BIOCHEMISTRY
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Biochemistry **2009,** *48,* 7519–7524 7519
DOI: 10.1021/bi900696x

Nucleotide Regulation of Soluble Guanylate Cyclase Substrate Specificity†

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Received April 22, 2009; Revised Manuscript Received June 13, 2009

ABSTRACT: Soluble guanylate cyclase (sGC) serves as a receptor for the signaling agent nitric oxide (NO). sGC synthesis of cGMP is regulated by NO, GTP, ATP, and allosteric activators such as YC-1. The guanylate cyclase activity and adenylate cyclase activity of full-length sGC and the sGC catalytic domain constructs ($\alpha1_{cat}\beta1_{cat}$) are reported here. ATP is a mixed-type inhibitor of cGMP production for both sGC and $\alpha1_{cat}\beta1_{cat}$, indicating that the C-terminus of sGC contains an allosteric nucleotide binding site. YC-1 did not activate $\alpha1_{cat}\beta1_{cat}$ or compete with ATP inhibition of cGMP synthesis, which suggests that YC-1 and ATP bind to distinct sites. $\alpha1_{cat}\beta1_{cat}$ and NO-stimulated sGC also synthesize cAMP, but this activity is inhibited by ATP via noncompetitive substrate inhibition and by GTP via mixed-type inhibition. Additionally, the adenylate cyclase activity of purified sGC was inhibited by PC12 lysate, suggesting that an intracellular small molecule or protein regulates this activity *in vivo*.

Soluble guanlyate cyclase (sGC)[1] is a heterodimeric hemoprotein that is essential to several physiological processes (*1*−*3*). sGC synthesis of cGMP from GTP is activated by the signaling agent nitric oxide (NO). NO activation of sGC is complex and is known to be influenced by the binding of GTP and ATP to an allosteric binding site on the protein (*4*, *5*). In addition to nucleotides, several small molecules have been shown to influence sGC activity (*6*, *7*). Despite several studies focused on understanding sGC regulation by nucleotides and allosteric activators, many questions pertaining to the mechanism of this regulation remain unresolved.

sGC consists of two homologous subunits, α1 and β1. Architectural information about sGC has been advanced by the expression and isolation of the minimal heme binding domain β1(1−194)[2] (*8*) and the catalytic domains α1(467−690) and β1(414−619) (*9*). The functional catalytic domains, termed $\alpha1_{cat}\beta1_{cat}$, contain a pseudosymmetric active site. This pseudosymmetric site contains residues known to be involved in nucleotide binding but lacks the amino acids required for catalysis (*10*). Although binding of a nucleotide to this pseudosymmetric site has not been directly observed, it is known that sGC binds 2 equivalents of substrate (*11*) and that nucleotide binding to an allosteric site influences activity (*5*, *12*).

Pharmaceutical screens have also identified sGC allosteric activators, including YC-1 (*7*), and its more soluble derivative BAY 41-2272 (*13*). These compounds activate the Fe^{II}-unligated sGC state weakly (2−4-fold) but significantly increase sGC activity when a ligand is bound at the Fe^{II} heme (*14*, *15*). This synergistic activation leads to a Fe^{II}−CO complex that is activated 100−400-fold and a Fe^{II}−NO complex that is activated 200−400-fold. While the precise mechanism of activation by these compounds is unknown, it has been proposed that they are GTP mimics that bind to the pseudosymmetric site (*10*, *11*).

Detailed spectroscopic and kinetic studies on the effects of GTP, ATP, and YC-1 on sGC have been previously reported (*4*, *11*, *16*−*19*); however, it remains unclear where these molecules bind and how they function to regulate sGC activity. These questions were addressed here by examining guanylate cyclase (GC) and adenylate cyclase (AC) activity of both full-length sGC and $\alpha1_{cat}\beta1_{cat}$. The kinetic study reported herein suggests that sGC and $\alpha1_{cat}\beta1_{cat}$ contain two nucleotide binding sites that are able to bind both GTP and ATP, thus localizing the allosteric GTP/ATP binding site to the C-terminus of sGC. We observed that GTP binding to this site leads to inhibition of ATP turnover, whereas ATP binding to this site leads to inhibition of both GTP and ATP turnover. Additionally, we observed that although $\alpha1_{cat}\beta1_{cat}$ contains a functional allosteric nucleotide binding site, it is unresponsive to YC-1 and BAY 41-2272. This work shows that sGC differentially responds to GTP and ATP at physiologically relevant nucleotide concentrations and indicates that distinct conformational changes occur within the catalytic

†Funding was provided by National Institutes of Health Grant GM077365 to M.A.M.

