THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 43, Issue of October 25, pp. 27083–27089, 1996
Printed in U.S.A.

The Photoreceptor Guanylate Cyclase Is an Autophosphorylating Protein Kinase*

(Received for publication, April 1, 1996, and in revised form, August 15, 1996)

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The photoreceptor membrane guanylate cyclase is a member of a family of proteins with a set of four structural motifs: an extracellular ligand binding domain, a transmembrane domain, an intracellular protein kinase-like domain, and an intracellular catalytic domain. Purified preparations of the photoreceptor guanylate cyclase have allowed us to explore the function of the protein kinase-like domain. ATP enhances the guanylate cyclase activity 2-fold in membranes stripped of peripheral proteins. The stimulation can be mimicked by ATPγS (adenosine 5′-*O*-(3-thiotriphosphate)), AMPPNP (5′-adenylyl β,γ-imidodiphosphate), and ADP, but not AMP. While this effect is lost by solubilizing guanylate cyclase, ATP binds the purified, solubilized enzyme in a site distinct from the catalytic GTP site as shown by specific labeling with 8-N_3[α-^{32}P]ATP. The enzyme has a protein kinase activity that is Mg^{2+}-dependent and autophosphorylates serine residues. Myelin basic protein serves as a substrate for the kinase and enables further characterization of the kinase properties. The K_m for ATP is 81 μM. The kinase activity is unaffected by calcium, cyclic nucleotides, and phorbol 12-myristate 13-acetate/L-α-phosphatidylserine/Ca^{2+} and is inhibited by high concentrations of staurosporine. These properties are distinct from other Ser/Thr kinases identified in rod outer segment preparations including protein kinase A, protein kinase C, and rhodopsin kinase. The observations offer the first biochemical evidence that a member of the receptor guanylate cyclase family has intrinsic protein kinase activity.

The membrane guanylate cyclase of vertebrate photoreceptor rod outer segments plays a key role in controlling the levels of cGMP, the intracellular second messenger that mediates visual signaling (1–5). In the dark-adapted photoreceptor, the resting cGMP levels are set by the balance of synthesis and degradation due to the basal activities of the guanylate cyclase and the cGMP phosphodiesterase. Throughout the processes of phototriggering, photorecovery, and light adaptation, the activity of the guanylate cyclase appears to be modulated to control cGMP levels. Several factors are known to regulate the guanylate cyclase activity. Intracellular calcium concentration in the range of 0.1 to 1 μM inhibits cyclase activity in the presence of small calcium-binding proteins, GCAPs[1] (6–8). In contrast, a protein of 40 kDa, composed of a multimer of 6–7-kDa peptides, activates the cyclase in a calcium-dependent manner with half-maximal activation occurring between 2 and 5 μM (9). In addition, guanylate cyclase activity has been reported to be modulated by ATP (10, 11) and by protein kinase C (12). The calcium regulation mediated by GCAPs is thought to be responsible for cyclase activation during photorecovery and light adaptation; however, the physiological significance of regulation by the other factors is unclear. In all cases, the mechanisms by which these factors control the activity of the photoreceptor guanylate cyclase are not understood.

The primary structure of the photoreceptor membrane guanylate cyclase has been deduced from cDNA sequences isolated from human, bovine, and rat retinas (13–15).[2] The information indicates that it is a member of the family of membrane guanylate cyclases that function as receptors for peptide ligands. Four functional domains are characteristic of these proteins: an amino-terminal "extracellular" domain, a transmembrane region, an intracellular protein kinase-like domain, and a carboxyl-terminal guanylate cyclase catalytic domain (17–19). In other members of the family, binding of peptide ligands to the extracellular amino-terminal domains induces stimulation of cytoplasmic guanylate cyclase activity. Molecules known to function in this manner include receptors for ANP (GC-A) and CNP (GC-B), guanylin and *Escherichia coli* enterotoxin (GC-C), and the sea urchin egg peptides, speract and resact. Although no ligand has been identified for the photoreceptor guanylate cyclase, its structural similarity to the receptor guanylate cyclases suggests that it may be regulated in a similar manner.

In this work, we have focused on the role of the kinase-like domain of the photoreceptor guanylate cyclase. Studies of other members of the receptor guanylate cyclase family clearly indicate that the kinase homology domain is required to transduce the ligand-binding signals and activate the guanylate cyclases (20–22). ATP is required for ligand-mediated activation, but nonhydrolyzable ATP analogues work as well (17). No kinase activity has been measured for any receptor guanylate cyclase, and autophosphorylation is not considered to be part of the signal transduction mechanism (17, 22). In the basal state, the

* This work was completed in partial fulfillment of the requirements for the degree of Doctor of Philosophy (to J. G. A.) and was supported by a National Institute of Mental Health, National Research Service Award MH1-164 (to J. G. A.), as well as grants from the National Eye Institute (EY04810) and from the RP/Gund Foundation (to M. L. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GCAP, guanylate cyclase activating protein; ROS, rod outer segment; PMA, phorbol 12-myristate 13-acetate; PS, L-α- phosphatidylserine; ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; GC-A, ANP receptor; GC-B, CNP receptor; GC-C guanylin and *E. coli* enterotoxin receptor; MBP, myelin basic protein; cAPK, catalytic subunit of cAMP-dependent protein kinase; DDM, dodecyl-β-D-maltoside; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMPPNP, 5′-adenylyl β,γ-imidodiphosphate; RSP, ROS-soluble protein; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

[2] J. P. Johnston, F. Farhangfar, J. G. Aparicio, S. Nam, and M. L. Applebury, manuscript in preparation.

kinase homology domain is suggested to be an inhibitory domain. Release of this inhibition upon ligand binding is proposed to activate the cyclase catalytic domain (20–22). The molecular mechanisms by which the kinase-like domain transduces the extracellular signal and stimulates the cyclase are unclear.

For the photoreceptor guanylate cyclase, the function of the kinase-like domain has not been assessed. We explored the role of ATP in modulating cyclase activity as an initial probe for function of the kinase-like domain. In addition, we examined the potential for kinase activity. Our studies show that a highly active, purified preparation of the photoreceptor guanylate cyclase has an ATP-binding site and an autophosphorylating protein kinase activity, and it will phosphorylate selected exogenous substrates. The observations suggest that the kinase-like domain has functional activity and provide the first biochemical evidence that a member of the membrane guanylate cyclase family has inherent kinase activity.

