Soluble guanylate cyclase is a heterodimeric hemoprotein composed of α- and β-subunits with a homologous motif to the nucleotide-binding sites of adenylate cyclases. Homology modeling of guanylate cyclase, based on the crystal structure of adenylate cyclase, reveals a single GTP-binding site and a putative second site pseudosymmetric to the GTP-binding site. However, the role of this pseudosymmetric site has remained unclear. Using equilibrium dialysis, we identified two nucleotide-binding sites with high and low affinity for ATP. The function of the low affinity site was examined solely to the high affinity site, indicating the role of this site as the catalytic site. The function of the low affinity site was examined using allosteric activators YC-1 and BAY 41-2272. YC-1 significantly reduced the affinity of 2′-dADP, probably by competing for the same site as 2′-dADP. BAY 41-2272 totally inhibited the specific binding of one molecule of 2′-dADP as well as GMP-CPP. This suggests that the activators compete with these nucleotides for the low affinity site. Infrared and EPR analyses of the enzymic CO- and NO-hemes also supported the suggested role of the low affinity site as a target for the activators. Our results imply that the low affinity site is the pseudosymmetric site, which binds NO. Soluble guanylate cyclase is a well characterized NO receptor involved in cell-cell signal transduction pathways associated with neuronal communication and vasodilation (1–7). Mammalian sGC is a heterodimeric (αβ) hemoprotein (8–10) in which the β subunit binds a stoichiometric amount of heme via a weak His-iron bond (11–13). The binding of NO to the heme markedly stimulates the enzymatic production of cGMP (9, 14–16). The NO-mediated stimulation of enzyme activity occurs in two steps: a six-coordinate NO complex is initially formed in the reaction with NO, which is then converted to an active five-coordinate NO complex, resulting in rupture of the weak His-iron bond (17, 18).

Guanylate cyclases, including soluble and particulate forms, have several functional and structural features common to those of adenylate cyclases. Both classes of enzymes catalyze the cyclization of chemically related compounds (i.e. GTP and ATP). Mammalian ACs contain two cytoplasmic catalytic domains, designated C1 and C2, that share 35% sequence identity (19–21). When mixed together, the isolated C1 and C2 domains form an active heterodimer, which is stimulated by forskolin, a specific activator of AC (22, 23). In contrast, neither the C2 nor the C1 homodimer display detectable enzyme activity. The crystal structure of the C1–C2 heterodimer reveals a shallow trough at the interfaccial region created by the head-to-tail association of the C1 and C2 domains (24). ATP-binding site and pseudosymmetric site are formed within the cleft of the interfaccial region: the former site where catalysis occurs and the latter site that binds forskolin. In contrast, the C2 homodimer contains two forskolin-binding sites within the cleft (25).

sGC consists of two similar subunits, α- and β-subunits, both of which contain a C-terminal catalytic domain with a nucleotide-binding motif homologous to that of the C1 and C2 domains of mammalian ACs (26). A modeling study, using the crystal structure of AC as a template, predicted that the association of the C-terminal regions of sGC creates a single GTP-binding site similar to that of AC, as well as a potential pseudosymmetric site at the interfaccial region (27). The GTP-binding site includes amino acid residues responsible for catalysis, whereas the pseudosymmetric site lacks several basic residues thought to be required for catalysis. By analogy with the forskolin-binding site of AC, one might predict that the pseudosymmetric site constitutes the binding site for YC-1 (3′-(5′-hydroxymethyl-3′-furyl)-1-benzylindazol), which is known to sensitize sGC to activation by NO and CO (28). Furthermore, Chang et al. (29) have shown that ATP analogue ATPS alters the cyclase activity presumably by acting at the pseudosymmetric site.

Unlike NO, YC-1 and BAY 41-2272 (3′-(4-amino-5-cyclopropypyrrolimidinyl-2′-furyl)-1′-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine) are non-heme-binding allosteric activator of sGC (28, 30). The NO-sensitizing action of YC-1 is due to the facilitational interactions of YC-1 and its analogues with the low affinity nucleotide-binding sites of sGC.

**Functional Characterization of Two Nucleotide-binding Sites in Soluble Guanylate Cyclase**

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Itated formation of an active five-coordinate NO-heme (31). Furthermore, the sensitizing action of YC-1 is explained by preventing the release of NO from the active NO-heme (32). Similarly, the CO sensitization by YC-1 and BAY 41-2272 is associated with the formation of five-coordinate CO-heme (31, 33).

In addition to these in vitro studies, the action of these allosteric activators has also been studied in vivo. Administration of BAY 41-2272 was found to initiate a physiological response in the NO-sGC signaling pathway (30, 34, 35). Although it has been reported that YC-1 occupied the same site as BAY 41-2272 (30), no direct experimental evidence for the location of this site has been obtained. For instance, several investigators proposed the N-terminal half of the α-subunit as a target for YC-1 (30, 36), whereas a recent mutational study supported the presence of a YC-1-binding site on the C-terminal part (37).

In the present study, we identified two nucleotide-binding sites/heterodimeric sGC by equilibrium dialysis. Until recently such kinds of experiments were not possible because of the limited quantity of purified protein. Functional analyses by kinetics together with infrared and EPR spectroscopic studies indicate that one of the two sites constitutes the substrate site responsible for catalysis. The other site was considered to be a pseudosymmetric site, which exclusively serves as the binding site for YC-1 or BAY 41-2272. Our model not only explains the data described in the present study but also consolidates the results from previous investigations.