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[1]Abbreviations: sGC, soluble guanylate cyclase; NO, nitric oxide; GTP, guanosine 5′-triphosphate; Sf9, *Spodoptera frugiperda*; DEA/NO, diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate; PROLI/NO, 1-(hydroxy-NNO-azoxy)-L-proline; YC-1, 3-(5′-hydroxymethyl-3′-furyl)-1-benzylindazole; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay.

[2]sGC amino acid numbering is that of the rat enzyme unless otherwise noted.

Published on Web 06/15/2009

domain depending on the occupation of the allosteric nucleotide binding site.

MATERIALS AND METHODS

Materials. sGC was expressed using a baculovirus/Sf9 expression system and purified as previously described (*19*). The sGC catalytic domains ($\alpha1_{cat}$ and $\beta1_{cat}$) were expressed and purified as previously described (*9*). Diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) was from Cayman Chemical Co. All other reagents were from Sigma, unless otherwise noted.

Full-Length sGC Activity Assays. sGC activity was examined in the presence of various allosteric regulators by performing duplicate end point assays at 37 °C. The assay mixture contained 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT. When present, YC-1 and BAY 41-2272 (both in DMSO) were at 150 and 20 μM, respectively, and the final concentration of DMSO was 2% (v/v), which was shown not to affect enzyme activity. The GTP concentration ranged from 0.0025 to 3 mM, and the ATP concentration ranged from 0 to 5 mM. In each assay, sGC (0.2 μg) was incubated with ATP for 1 min at 37 °C before the reaction with GTP was initiated. All assays were conducted in a final volume of 100 μL. Reactions were quenched after 2 min by the addition of 400 μL of 125 mM $Zn(CH_3CO_2)_2$ and 500 μL of 125 mM Na_2CO_3. cGMP quantification was conducted using a cGMP enzyme immunoassay kit (Assay Designs), per the manufacturer's instructions. The resulting data were fit to the Michaelis−Menten equation [$v = V_{max}[GTP]/(K_M + [GTP])$] to obtain K_M and V_{max} in the presence of ATP. The experiments were repeated three times to ensure reproducibility.

Adenylate cyclase assays were preformed as described above but with the following differences. In each assay, sGC (0.2 μg) was incubated with or without GTP (0.5 mM) for 1 min at 37 °C before the reaction was initiated with ATP (0−10 mM). Reactions were quenched after 5 min, and cAMP quantification was conducted using a cAMP enzyme immunoassay kit (Assay Designs), per the manufacturer's instructions. The resulting data were fit to the equation for noncompetitive substrate inhibition to obtain K_M, K_I, and V_{max}, where α is the maximum velocity in the presence of saturating substrate (*20*).

$$v = \frac{V_{max}\left(\frac{[ATP]}{K_M} + \frac{[ATP]^2}{\alpha K_M KI}\right)}{1 + \frac{[ATP]}{K_M} + \frac{[ATP]}{K_I} + \frac{[ATP]^2}{\alpha K_M K_I}} \quad (1)$$

The experiments were repeated three times to ensure reproducibility.

$\alpha1_{cat}\beta1_{cat}$ Activity Assays. The sGC catalytic domains were assayed as described above but with the following exceptions. $\alpha1_{cat}\beta1_{cat}$ (10 μg of heterodimer) was assayed in 50 mM Hepes (pH 7.4), 6 mM $MnCl_2$, 2 mM DTT, and varying amounts of GTP or ATP as indicated. Reactions were quenched after 16 min, and cGMP or cAMP quantification was conducted as previously described.