EXPERIMENTAL PROCEDURES

Materials—8-N_3[α-^{32}P]ATP was purchased from ICN. [γ-^{32}P]ATP was purchased from Amersham Corp. Dithiothreitol, okadeic acid, and DDM were purchased from Calbiochem. Na_2GTP, aprotinin, pepstatin, and leupeptin were purchased from Boehringer Mannheim. Staurosporine and PMA were purchased from LC Laboratories. TLC plates (type 100 cellulose), protein kinase A, and all other reagents were obtained from Sigma.

ROS Membranes and Guanylate Cyclase—Isolation of bovine ROS membranes, purification of the photoreceptor guanylate cyclase, and assay of guanylate cyclase activity were described previously (23). Washed membranes refer to ROS stripped of peripheral membrane proteins with a hypotonic buffer containing GTP (23). Greater than 95% of the protein in purified preparations is guanylate cyclase; no other protein bands are visible when SDS gels containing 1 μg of purified protein bands are Coomassie-stained (23).

Reconstitution of Guanylate Cyclase into Phosphatidylcholine Vesicles—Guanylate cyclase was reconstituted into membrane vesicles using a dilution method similar to that of Hebdon *et al*. (24). Phosphatidylcholine, NaCl, $MgCl_2$, and Hepes, pH 7.6, were added to a concentrated fraction of purified guanylate cyclase in 2.5 mM DDM, 0.025% phosphatidylcholine, 20% glycerol, 120 mM NaCl, 6 mM EGTA, 10 mM Hepes, pH 7.6, to make a final concentration of 5 mg/ml phosphatidylcholine, 120 mM NaCl, 5 mM $MgCl_2$, and 10 mM Hepes. After 30 min on ice, the sample was diluted 50-fold with dilution buffer (120 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.6), and centrifuged at 300,000 $\times$ *g* for 1 h at 4 °C. The pellet was gently resuspended in an equivalent 50-fold volume of dilution buffer and recentrifuged as above. The final pellet was resuspended in a small volume (<100 μl or a volume appropriate for use in assays) of dilution buffer minus $MgCl_2$. Roughly one-third of the original protein was recovered in the pellet based on the intensity of Coomassie-stained protein in gels.

8-N_3[α-^{32}P]ATP Labeling—The protocol for photolabeling with 8-N_3ATP was adopted from that described in an ICN publication (25) and by Czarnecki *et al*. (26). Dithiothreitol, which destroys the photoactivity of 8-N_3ATP, was removed from the purified guanylate cyclase using a G-50 Sephadex column equilibrated in 20 mM sodium phosphate, pH 7.0, 50 mM NaCl, 0.1% DDM, 0.025% phosphatidylcholine, and 1 μg/ml each aprotinin, leupeptin, and pepstatin. Guanylate cyclase (1 μg) was added to a reaction mix containing 20 μM 8-N_3[α-^{32}P]ATP (2 Ci/mmol), 20 mM sodium phosphate, pH 7.0, 4 mM Na_2GTP, 2.0 mM Na_2ADP, 2.0 mM NaAMP, 80 mM NaCl, and 12 mM $MgCl_2$. The final reaction volume was 20 μl. After a 15 s preincubation, the 8-N_3[α-^{32}P]ATP was activated by irradiating for 1 min, at 12 °C, using a short wave UV light at an intensity of 1000 microwatts/cm^2. The reaction was quenched immediately with 10 μl of SDS-PAGE sample buffer containing dithiothreitol, protein was separated from unincorporated radioactivity by SDS-PAGE, and the incorporated radioactivity was detected by autoradiography.

Kinase Reactions—Kinase assays were performed by adding enzyme to a reaction mix containing 30 μM [γ-^{32}P]ATP (~5000 cpm/pmol), 20 mM MOPS, pH 7.1, 100 mM NaCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml each aprotinin, pepstatin, and leupeptin. Reactions of 25 μl (see Fig. 3) or 10 μl (see Figs. 4–6) were incubated at room temperature for the time indicated in figure legends, and quenched with 12.5 μl or 5 μl of 3 $\times$ quench solution (3 $\times$ SDS-PAGE sample buffer containing 30 mM EDTA and 15 mM ATP). Protein was separated from unincorporated radioactivity by SDS-PAGE, and incorporation of ^{32}P into proteins was monitored by autoradiography or excision of the protein bands following Coomassie staining and counting the rate of ^{32}P disintegration.

TABLE I
Effect of ATP on guanylate cyclase activity

Reaction conditions for the guanylate cyclase assay are described in the legend of Fig. 1, except for "Mn^{2+}" where 2 mM $MnCl_2$ + 1 mM $MgCl_2$ is substituted for 6 mM $MgCl_2$.

Source of enzyme	Cation	Stimulation by ATP[a] (0.5 or 1.0 mM)	
		-fold	*n*
1. Bleached, washed ROS membranes[b]	Mg^{2+}	2.4 ± 0.4	14
	Mn^{2+}	1.1 ± 0.3	2
2. Dark, washed ROS membranes[c]	Mg^{2+}	2.0 ± 0.5	4
3. Bleached, washed ROS membranes[d]	Mg^{2+}		
10 nM [Ca^{2+}]		1.9 ± 0.3	2
100 nM [Ca^{2+}]		2.0 ± 0.3	2
1 μM [Ca^{2+}]		1.8 ± 0.2	2
4. Dark, washed ROS membranes[d]	Mg^{2+}		
10 nM [Ca^{2+}]		2.1 ± 0.1	2
100 nM [Ca^{2+}]		2.1 ± 0.6	2
1 μM [Ca^{2+}]		1.8 ± 0.1	2
5. Washed ROS membranes solubilized in dodecyl maltoside	Mg^{2+}	1.0 ± 0.1	4
6. Purified guanylate cyclase in dodecyl maltoside	Mg^{2+}	0.79 ± 0.09	10
7. Purified guanylate cyclase reconstituted into phosphatidylcholine	Mg^{2+}	0.82 ± 0.10	6

[a] The data is given as the mean ± the standard deviation except for cases where $n = 2$ in which the mean ± the range is shown.
[b] Data include use of both stripped and washed ROS membranes as defined in Aparicio and Applebury (23).
[c] Dark, washed ROS membranes were prepared from crude ROS by washing one time with 4 mM MOPS as described by Koch and Stryer (6).
[d] Calcium concentration was buffered with EGTA following the method of Tsien and Pozzan (16).

