et al., 1973; Weller et al., 1975; Frank & Buzney, 1975; Farber et al., 1978; Shichi & Somers, 1978). This solubility is dependent on the state of

light adaptation of the outer segments (Kuhn, 1978). In the present study, we have utilized only light-adapted, highly purified outer segment membranes (Krishna et al., 1976) and thus have examined control of kinase activity when the enzyme is membrane bound or "membrane associated". Under these conditions, the enzyme might be expected to be in a somewhat physiological milieu in (or on) the membrane in relation to other proteins and lipids and thus be subject to at least some of the control mechanisms or constraints imposed upon the enzyme in vivo.

Phosphorylation with GTP has been less well studied, although our findings are in agreement with those of Farber et al. (1979) indicating high GTP-kinase activity when the enzyme is membrane bound but lower when it is solubilized. Our values obtained with the "soluble" enzyme are comparable to those reported by Frank & Buzney (1977) under relatively similar conditions. Shichi & Somers (1978) have also reported significant GTP-kinase activity with solubilized, purified rhodopsin kinase with K_m values for ATP and GTP similar to those of the membrane-bound activity we previously reported (Chader et al., 1976). Varying activities (e.g., V_{max}) reported in several laboratories can probably be ascribed to differences in enzyme activities in soluble vs. membrane-bound forms and to the differing experimental conditions under which the assays were conducted, as discussed below. In spite of these differences, however, it seems clear that significant GTP-kinase activity could be expressed in the photoreceptor unit under physiological conditions since deAzeredo et al. (1978) have found high endogenous GTP concentrations (170 to 400 μM) in frog outer segments, concentrations well within the range of reported K_m values.

In the present study, we have found at least four distinct differences in ATP- and GTP-kinase activities that may be significant in the control of phosphorylation in vivo. These involve (1) cation concentration, (2) pH effect, (3) calcium ion, and (4) nucleoside concentration.

(*1*) *Cation Concentration.* In studying photoreceptor protein kinase activity, investigators have used Mg^{2+} concentrations from 0 to 5 mM with various concentrations of ATP and have included up to 3 mM Ca^{2+} in the assay buffer (Kuhn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Weller et al., 1975; Chader et al., 1976; Miller & Paulsen, 1975; Shichi & Somers, 1978; Farber et al., 1979). This variation at least in part could explain the wide differences in reported ATP-kinase activities. Under our conditions, not only is Mg^{2+} the preferred divalent cation for ATP-kinase activity, but also an equimolar concentration of Mg^{2+} and ATP yields the highest activity. The high Mg^{2+} to ATP ratios as used in some studies (e.g., Shichi & Somers, 1978) are inhibitory under our conditions. A similar effect is observed with adenylate cyclase activity in many tissues where a Mg^{2+}–ATP complex appears to be the actual substrate for the enzyme with inhibition of enzyme activity observed at higher cation/nucleoside triphosphate ratios. Outer segment guanylate cyclase activity is also maximal at equimolar concentrations of Mn^{2+} and GTP (Krishnan et al., 1978). It is interesting that Mn^{2+} supports ATP-kinase activity in a lower concentration range than Mg^{2+}. This offers the possibility of having significant kinase activity over a wide range of concentration of the two cations and possibly under separate control. The significance of the effect of Co^{2+} on ATP- but not on GTP-kinase activity is not known, but a similar effect has been reported on aortic cyclic GMP-dependent protein kinase by Kuo and co-workers (Shoji et al., 1977). In contrast to ATP-kinase activity, GTP-kinase activity is more active in the presence of Mn^{2+} than Mg^{2+} and demonstrates a completely different pattern of cation dependency (10^{-6} to 10^{-1} M). Both Mg^{2+} and Mn^{2+} GTP-kinase activities are maximally expressed with 5 mM cation, a cation/nucleoside triphosphate ratio of 50:1 in the present study.