PC12 Cells. Semi-adherent PC12 cells were obtained from American Type Culture Collection (ATCC) and were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were counted with a hemocytometer, and viability was routinely assessed by trypan blue staining and found to be >90%. PC12 cells in 150 mm × 15 mm Petri dishes were washed extensively with ice-cold assay buffer [50 mM Hepes (pH 7.4) and 50 mM NaCl] and harvested with a rubber policeman. Cells were collected by centrifugation at 4 °C and gently resuspended in ice-cold assay buffer with 1 mM 3-isobutyl-1-methylxanthine (IBMX). Whole cell assays were initiated via addition of 1 μM PROLI/NO (Cayman Chemicals) to 100 μL of cells (50−100 μg of total protein) at 37 °C. Reactions were quenched 5 s after addition of NO with 1 volume of 133 mM HCl. For lysate assays, cells were resuspended in 50 mM Hepes (pH 7.4), 50 mM NaCl, 5 mM DTT, 1 mM benzamidine, 1 mM Pefabloc (Roche), 1 mM IBMX, and Complete EDTA-free protease inhibitor cocktail (Roche) and lysed by sonication. The cell lysate was equilibrated at 37 °C for 10 s before the reaction was initiated with 1 mM GTP or ATP and 3 mM $MgCl_2$. Assays contained 100 μM DEA/NO and/or 13 nM purified sGC where indicated and were quenched after 5 min by addition of 133 mM HCl. The protein concentration was measured in each sample using the Bradford Microassay (Bio-Rad Laboratories) against a bovine serum albumin standard, and cGMP or cAMP was quantified using an enzyme immunoassay kit (Assay Designs), per the manufacturer's instructions. The detection limit of cAMP was 0.078 pmol/mL, and the detection limit of cGMP was 0.08 pmol/mL; all the data reported in this work were above these limits. Each assay point was determined in duplicate, and all experiments were repeated three times to ensure reproducibility.

RESULTS

sGC Activity. The effect of ATP on sGC activity was examined with both full-length sGC (Figure 1A) and $\alpha1_{cat}\beta1_{cat}$ (Figure 1B). Figure 1A shows that ATP is a mixed-type inhibitor of Fe^{II}-unligated sGC as indicated by an increase in the apparent K_M and a decrease in the V_{max}. This is in agreement with previously published reports (*5*, *12*) and indicates that ATP inhibits full-length sGC by binding to both the catalytic site and an allosteric nucleotide binding site. Kinetic data from previous reports show that this mechanism of ATP inhibition is observed with NO-stimulated sGC (*5*, *12*, *21*). This indicates that both nucleotide binding sites are accessible in the stimulated and basal sGC states. Figure 1B shows that ATP is also a mixed-type inhibitor of $\alpha1_{cat}\beta1_{cat}$. $\alpha1_{cat}\beta1_{cat}$ activity was measured in the presence of Mn^{2+} because the complex is 50-fold more active in the presence of Mn^{2+} than in the presence of Mg^{2+} (*9*). $\alpha1_{cat}\beta1_{cat}$ consists of the C-terminus of both $\alpha1$ and $\beta1$ (residues 467−690 of $\alpha1$ and 414−619 of $\beta1$) and contains the catalytic site and the pseudosymmetric substrate binding site. This result localizes the allosteric nucleotide binding site to the C-terminus of sGC and establishes that the catalytic domains can be further used as a model to study the allosteric nucleotide binding site.

Different binding sites have been proposed for YC-1, including the N-terminus of the $\alpha1$ subunit (*13*, *22*) and the pseudosymmetric substrate site (*11*, *23*, *24*). Others have proposed that both ATP and YC-1 bind to the pseudosymmetric site (*10*, *11*). To examine this possibility, full-length sGC and $\alpha1_{cat}\beta1_{cat}$ activity were examined in the presence of both ATP and YC-1. If YC-1 and ATP compete for the same site, then ATP should inhibit activation of sGC by YC-1. Figure 2A shows that YC-1 activates full-length sGC 10−15-fold regardless of the presence of ATP (2.5 mM). Furthermore, there was no decrease in the degree of activation of sGC by YC-1 at any concentration of ATP