Phosphoamino Acid Analysis—Purified guanylate cyclase (1 μg) was phosphorylated by incubating with [γ-^{32}P]ATP in a kinase reaction. After separation from unincorporated radioactivity on a 7.5% Laemmli gel, the protein was transferred to Immobilon-P for 1 h at 0.4 mA/cm^2 in a semidry blot apparatus (Hoeffer) as described (27) using 20% methanol, 0.005% SDS, 25 mM Tris, and 125 mM glycine as the transfer buffer. Using autoradiography for detection of the ^{32}P-labeled guanylate cyclase band, the membrane area containing the labeled guanylate cyclase protein was excised and hydrolyzed *in situ* on the Immobilon-P membrane for 1 h at 110 °C with 6 N HCl using the method of Kamps and Sefton (28). The hydrolysate was mixed with phosphoserine, phosphothreonine, and phosphotyrosine standards (5 μg each) and electrophoresed (20 min, 1000 V) in one dimension in pH 3.5 buffer on a 10-cm cellulose TLC plate as described previously (29).

Other Methods—Methods described in Ausubel *et al*. (27) were used for SDS-PAGE (Laemmli system) and Coomassie staining of polyacrylamide gels. Protein concentrations were measured by the Bradford (Bio-Rad) assay or the method of Kaplan and Pederson (30) using bovine serum albumin as a standard. The latter method was used to determine protein in purified guanylate cyclase samples.

RESULTS

ATP Stimulation of Guanylate Cyclase Activity—To investigate the function of the photoreceptor guanylate cyclase kinase homology domain we initially tested whether ATP has any effect on guanylate cyclase activity in photoreceptor membranes. Addition of ATP to washed bovine photoreceptor outer segment membranes stimulates the guanylate cyclase activity in these membranes in the presence of Mg^{2+}, but not in the presence of Mn^{2+} (Table I). The twofold activity increase is observed in both bleached and dark adapted, washed ROS membranes and is independent of calcium concentration. In the presence of ATP, the apparent K_m for the cyclase's substrate, MgGTP, decreases from 2.2 ± 0.3 mM to 1.0 ± 0.1 mM, and the V_{max} increases from 11.7 ± 0.8 nmol of cGMP min^{-1} mg^{-1} to 17.1 ± 0.8 nmol of cGMP min^{-1} mg^{-1} (data not shown). This stimulation of activity by ATP is lost when the

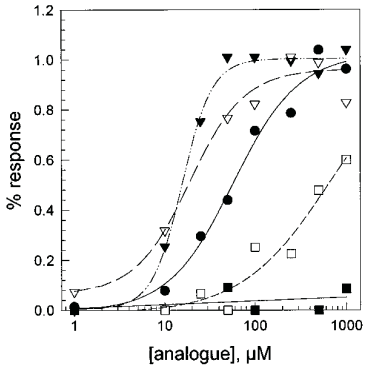

FIG. 1. **Effect of ATP analogues on the native ROS membrane guanylate cyclase activity.** Washed ROS membranes (16 μg) were assayed for guanylate cyclase activity in a 100 μl reaction containing 6 mM $MgCl_2$, 2 mM Na_2GTP, 30 mM MOPS, pH 7.1, 1 mM isobutylmethylxanthine, 1 mM 8-bromo-cGMP, 100 mM NaCl, and the indicated concentration of ATPγS (▼), AMPPNP (▽), ATP (●), ADP (□), or AMP (■). Isobutylmethylxanthine and 8-bromo-cGMP are included to inhibit any cGMP phosphodiesterase activity. Plotted points are the mean values of assays performed in triplicate. The standard error did not exceed 15% of the mean. To calculate the percent response for ADP and AMP, the maximum response to ATP was used as the maximum. To determine the EC_{50} values for ATPγS, AMPPNP, ATP, and ADP and to generate the drawn curves, the data were fit to the equation $\%R = [A]^n/(EC_{50}^n + [A]^n)$ using Sigma Plot (Jandel Corporation). AMP data were fit to a linear regression.

guanylate cyclase is solubilized in DDM. Moreover, stimulation is not restored when purified guanylate cyclase is reconstituted into phosphatidylcholine (Table I).

To determine whether the stimulation of activity requires ATP hydrolysis, we examined the effect of ATP analogues and adenosine containing compounds on the guanylate cyclase activity in washed ROS membranes. As shown in Fig. 1, increasing concentrations of ATPγS, AMPPNP, ATP, and ADP, but not AMP, stimulate guanylate cyclase activity. Both ATPγS and AMPPNP were more effective than ATP in stimulating guanylate cyclase; the nonhydrolyzable analogues exhibit EC_{50} values of ~16 and ~19 μM, respectively, as compared to an EC_{50} of ~58 μM for ATP. ADP is much less effective, but will stimulate the guanylate cyclase at concentrations of 100 μM or greater. The mechanism of action of ATP appears to be independent of hydrolysis since the nonhydrolyzable analogue, AMPPNP (31), and ADP both stimulate the guanylate cyclase.

The Guanylate Cyclase Binds ATP—We considered that the mechanism of ATP activation of guanylate cyclase could involve direct binding to an allosteric site or binding to other factors in ROS membranes. To determine whether ATP binds the guanylate cyclase directly we examined whether purified guanylate cyclase can be photoaffinity labeled by 8-N_3[α-^{32}P]ATP. When photolyzed by UV light, this compound becomes a highly reactive nitrene that forms covalent bonds with amino acids in a protein-binding site. In the presence of 4 mM GTP, 2 mM ADP, 2 mM AMP, and 20 mM P_i, included to block nonspecific binding of 8-N_3[α-^{32}P]ATP, and 20 μM 8-N_3[α-^{32}P]ATP, the ^{32}P label is incorporated into the guanylate cyclase (Fig. 2, *lane 3*). No labeling is observed in the absence of UV irradiation or when protein is added after UV light stimulation (Fig. 2, *lanes 1* and *2*). The 8-N_3[α-^{32}P]ATP labeling is competed partially by 10 μM ATPγS and effectively by an excess of ATPγS (100 μM) (Fig. 2, *lanes 6* and *7*). The 8-N_3[α-^{32}P]ATP is unlikely to label the GTP-binding site of the guanylate cyclase since the labeling reaction contained 4 mM GTP to saturate the GTP site. Moreover, removal of GTP does not increase labeling, which would be predicted for removal of a competitor. Additionally, the 8-N_3[α-^{32}P]ATP concentration used in the labeling reaction was 20 μM, a value close to the