(*2*) *pH Effect.* The broad pH optimum curve for ATP-kinase activity differs considerably from the pattern seen with GTP. Above pH 7.5 in particular, the activity patterns are sharply different, with ATP-kinase activity only slowly decreasing while GTP-kinase activity drops quickly. GTP phosphorylation is thus much more sensitive to pH changes, again demonstrating the separate nature of the two activities. Other factors in concert with pH (e.g., ionic strength) will undoubtedly be found to affect the kinase activities as has recently been reported for ribosomal GTPase activity (Beaudry et al., 1979).

(*3*) *Calcium Ion.* Calcium has been implicated in the visual process and is thought to be in high concentration in photoreceptor outer segments (Hess, 1974; Hendriks et al., 1974; Hagins & Yoshikami, 1975) and pigment epithelium (Hess, 1974). Farber & Lolley (1976) have calculated that rodent photoreceptor cells contain 20–30 mol of Ca^{2+} and 30–40 mol of Mg^{2+} per mol of rhodopsin. We have used Ca^{2+}, however, in the present study as a ready tool for defining differences between the ATP- and GTP-kinase activities. ATP-kinase activity is particularly susceptible to inhibition by Ca^{2+} under the present conditions. Similarly, GTP kinase is drastically inhibited by Ca^{2+} when Mg^{2+} is used as the supporting cation. When Mn^{2+} is used, however, GTP-kinase activity is only minimally affected by Ca^{2+}, affording another interesting possibility for differential control of phosphorylation by ATP- and GTP-kinase activities in the photoreceptor unit, with GTP kinase considerably more active at high Ca^{2+} concentration than ATP kinase.

(*4*) *Nucleoside Concentration.* Membrane-associated ATP-kinase activity is unaffected by GTP, cyclic AMP, or cyclic GMP when the concentration of supporting cation is optimized. The inhibition exerted by adenosine is therefore interesting and appears to be not merely a general effect exerted by all adenine nucleotides. This is in line with the special regulatory role played by adenosine in several biological systems (Baer & Paton, 1978). In contrast, the more general inhibitory effect of adenine nucleotides on GTP-kinase activity is striking, with ATP a particularly potent inhibitor. High concentrations of ATP may thus effectively inhibit phosphorylation by GTP in photoreceptor membranes, but, as ATP levels drop (energy utilization, etc.), increased GTP-kinase activity could be an effective alternate signal.

We have utilized the finding that outer segment protein (presumably mainly opsin) aggregates with heating (Saari, 1974) to some advantage in attempting to determine if similar protein species are phosphorylated by ATP and GTP (Figure 4). Patterns of phosphorylation by ATP and GTP are quite similar in both unheated and heated samples, indicating that although kinase activity may be under differential control, the same (or very similar) protein species appear to be phosphorylated. Shichi & Somers (1978) have suggested that the high GTP-kinase activity reported from our laboratory may be due to "an unidentified kinase" used "for the phosphorylation of protein other than opsin". Whether the present kinase activity is "identified" or not, it is light activated, has a similar K_m (for both ATP and GTP) to that reported for "purified" rhodopsin kinase (Shichi & Somers, 1978), and phosphorylates similar protein species with ATP and GTP.

As previously shown (Chader et al., 1976), only ATP and GTP are ready phosphate donors in the light-activated protein

kinase reaction of the photoreceptor unit. We also found negligible P_i transfer from GTP to ATP (nucleoside diphosphokinase activity), and thus the phosphorylation of membrane protein by GTP appears to be specific and direct and not through an ATP intermediate. Moreover, the initial rates of phosphorylation are greater with GTP than with ATP (Chader et al., 1975). The role of phosphorylation by ATP or GTP in the process of photoreception is yet unknown, although it seems likely that a soluble cyclic GMP dependent protein kinase activity (Farber et al., 1978) may very well play a role in the process. It is clear from the present and other (Kuhn, 1978; Farber et al., 1979) studies, however, that complex factors of cation and nucleoside triphosphate availability as well as light or dark adaptation can profoundly affect protein kinase activity both qualitatively and quantitatively in the photoreceptor unit.

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