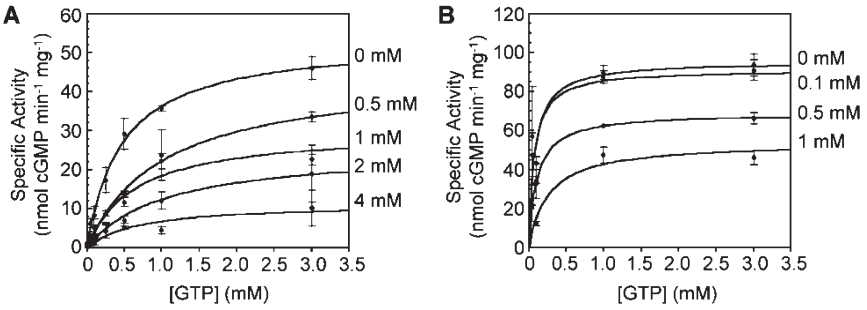

FIGURE 1: ATP inhibition of sGC (A) and $\alpha1_{cat}\beta1_{cat}$ (B) activity. The specific activity vs the concentration of substrate (GTP) was plotted in the presence of various ATP concentrations, which are indicated at the right sides of panels A and B. sGC (0.2 μg) was assayed in 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT. $\alpha1_{cat}\beta1_{cat}$ (10 μg of heterodimer) was assayed in 50 mM Hepes (pH 7.4), 6 mM $MnCl_2$, and 2 mM DTT. Assays were conducted at 37 °C. Each curve was fit to a standard saturation equation [$v = V_{max}[GTP]/(K_M + [GTP])$] to obtain K_M and V_{max} in the presence of ATP.

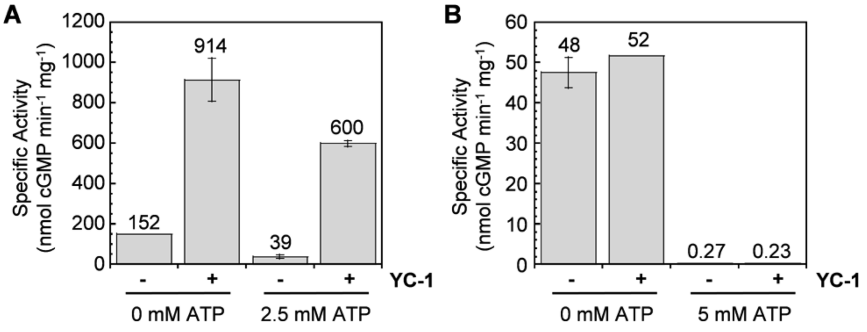

FIGURE 2: Effect of YC-1 on the inhibition of sGC and $\alpha1_{cat}\beta1_{cat}$ guanylate cyclase activity in the presence of ATP. The effect of YC-1 (150 μM) on sGC (A) and $\alpha1_{cat}\beta1_{cat}$ (B) was examined in the presence and absence of ATP at 37 °C. sGC (0.2 μg) was assayed in 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, and 1.5 mM GTP, and $\alpha1_{cat}\beta1_{cat}$ (10 μg of heterodimer) was assayed in 50 mM Hepes (pH 7.4), 6 mM $MnCl_2$, 2 mM DTT, and 3 mM GTP.

examined (0−5 mM). The sGC catalytic domain constructs, which contain the allosteric ATP binding site, were insensitive to YC-1 activation, and YC-1 had no effect on the ATP-induced inhibition (Figure 2B). Figure 2 shows full-length sGC and $\alpha1_{cat}\beta1_{cat}$ activity at a single ATP concentration, but we observed consistent results at several nucleotide concentrations (0.1, 0.5, 1.0, 2.5, and 5.0 mM ATP). Additionally, the more soluble YC-1 derivative, BAY 41-2272, had no effect on the guanylate cyclase activity of $\alpha1_{cat}\beta1_{cat}$ (data not shown). The overall conclusion is that ATP and the allosteric activators YC-1 and BAY 41-2272 bind to distinct sites on sGC.

sGC Adenylate Cyclase Activity. The mixed-type inhibition observed for both full-length sGC and $\alpha1_{cat}\beta1_{cat}$ (Figure 1A, B) indicates that ATP binds to the sGC catalytic site in addition to an allosteric nucleotide binding site and indicates the potential for ATP turnover by the enzyme. sGC-catalyzed adenylate cyclase (AC) activity has been reported (*25*, *26*); however, a thorough kinetic characterization of this activity had not been previously performed. Therefore, cAMP formation at various ATP concentrations for both NO-stimulated sGC and $\alpha1_{cat}\beta1_{cat}$ was assessed.