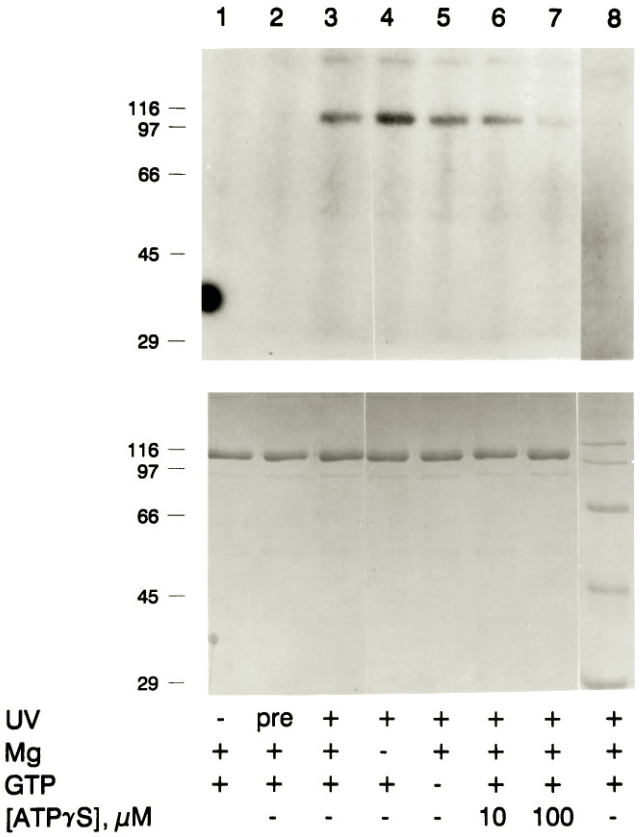

FIG. 2. **8-N_3[α-^{32}P]ATP labeling of purified guanylate cyclase.** Purified guanylate cyclase (*lanes 1–7*) or a protein standard mixture (Sigma) containing β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase (*lane 8*), were reacted with 8-N_3[α-^{32}P]ATP as described under "Experimental Procedures." An autoradiogram (*top*) and the corresponding Coomassie R-250-stained 9% polyacrylamide gel (*bottom*) are shown. As indicated, $MgCl_2$ (*lane 4*) or GTP (*lane 5*) was omitted from some reactions, and 10 (*lane 6*) or 100 μM (*lane 7*) ATPγS was included in some reactions. *Pre* (preirradiated, *lane 2*) means the 8-N_3[α-^{32}P]ATP compound was irradiated for 1 min prior to the addition of protein.

EC_{50} for ATP (Fig. 1) and 50-fold lower than the guanylate cyclase's K_m for MgGTP of 1 mM (23). Mg^{2+} was not required for labeling; in fact, greater labeling was observed consistently when Mg^{2+} was not added to the reaction mixture (Fig. 2, *lane 4*). Labeling of the cyclase is not due to nonspecific reaction of the 8-N_3[α-^{32}P]ATP since five additional proteins failed to be labeled with photolyzed 8-N_3[α-^{32}P]ATP under identical conditions used for labeling of the guanylate cyclase. The labeling of the guanylate cyclase protein by the photolyzed 8-N_3[α-^{32}P]ATP is consistent with the guanylate cyclase having an ATP-binding site independent of its GTP substrate-binding site. The photoaffinity labeled site could be identical to the ATP activating site, since the concentrations of 8-N_3[α-^{32}P]ATP and the competitor, ATPγS, that effectively label the cyclase are similar to those that stimulate activity (Fig. 1). Although other factors in ROS membranes could also bind ATP and affect the cyclase activity, we turned our attention to what role the direct ATP binding might play.

Purified Guanylate Cyclase Has a Phosphoryl Transfer Activity and Phosphorylates Serine Residues of the Guanylate Cyclase—The above evidence for ATP binding and the structural homology to protein kinases prompted us to examine whether the guanylate cyclase undergoes autophosphorylation. Initial experiments were performed using purified guanylate cyclase solubilized in DDM, as well as protein reconstituted into phosphatidylcholine vesicles. In both cases the purified