Adenylate cyclase activity was observed for both NO-stimulated full-length sGC and $\alpha1_{cat}\beta1_{cat}$ (Figure 3A,B). The amount of turnover observed for sGC in the absence of NO was very small (0.1 nmol of cAMP min^{-1} mg^{-1} at 1 mM ATP). Because of the low specific activity of the unligated enzyme and limitations in acquiring purified protein, the sGC K_M for ATP in the absence of NO was not determined; however, it is clear that the adenylate cyclase activity of purified sGC is significantly activated by the presence of NO. This observation is in agreement with an earlier

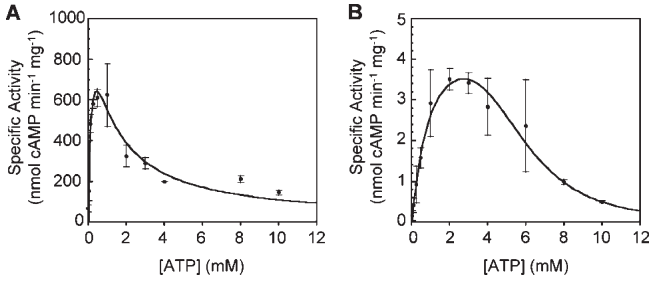

FIGURE 3: sGC adenylate cyclase activity. Substrate inhibition by ATP of sGC in the presence of 100 μM DEA/NO (A) and $\alpha1_{cat}\beta1_{cat}$ (B) at 37 °C. sGC (0.2 μg) was assayed in 50 mM Hepes (pH 7.4), 20 mM $MgCl_2$, and 1 mM DTT, and $\alpha1_{cat}\beta1_{cat}$ (10 μg of heterodimer) was assayed in 50 mM Hepes (pH 7.4), 25 mM $MnCl_2$, and 2 mM DTT. The data were fit to a noncompetitive substrate inhibition equation to obtain K_M(ATP), K_I(ATP), and V_{max}.

study with protein isolated from various rat tissues (*26*). The presence of YC-1 also stimulated the AC activity of full-length sGC ~10-fold (data not shown). Substrate inhibition of adenylate cyclase activity was observed at high concentrations of ATP (0.5−10 mM) in both NO-stimulated full-length sGC and $\alpha1_{cat}\beta1_{cat}$. Fitting the plots in Figure 3 to a noncompetitive substrate inhibition equation yields a K_M(ATP) of 52 μM and a K_I(ATP) of 1.4 mM for full-length sGC. $\alpha1_{cat}\beta1_{cat}$ exhibits a decreased affinity for ATP relative to sGC as the K_M(ATP) and K_I(ATP) were determined to be 459 μM and 5.4 mM, respectively (Table 1). This suggests that ATP binding to the allosteric site leads to a conformational change at the catalytic site to inhibit turnover of both ATP and GTP. Additionally, the observation of

this kinetic phenomenon in both sGC and $\alpha1_{cat}\beta1_{cat}$ confirms that the allosteric nucleotide binding site is contained on the C-terminus of $\alpha1$ and $\beta1$.

GTP inhibition of adenylate cyclase activity was also examined. Figure 4 shows that the presence of GTP (0.5 mM) decreased the V_{max} for both NO-stimulated full-length sGC and $\alpha1_{cat}\beta1_{cat}$ AC activity. This indicates that GTP is not strictly a competitive inhibitor of ATP and suggests that GTP also binds to two sites on full-length sGC and $\alpha1_{cat}\beta1_{cat}$. Due to the low yields of both full-length sGC and the sGC catalytic domains several purifications were required to complete the experiments in Figures 1−4. The data in each figure was obtained on the same day with the same protein; however, the data in separate figures were obtained with different protein preparations, hence the apparent variability in V_{max} between figures.

Endogenous sGC Activity. On the basis of the ability of sGC to cyclize ATP *in vitro*, we sought to determine if NO-induced adenylate cyclase activity is observed *in vivo*. Using PC12 cells, an immortalized cell line derived from a rat pheochromocytoma which is known to express sGC, we found that sGC does not produce cAMP in response to NO *in vivo*. In both intact cells and PC12 lysate, there was no increase in the level of cAMP in response to NO, whereas cGMP levels increased several hundred-fold in response to NO (Table 2). This suggested that a small molecule or protein present in PC12 cells could be inhibiting sGC adenylate cyclase activity. To test this possibility, the activity of purified sGC in the presence and absence of PC12 lysate was measured. Figure 5 shows that the NO-induced guanylate cyclase activity of purified sGC (~370-fold) is not inhibited by the presence of PC12 lysate, but the NO-induced adenylate cyclase activity of purified sGC is completely inhibited by PC12 lysate, suggesting that ATP turnover by sGC is regulated by an intracellular protein or small molecule.