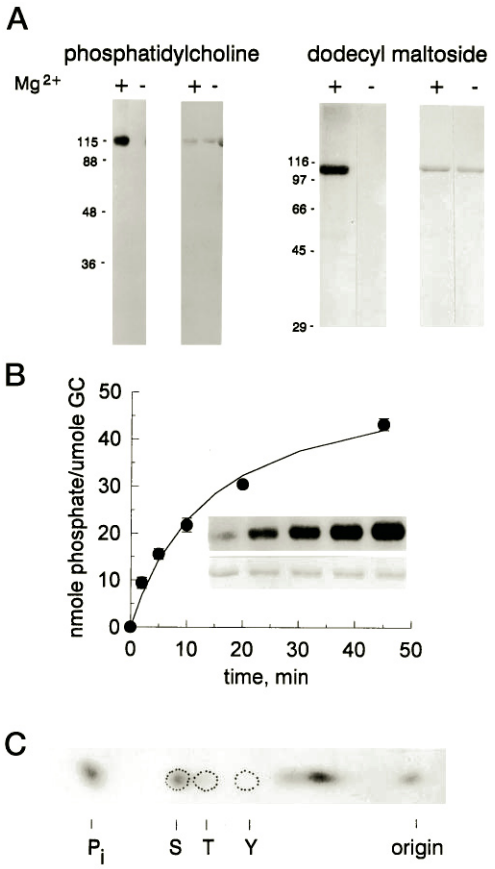

FIG. 3. **Mg^{2+}- and time-dependent [γ-^{32}P]ATP labeling of purified guanylate cyclase.** *A*, autoradiograms of a Mg^{2+}-dependent incorporation of ^{32}P into the guanylate cyclase in either a lipid or detergent environment are shown on the left. The corresponding Coomassie-stained gels are shown on the right. Purified guanylate cyclase, in buffer B (23) or reconstituted into phosphatidylcholine, was added to a kinase reaction mixture containing [γ-^{32}P]ATP in the presence or absence of added $MgCl_2$ and incubated for 45 min. The phosphatidylcholine samples (containing ~1.5 μg of guanylate cyclase prior to reconstitution) were separated on a 10-cm 10% polyacrylamide gel, and the dodecyl maltoside samples (0.6 μg of guanylate cyclase) were separated on a 6-cm 9% polyacrylamide gel. *B*, time-dependent ^{32}P incorporation into the purified guanylate cyclase. Kinase reactions included 0.3 μg of guanylate cyclase and were incubated for the times indicated. After autoradiography, the guanylate cyclase band was excised and counted for ^{32}P, and the moles of phosphate were calculated using the specific activity of the [γ-^{32}P]ATP. The data plotted is the average of duplicate reactions. The *inset* shows an autoradiogram (*top*) and the corresponding Coomassie-stained gel (*bottom*) with the guanylate cyclase protein. *C*, purified guanylate cyclase was phosphorylated and subjected to a phosphoamino acid analysis. Shown is an autoradiogram of a TLC plate loaded with 792 cpm of protein hydrolysate on which the migration of standard phosphoamino acids detected with ninhydrin (29) are marked. All kinase reactions carried out for this figure included 1 mM Na_2GTP.

guanylate cyclase was labeled following incubation with [γ-^{32}P]ATP (Fig. 3*A*). The reaction is dependent on Mg^{2+}. Since reactions with DDM-solubilized guanylate cyclase are technically simpler and require less purified enzyme, all subsequent experiments were performed using the purified, solubilized protein. The incorporation of labeled phosphate was time-dependent and approached saturation as is expected of an enzymatic kinase activity in the presence of limiting substrate (Fig. 3*B*). These observations indicate that the purified guanylate cyclase has a Mg^{2+}-dependent autophosphorylating activity.

To examine what type of amino acid residue is phosphorylated, a phosphoamino acid analysis was performed. The ^{32}P-labeled, phosphorylated guanylate cyclase was subjected to acid hydrolysis. The protein hydrolysate was separated by thin layer electrophoresis, and the migration of the ^{32}P-label was compared to that of standard phosphoamino acids. The ^{32}P label incorporated into the guanylate cyclase comigrated with phosphoserine in two independent guanylate cyclase preparations, one of which is shown in Fig. 3*C*. No incorporation into phosphothreonine or phosphotyrosine is detected. Thus, the phosphoryl transfer activity is that of a serine kinase.

Our initial attempts to determine whether phosphorylation affected cyclase activity revealed no significant difference between the unphosphorylated or phosphorylated molecules (data not shown). However, these experiments are compromised by relatively small difference in phosphorylation levels of the phosphorylated *versus* unphosphorylated molecules due to the low stoichiometry in the autophosphorylation reaction. An estimate of the stoichiometry of autophosphorylation of the guanylate cyclase calculated from the data in Fig. 3*B*. suggests that ~1 in 20 guanylate cyclase molecules are phosphorylated. Higher levels of phosphorylation may be prevented by the presence of residues already phosphorylated in the isolated preparation, a significant fraction of inactivated kinase domains, and/or disruption of an oligomeric structure required for intermolecular or *trans* phosphorylation.

Exogenous Kinase Substrates—In autophosphorylation reactions the protein substrate concentration cannot be manipulated independently of the kinase activity; therefore, the protein substrate concentration cannot be controlled and the sensitivity of the kinase reaction is limited. To find a convenient substrate to use for *in vitro* characterization of the kinase and to crudely assess substrate specificity, we examined whether the kinase activity in the purified guanylate cyclase preparation could phosphorylate common substrates of Ser/Thr kinases. MBP, β-casein, histones (Sigma type II-S and V-S), protamine sulfate, and a poly(Arg/Ser) (3:1) peptide were incubated with purified guanylate cyclase in the presence of [γ-^{32}P]ATP. Unincorporated label was separated from substrates by SDS-PAGE and ^{32}P-labeling of the proteins was examined by autoradiography. MBP and protamine sulfate were found to serve as the best substrates, whereas β-casein and histones (both Sigma type II-S and V-S) are much poorer substrates. A longer film exposure revealed low level phosphorylation of histones II-S (data not shown). No phosphorylation of the poly(Arg/Ser) peptide is detected (Fig. 4) even upon longer film exposures. To control for kinase contamination of the substrates themselves, parallel experiments were run in the absence of guanylate cyclase. No phosphoryl transfer activity is detected for MBP, β-casein, histone II-S, protamines, or the poly(Arg/Ser) peptide; however histone V-S has some endogenous activity (Fig. 4). As an independent positive control all substrates can be shown to be phosphorylated upon addition of protein kinase A[3] (Fig. 4).

Kinase Activity in the Presence of Activators and Inhibitors—It is extremely difficult to exclude absolutely the possibility of residual kinase contaminants in a biochemical preparation. However, the identification of a convenient substrate enabled us to characterize cyclase kinase activity in the presence of several known activators and inhibitors of protein kinases and distinguish this activity from the most probable kinase contaminants, Ser/Thr kinase activities of the photoreceptor outer segments. Cyclic nucleotides or a mixture of PMA, phosphatidylserine, and calcium (PMA/PS/Ca^{2+}) were used to test specifically whether the properties of the cyclase kinase were consistent with a cyclic nucleotide activated protein ki-

[3] Protein kinase A refers to a bovine heart cAMP-dependent protein kinase from Sigma, and cAPK refers to the catalytic subunit of the cAMP-dependent protein kinases for which the crystal structure has been solved.