Table 1: Kinetic Parameters of ATP and GTP Binding to sGC[a]

protein	substrate	k_{cat} (min^{-1})	K_M (μM)	K_I (mM)
Fe^{II}-unligated sGC	Mg^{2+}-GTP	8.4 ± 1.5	130[c] ± 20	—
Fe^{II}−NO sGC	Mg^{2+}-GTP	1745 ± 168	44[c] ± 2	—
	Mg^{2+}-ATP[b]	93 ± 5.1	52 ± 5	1.4 ± 0.2
$\alpha1_{cat}\beta1_{cat}$	Mn^{2+}-GTP	2.7 ± 0.4	85[d] ± 18	—
	Mn^{2+}-ATP[b]	0.19 ± 0.04	459 ± 166	5.4 ± 1.5

[a] Values determined at 37 °C. [b] Data were fit to the noncompetitive substrate inhibition equation to obtain K_M(ATP) and K_I(ATP). [c] Values from ref *16*. [d] Values from ref *9*.

DISCUSSION

sGC is a critical cellular receptor for the gaseous signaling agent NO. *In vivo*, sGC is regulated by a complex interplay among NO, GTP, and ATP, and this regulation is essential for the physiological functions controlled by cGMP. Understanding

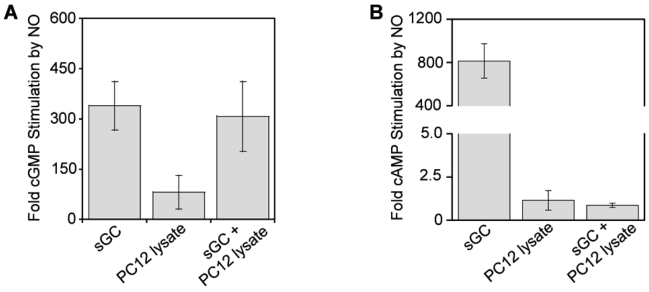

FIGURE 5: Inhibition of sGC adenylate cyclase activity in PC12 cells. NO-stimulated guanylate cyclase (A) and adenylate cyclase (B) activity of purified rat sGC in the presence and absence of PC12 lysate (~50 μg). PC12 lysate inhibits cAMP synthesis in response to NO (100 μM DEA/NO), but not cGMP synthesis.

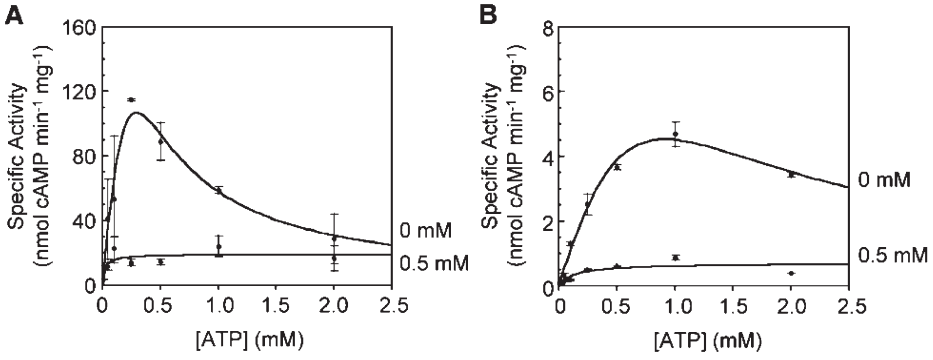

FIGURE 4: GTP inhibition of sGC adenylate cyclase activity. The specific activity vs the concentration of substrate (ATP) was plotted in the presence and absence of 0.5 mM GTP for sGC in the presence of 100 μM DEA/NO (A) and $\alpha1_{cat}\beta1_{cat}$ (B) at 37 °C. sGC (0.2 μg) was assayed in 50 mM Hepes (pH 7.4), 20 mM $MgCl_2$, and 1 mM DTT, and $\alpha1_{cat}\beta1_{cat}$ (10 μg of heterodimer) was assayed in 50 mM Hepes (pH 7.4), 25 mM $MnCl_2$, and 2 mM DTT. The data in the absence of GTP were fit to a noncompetitive substrate inhibition equation, and the data in the presence of 0.5 mM GTP were fit to a standard saturation equation.