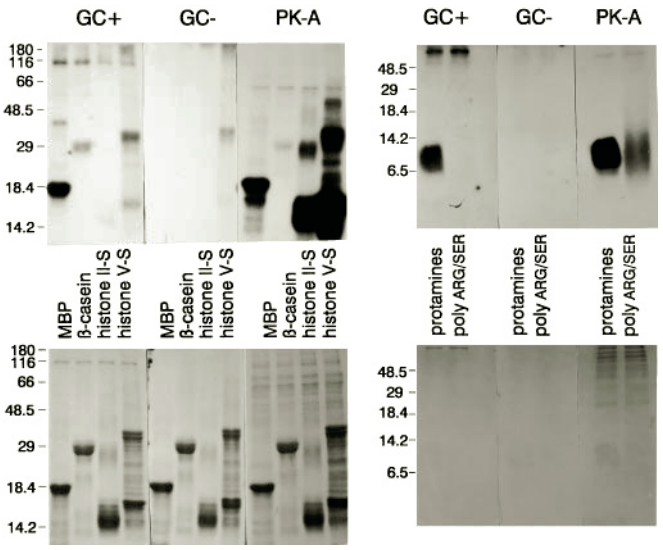

FIG. 4. **Substrate analysis of the guanylate cyclase kinase.** Kinase reactions were performed with ~0.05 μg of purified guanylate cyclase (*GC+*) or no addition (*GC−*) for 10 min, or 6 ng of protein kinase A (*PK-A*) in the presence of 1 mM cAMP for 5 min. MBP (0.9 μg/μl), β-casein (1.25 μg/μl), histone II-S (1 μg/μl), histone V-S (0.7 μg/μl), protamine sulfate (1 μg/μl), or a poly(Arg/Ser) (3:1) peptide (5 μg/μl) were used as substrates. Autoradiograms are shown on the top and the corresponding Coomassie-stained gels are shown on the *bottom*. The molecular mass of MBP, β-casein, histone II-S, and histone V-S is 18.5, 25, 14, and 16 kDa respectively. The poly(Arg/Ser) peptide and the protamines do not stain well with Coomassie.

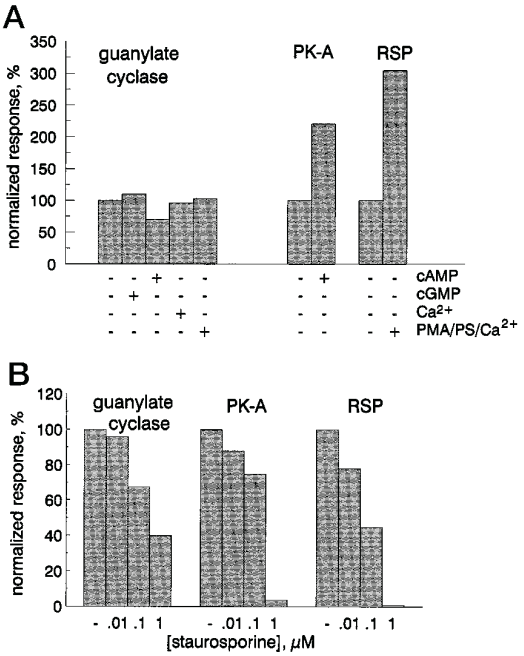

FIG. 5. **Effect of calcium, cyclic nucleotides, PMA/PS/Ca^{2+}, and staurosporine on the guanylate cyclase kinase activity.** *A*, the kinase activity of the guanylate cyclase sample, protein kinase A or RSP was assayed in the presence or absence of 1 mM cAMP, 1 mM cGMP, 0.5 mM Ca^{2+}, and 0.1 ng/μl PMA, 0.3 μg/μl PS, and 0.5 mM Ca^{2+} (*PMA/PS/Ca*$^{2+}$) as indicated. *B*, kinase activity for guanylate cyclase samples, protein kinase A (plus 1 mM cAMP), and ROS-soluble proteins (plus PMA/PS/Ca^{2+}) were assayed in the presence of staurosporine at the indicated concentration. For both *A* and *B*, kinase reactions were incubated for 10 min. ROS-soluble proteins are a concentrated sample of the hypotonic wash from the ROS preparation described in Aparicio and Applebury (protocol 2) (23). Each reaction contained 0.05 μg of guanylate cyclase, 6 ng of protein kinase A, or 2 μg of ROS-soluble proteins. MBP was used as the substrate for all the kinase activity measurements. The activity was determined by counting ^{32}P incorporated into MBP following electrophoretic separation and normalizing to the control (100%). PMA and staurosporine were dissolved in Me_2SO, and the final concentration of Me_2SO in all reactions in *A* and *B* was 0.05%.

nase or protein kinase C. Phosphorylation of MBP by the guanylate cyclase kinase is not stimulated by cGMP, cAMP, or PMA/PS/Ca^{2+} (Fig. 5*A*). As positive controls, protein kinase A or a ROS-soluble protein extract (RSP)[4] containing protein kinase C are shown to phosphorylate MBP in a cAMP or PMA/PS/Ca^{2+} stimulated manner. Calcium had no effect on the guanylate cyclase kinase activity when added to 0.5 mM (Fig. 5*A*).

The effect of a general protein kinase inhibitor, staurosporine, on the guanylate cyclase kinase was also examined. At 1 μM the guanylate cyclase kinase is only partially inhibited (Fig. 5*B*). For comparison, phosphorylation of MBP by protein kinase A and ROS-soluble protein kinase activity(ies) (RSP) was measured in the presence of staurosporine as well. Protein kinase A and all protein kinase activities in a ROS-soluble extract (RSP) were completely inhibited by 1 μM staurosporine (Fig. 5*B*). Consistent with these observations, autophosphorylation of the purified guanylate cyclase is partially inhibited by 1 μM staurosporine and not affected by cGMP, cAMP, PMA/PS/Ca^{2+}, or calcium either 0.5 mM or between 90–540 nM, the range over which calcium changes in photoreceptors (data not shown).

Kinase Kinetics—Protein kinases are bisubstrate enzymes utilizing both MgATP and a polypetide as substrates. We determined the K_m and V_{max} using excess MBP as the protein substrate. The velocity *versus* ATP concentration plot is hyperbolic and transforms into a linear double reciprocal plot indicative of simple Michaelis-Menton kinetic behavior (Fig. 6). The data were fit to the Michaelis-Menten velocity equation yielding a K_m for ATP of 81 ± 2 μM. The V_{max} of 2.1 ± 0.2 nmol min^{-1} m^{-1} corresponds to a turnover number of 0.004 s^{-1}.