Table 2: Nucleotide Specificity of sGC *in Vivo*[a]

	GC activity (pmol of cGMP min^{-1} mg^{-1})		AC activity (pmol of cAMP min^{-1} mg^{-1})	
sample	without NO	with NO	without NO	with NO
PC12 cells	1.15 ± 0.34	779 ± 166	16.4 ± 4.57	11.3 ± 0.87
PC12 lysate	0.44 ± 0.31	1629 ± 122	0.57 ± 0.06	0.81 ± 0.21
purified sGC	(1.61 ± 0.32) × 10^4	(547 ± 43.3) × 10^4	111 ± 19	(8.06 ± 3.60) × 10^4

[a] Values determined at 37 °C; GC assays with 3 mM $MgCl_2$ and 1.5 mM GTP and AC assays with 3 mM $MgCl_2$ and 1 mM ATP.

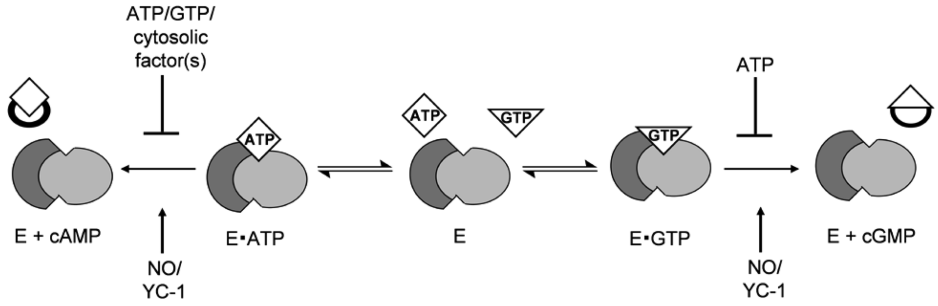

FIGURE 6: Model of sGC regulation by nucleotides. The sGC catalytic domains contain a catalytic site and an allosteric nucleotide binding site that can bind both GTP and ATP. ATP binding to the allosteric site leads to a general inhibition of catalytic activity, while GTP binding to the allosteric site selectively inhibits ATP turnover. NO and YC-1 are activators of cGMP and cAMP synthesis.

sGC regulation by both nucleotides and synthetic allosteric activators, such as YC-1 and BAY 41-2272, is necessary for the development of therapeutic agents to treat diseases related to dysfunction in sGC. To this end, we examined the effects of ATP, GTP, and YC-1 on the guanylate and adenylate cyclase activity of full-length sGC and $\alpha1_{cat}\beta1_{cat}$.

Several groups have studied the interaction of ATP with sGC. These detailed studies provided kinetic and thermodynamic data to support the existence of two distinct nucleotide binding sites on sGC: a catalytic site and an allosteric nucleotide binding site (*5*, *11*). On the basis of primary sequence analysis, the C-terminus of sGC is predicted to contain the allosteric nucleotide binding site, but limitations in acquiring purified full-length protein and the lack of sensitive tools for studying nucleotide binding have hindered the identification of this site. By using the sGC catalytic domains, $\alpha1_{cat}\beta1_{cat}$, we were able to localize the allosteric nucleotide binding site to the C-terminus of sGC. Specifically, we found that ATP is a mixed-type inhibitor for $\alpha1_{cat}\beta1_{cat}$ (Figure 1) which indicates that ATP inhibits cGMP production by binding to two different sites on the C-terminus of sGC. We then used the catalytic domains as a probe to investigate the YC-1 binding site. We found that $\alpha1_{cat}\beta1_{cat}$ was unresponsive to YC-1 and that ATP did not alter the degree of activation of full-length sGC induced by YC-1 (Figure 2). These observations suggest that YC-1 does not bind to the allosteric nucleotide binding site that is contained on the C-terminus of sGC. The N-terminus of the α1 subunit has been shown to bind YC-1 (*13*). YC-1 binding to a hydrophobic pocket within this domain (*22*) could induce a conformational change that affects both the sGC heme environment and the catalytic domain. This model of YC-1 activation is consistent with our current results and our previously reported spectroscopic studies (*17*, *19*).

While researchers have studied the inhibition of sGC by ATP for more than 30 years, the potential for adenylate cyclase activity with purified protein had not been thoroughly investigated. Recently, the catalytic domain from a bacterial guanylate cyclase, Cya2, was shown to cyclize both GTP and ATP (*27*). Cya2 shows specificity for GTP versus ATP, and sequence analysis predicts that this mechanism of nucleotide specificity varies from that of eukaryotic sGCs (*27*). We have now found that both NO-stimulated sGC and $\alpha1_{cat}\beta1_{cat}$ cyclize ATP to cAMP (Figure 3). The AC activity of full-length sGC was significantly lower in the absence of NO (Table 2), indicating that NO stimulates cAMP synthesis several hundred-fold. YC-1 also stimulates cAMP synthesis ~10-fold (data not shown), suggesting that NO and YC-1 are not selective for GTP-bound sGC. Interestingly, AC activity is inhibited at high ATP concentrations due to substrate inhibition. This phenomenon was not observed in the Cya2 homodimer and represents a unique effect of ATP binding to the allosteric nucleotide binding site on the eukaryotic heterodimeric enzyme.