DISCUSSION

The photoreceptor membrane guanylate cyclase, like other members of the receptor guanylate cyclase family, has a cytoplasmic kinase-like domain that is contiguous with its catalytic domain. The sequence of this region (13, 14) is easily aligned with Ser/Thr kinases and tyrosine kinases (32–34) showing that 24 of the 33 highly conserved amino acids important for proper structure and function are present in the photoreceptor guanylate cyclase kinase domain. Six different high resolution crystal structures of diverse members of the protein kinase family, including the catalytic subunit of cAPK[3] and that of the tyrosine kinase domain of the human insulin receptor, provide structural frameworks that predict ATP-binding sites and kinase activity (34–39). Using this information as guidance, we undertook studies to examine whether these predicted properties are present in the photoreceptor membrane guanylate cyclase.

ATP Stimulates Guanylate Cyclase Activity—Our initial observations showed that ATP stimulated guanylate cyclase activity 2-fold in washed ROS membranes. The stimulation is light and calcium independent and mimicked by nonhydrolyzable ATP analogues, but the effect is lost upon replacement of Mg^{2+} by Mn^{2+} and upon solubilization of the ROS membranes (Table I, Fig. 1). These data confirm studies of Gorczyca *et al.* (11) who reported 2-fold activation, similar effects of nonhydrolyzable analogues, and calcium independence. A modest 20–30% inhibition of cyclase activity reported by Sitaramayya *et al.* (10) is likewise consistent with our observations that ATP stimulation is lost or even slightly inhibited upon membrane solubilization. Their studies were carried out with Nonidet P-40-solubilized and partially purified guanylate cyclase. Wolbring and Schnetkamp (12) have also recently reported a 2-fold

[4] ROS contain a PMA/PS/Ca^{2+}-stimulated protein kinase C activity (49).

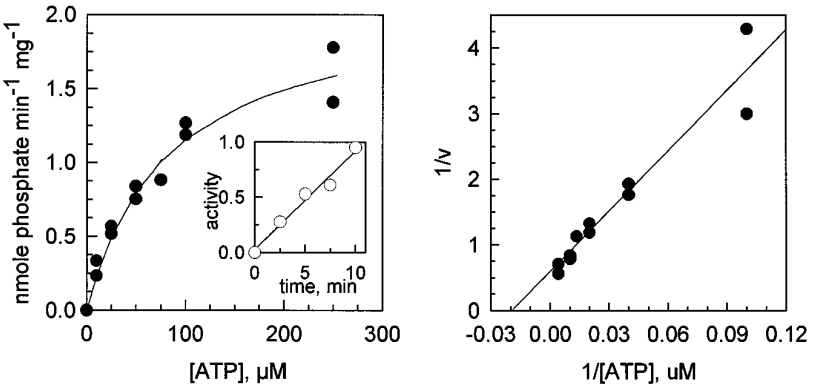

FIG. 6. **Kinetic analysis of the guanylate cyclase kinase.** Guanylate cyclase kinase (0.05 μg) was added to kinase reactions in which 0.9 μg/μl MBP served as the phosphate acceptor protein. Kinase reactions were performed as described under "Experimental Procedures" except that the [γ-^{32}P]ATP concentration was varied and the reaction times were 5 min. After separation from unincorporated radioactivity by SDS-PAGE, the MBP band was excised and counted for ^{32}P, and the specific activity (velocity) was calculated from the specific activity of [γ-^{32}P]ATP. The *inset* shows that transfer of phosphate to MBP was linear for at least 10 min in the presence of 0.9 mg/ml MBP and 500 μM ATP, indicating neither substrate was limiting after a 10-min reaction. The activity in the *inset* is given in picomoles of phosphate transferred per 0.05 μg of guanylate cyclase kinase.

stimulation of cyclase activity by ATP. However, in their study nonhydrolyzable ATP analogues do not substitute for ATP and the EC_{50} for ATP is <10 μM, in contrast to the EC_{50} of ~58 μM for ATP stimulation observed in our work. Wolbring and Schnetkamp show that soluble factors are required to achieve stimulation and conclude the effect occurs through phosphorylation by protein kinase C. It is possible that these authors did not observe stimulation by ATP or nonhydrolyzable analogues in the absence of soluble proteins because their ROS membranes were washed with streptolysin S, an agent that disrupts the integrity of membranes. Their work does point out the complexity of examining effects of ATP and phosphorylation upon guanylate cyclase activity and together with this work emphasizes that both extrinsic and intrinsic phosphorylation could modulate the cyclase in the photoreceptor.

The above observations are consistent with studies of other members of the receptor guanylate cyclase. The basal activities of the ANP, CNP, and guanylin receptors are stimulated up to 5-fold by ATP (10, 40–42). Nonhydrolyzable analogues and ADP will substitute for this action. No stimulation is observed when Mn^{2+} replaces Mg^{2+}. Moreover, the ability of ATP to stimulate the ANP receptor guanylate cyclase activity is lost upon solubilization in a nonionic detergent, as we observed for the photoreceptor enzyme. ATP, nonhydrolyzable ATP analogues, or ADP also enhance ligand-induced cyclase activation (40, 41, 43, 44). Thus, ATP potentiates both basal cyclase activity and ligand stimulation throughout the family of receptor guanylate cyclases. The ATP-binding site predicted for the kinase homology domain would be a good candidate to achieve these affects.

The Guanylate Cyclase Has an ATP-binding Site—Photoaffinity labeling with 8-N_3[α-^{32}P]ATP was carried out to show that the photoreceptor guanylate cyclase has an inherent ATP-binding site. Purified, DDM-solubilized guanylate cyclase is effectively labeled at a site independent of the GTP-binding site (Fig. 2). This site was labeled using 20 μM 8-N_3[α-^{32}P]ATP, a concentration that favorably compares with the effective range for ATP stimulation of basal activity (Fig. 1). Thus, the data are consistent with the hypothesis that the site identified by photoaffinity labeling is the same as the ATP-stimulation site. Confounding this last interpretation is the observation that the ATP stimulation is no longer apparent upon DDM solubilization. Although solubilization by DDM could suggest the loss of an associated activating factor, this detergent modestly stimulates guanylate cyclase activity (~1.4 fold) in the absence of ATP (23). Thus, it is possible that DDM promotes an active conformation roughly equivalent to that induced by ATP, which masks our ability to detect further stimulation due to ATP binding. This idea is supported by the fact that ATP decreases the K_m for MgGTP from 2 mM to 1 mM. The latter value is the same as that measured for the purified enzyme in DDM in the absence of ATP (1.07 mM) (23). Finally, the interpretation is consistent with the function of ATP stimulation in other members of the family, which is thought to be mediated by the nucleotide binding site in the kinase homology domain and not via a factor present with the guanylate cyclases in membranes (20, 22, 43).