As evaluated by the k_{cat} for each nucleotide, sGC exhibits a high specificity for GTP as a substrate, but the observed AC activity enabled us to further investigate the allosteric nucleotide binding site. By fitting the plots in Figure 3 to the noncompetitive substrate inhibition equation, we obtained both K_M(ATP) and K_I(ATP). These values provide a means of estimating the binding affinity of ATP at the catalytic site (K_M) and the allosteric nucleotide binding site (K_I). From this, we observe that the allosteric nucleotide binding site has a lower affinity for ATP (Table 1), which is in agreement with K_d estimations based on equilibrium dialysis experiments with ATP analogues (*11*). Since substrate inhibition of GC activity is not observed with GTP for either full-length sGC or $\alpha1_{cat}\beta1_{cat}$, it suggests that either GTP has a significantly lower affinity for the allosteric site or GTP binding does not inhibit GTP turnover. To evaluate these possibilities, we tested the mechanism of GTP inhibition of AC activity. Figure 4 shows that GTP decreases the V_{max} of cAMP formation for both NO-stimulated full-length sGC and $\alpha1_{cat}$-$\beta1_{cat}$. This indicates that GTP is a mixed-type inhibitor of AC activity and suggests that both nucleotides bind to both the catalytic site and the allosteric nucleotide binding site. Interestingly, GTP binding to the allosteric nucleotide binding site inhibits ATP turnover but not GTP turnover. This differential effect indicates that distinct conformational changes occur within the catalytic site depending on the occupation of the allosteric nucleotide binding site. Furthermore, this differential nucleotide regulation reflects a broken symmetry that would not be possible with a homodimeric enzyme. Understanding these conformational changes is essential to elucidating the mechanism of nucleotide regulation of sGC.

To evaluate the role of ATP in regulating sGC activity *in vivo*, we examined the effect of PC12 lysate on both GC and AC sGC activity. sGC produced endogenously in PC12 cells synthesizes cGMP in response to NO but not cAMP (Table 2). This discrepancy between AC activity in purified protein and PC12 cells led us to question if an endogenously produced small molecule or protein could be influencing sGC's ability to discriminate between ATP and GTP *in vivo*. To test this proposal, we assayed the AC and GC activity of purified sGC in the presence and absence of PC12 lysate. Interestingly, we observed the selective inhibition of NO-stimulated AC activity in the presence of PC12 lysate. It is unlikely that endogenous ATP or GTP in PC12 lysate could account for this inhibition as sGC maintains partial NO-stimulated activity even with saturating nucleotide (Figures 3 and 4). Additionally, we observed that salt

(50 mM NaCl) selectively inhibits sGC AC activity (data not shown). While the degree of sGC inhibition induced by the presence of salt is not enough to account for the effect observed in PC12 lysate, it lends support to the proposal that other small molecules may also influence nucleotide specificity. These intriguing data resolve a discrepancy in the literature between the absence of adenylate cyclase activity of sGC observed in COS-7 cells overexpressing sGC (*28*) and the activity observed with purified protein (*25*).

Here, we report the effects of GTP and ATP binding to both the catalytic site and the allosteric site *in vitro*. These nucleotides function as substrates as well as allosteric modulators that regulate the activity of the purified protein (Figure 6). However, it is apparent that additional factors are involved in regulating sGC activity *in vivo*. Additionally, this work localizes the allosteric nucleotide binding site to the C-terminus of sGC and strongly supports the existence of the proposed pseudosymmetric site. Clearly mapping the sites at which GTP and ATP bind to sGC or $\alpha 1_{cat}\beta 1_{cat}$ is essential to confirming if this proposed pseudosymmetirc site is important for regulating cGMP synthesis.

ACKNOWLEDGMENT

We thank Dr. Jacquin Niles, Dr. Jonathan Winger, and Joshua Woodward for helpful discussions. Additionally, we thank Dr. Jonathan Winger for providing $\alpha 1_{cat}\beta 1_{cat}$ for preliminary experiments.

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