The Guanylate Cyclase Is a Kinase—In the presence of [γ-^{32}P]ATP and Mg^{2+}, the purified guanylate cyclase exhibits a protein kinase activity as shown by ^{32}P autophosphorylation (Fig. 3, *A* and *B*). Phosphoamino acid analysis indicates that Ser residues of the guanylate cyclase are labeled (Fig. 3*C*), placing the kinase in the family of Ser/Thr protein kinases. To support the observation and to characterize the activity further, exogenous substrates for the kinase were identified. Upon addition of the purified cyclase, ^{32}P labeling of MBP, protamines, β-casein, and histones is detected (Fig. 4). The conclusion that the activity is inherent to the guanylate cyclase molecule is supported by several arguments. The guanylate cyclase has an ATP-binding site required for a kinase as shown through 8-N_3[α-^{32}P]ATP labeling (Fig. 2). The possibility that the observed activity is due to contamination by Ser/Thr kinases that have been identified in ROS, including rhodopsin kinase, protein kinase A, and protein kinase C, was eliminated by showing that the guanylate cyclase kinase has different properties. Rhodopsin kinase has a very limited substrate specificity and will not phosphorylate histones, β-casein, or protamines (45), yet all of these serve as substrates for the guanylate cyclase kinase (Fig. 4). The guanylate cyclase kinase exhibits no sensitivity to cyclic nucleotides or PMA/PS/Ca^{2+}. The guanylate cyclase kinase appears to be less sensitive to staurosporine than both protein kinase A and C (Fig. 5). Moreover, the levels of kinase activity detected in different purified preparations of guanylate cyclase are consistently uniform. Ultimately, mutagenesis of the kinase domain is needed to demonstrate irrefutably that the kinase is inherent to the guanylate cyclase. Such studies are in progress.

Comparison of the sequence of the photoreceptor guanylate cyclase with sequences of members of the extensive protein kinase family shows that most of the amino acids essential for ATP binding and kinase activity are conserved in the guanylate cyclase kinase. A notable exception is an Asp corresponding to

that located within the catalytic loop of cAPK (Asp-166), which is proposed to be a catalytic base for phosphoryl transfer in protein kinases (34). Although the region is generally conserved, the Asp residue appears to be deleted and possibly replaced by an adjacent Arg (13, 14, 18). The absence of the Asp raises the possibility that the kinase has no catalytic activity. However, when Asp-166 in cAPK is mutated to Ala, the catalytic power of cAPK-α was dramatically reduced, but not eliminated. The turnover number was reduced from 16.7 s^{-1} to 0.05 s^{-1} (46). Using a nonphysiological substrate, myelin basic protein, we measured a turnover number of 0.004 s^{-1} for the photoreceptor guanylate cyclase kinase. This value is at the low end of the range of turnover numbers reported for protein kinases which extends from 433 s^{-1} for phosphorylase kinase to 0.005 s^{-1} for isocitrate dehydrogenase kinase (47). The relatively low activity level of the photoreceptor guanylate cyclase kinase may reflect a function where high activity is not required; alternatively, measurement of a higher rate may be compromised by a fraction of kinase domains that are inactive, or by a lack of a physiological substrate or activator.

The data in this work provide the first biochemical evidence that a member of the membrane receptor guanylate cyclase family has protein kinase activity. Other studies of the kinase region have been carried out primarily with GC-A. This member of the cyclase family is not considered to be an active kinase because no kinase activity has been detected and the proposed catalytic base for protein kinases (the equivalent of cPKA's Asp-166 discussed above) is not conserved (22, 48). Given the finding that the photoreceptor guanylate cyclase has kinase activity, it might be worthwhile to readdress this issue in GC-A or other members of the family based on the following considerations. As discussed above, the lack of the conserved Asp in the catalytic loop would likely reduce, but not eliminate, catalytic activity. Interestingly, when Koller *et al*. (22) performed alanine-scanning mutagenesis of conserved residues considered to be involved in ATP binding and kinase activity, phosphorylation of GC-A was dramatically reduced or eliminated. The decreased phosphorylation has been attributed to tertiary structural changes that masked phosphorylation sites for extrinsic kinases (22). However, an alternative explanation that autophosphorylating activity was destroyed seems possible. Finally in the GC-A systems studied, kinase activity may be difficult to detect due to saturation of autophosphorylation sites, relatively low activity levels, or lability of the activity. It will be interesting to learn whether other members of the receptor guanylate cyclase family have kinase activity or whether this property is unique to the photoreceptor cyclase.

Is the ATP Site for Stimulation and Phosphoryl Transfer the Same?—The data accumulated so far suggest that there is a single ATP-binding site within the protein kinase domain that both stimulates cyclase activity and catalyzes phosphoryl transfer. In support of this hypothesis, the EC_{50} measured for ATP stimulation (58 μM) and the K_m for ATP of the kinase (81 μM) are similar, and both are consistent with the concentrations of 8-N_3[α-^{32}P]ATP that effectively label the cyclase (Figs. 1, 2, and 6). The relative potency of stimulation by the ATP analogues (Fig. 1) is consistent with an ATP-binding site in which hydrolysis or phosphoryl transfer occurs. The effective concentration required for ATP stimulation ($EC_{50} \sim 58$ μM) appears to be intermediate between that of the nonhydrolyzable or slowly hydrolyzable ATP analogues, AMPPNP ($EC_{50} \sim 19$ μM) and ATPγS ($EC_{50} \sim 16$ μM), and ADP ($EC_{50} \gg 100$ μM). For a single site model, one may postulate that ATP stimulation arises from conformational changes induced upon ATP or analogue binding. This active conformation may be maintained by ADP following hydrolysis. Only upon release of ADP would the initial conformation be restored. The observations in this work raise the question of the function of autophosphorylation. An active preparation of the guanylate cyclase in which the endogenous phosphorylation can be well controlled will help us better assess effects on cyclase activity. Identification of the site of autophosphorylation and a better understanding of the structure and function of the cyclase domains may help define the mechanisms by which the kinase homology domain regulates the production of cGMP in the photoreceptor outer segment.

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