EXPERIMENTAL PROCEDURES

Enzyme Purification—Fresh bovine lung (5 kg) was minced and homogenized using a Waring blender in 12 liters of 50 mM potassium phosphate buffer, pH 7.4, containing a mixture of protease inhibitors of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM EDTA, and 55 mM β-mercaptoethanol. Protease inhibitors and β-mercaptoethanol were included in all the buffers throughout the purification unless stated otherwise. The successive purification steps of the enzyme were the same as those described earlier (31). The purified enzyme preparations were stored in liquid nitrogen until use.

Spectral Measurements—Optical absorption spectra were recorded on a PerkinElmer Life Sciences Lambda 18 spectrophotometer. The temperature of the cuvette holder was controlled by thermomodule elements. The buffer used was 40 mM Hepes, pH 7.5, containing 50 mM NaCl and 5% (v/v) glycerol. EPR spectra were measured on a Varian E-12 X-band EPR spectrometer (Varian, Palo Alto, CA) with 100-kHz field modulation at 77 K, as described previously (17). The infrared spectra were measured at 27 °C on a PerkinElmer Life Sciences Spectrum One Fourier transform infrared spectrophotometer fitted with a mercury-cadmium-telluride detector. The cell had CaF₂ windows with a light path length of 0.1 mm.

Equilibrium Dialysis—The buffer used for equilibrium dialysis was 50 mM Hepes, pH 7.5, containing 50 mM NaCl and 5% (v/v) ethylene glycol supplemented with 4% (v/v) DMF to maintain the desired concentration of the poorly soluble YC-1. The concentration of DMF was increased to 7% (v/v) in experiments to test the effects of BAY 41-2272, because this compound had lower aqueous solubility than YC-1. The additions of 5% ethylene glycol and 4% or 7% DMF did not significantly affect the enzyme activity. A five-cell equilibrium dialyzer (Spectrum Laboratories, Rancho Dominguez, CA) was used for dialysis. Dialysis half-cells (250 μl) were separated by dialysis membrane with a cut-off of 14 kDa. One half-cell was filled with the ferrous enzyme, and the opposite half-cell contained the desired amount of nucleotides and/or effectors, YC-1 and BAY 41-2272. Other details were given in the appropriate figure legends. The dialysis chamber was shaken at a constant rate of 94 strokes/min at 4 °C or 69 strokes/min at 25 °C. The reaction achieved equilibrium within 12 and 6 h at 4 and 25 °C, respectively. After dialysis for 16 h at 4 °C and 7–8 h at 25 °C, nucleotides in each half-cell were quantified by HPLC analysis using a C18 or an anion exchange column at a constant flow rate of 1.0 ml/min. An aliquot of the sample was used for spectrophotometric determination of the enzymic heme concentration. The amount of enzyme-bound nucleotide was estimated from the difference in the concentration between the two half-cells, with and without enzyme.

Equilibrium binding of nucleotides with ferrous sGC was analyzed by saturation binding and Scatchard plots (38). Dissociation constant (Kd) and the maximum number of the binding sites (Bmax) for the binding of nucleotides were determined by nonlinear regression analyses of saturation binding plots and by linear or nonlinear analysis of Scatchard plots. In Scatchard plot analyses, the B/(nucleotide)free was plotted on the ordinate against fractional saturation (B = (nucleotide)bound/(sGC)total) on the abscissa. The plot yields a straight line with a slope of −1/Kd and an intercept on the horizontal axis that gives the maximum number of bound nucleotide (Bmax). The Kd and Bmax were determined by KELL or GraphPad Prism.

Kinetic Measurements—Inhibition of guanylate cyclase activity by ATP analogues was kinetically analyzed in the presence of a desired amount of these analogues. The buffer used was 50 mM Hepes, pH 7.5, containing 5% (v/v) glycerol, 5 mM Mn2⁺, 5 mM DTT, and 50 mM NaCl. The reaction mixture (150 μl) included 0.014 μM sGC. The reaction was started by the addition of 5 μl of S-nitroso-N-acetyl-D,L-penicillamine (4 mM) and was incubated for 10 min at 27 °C. After terminating the reaction by the addition of 5 μl of 30% (v/v) acetic acid, the concentration of cGMP was determined by HPLC analysis as described previously (17). The initial velocity was linear with time for 15 min irrespective of the concentration of GTP. All of the experimental points were averages of three independent assays.

Reagents—GMP-CPP was purchased from Jena Bioscience GmbH (Jena, Germany) and used without further purification. All of the other nucleotides were purchased from Sigma-Aldrich Japan (Tokyo, Japan). We further purified these nucleotides on a preparative C18 HPLC column to avoid ambiguity caused by impurities included in the commercially available preparations. The final purity of each nucleotide was >95%. YC-1 and BAY 41-2272 were purchased from ALEXIS (San Diego, CA). Other chemicals, purchased from Wako Chemicals Co. (Tokyo, Japan), were of the highest commercial grade.

RESULTS

Stoichiometry of GMP-CPP Binding—The stability of several GTP analogues during dialysis was checked. Of nucleotides
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FIGURE 1. Saturation plot of equilibrium binding between GMP-CPP and sGC. The equilibrium dialysis was performed as described under “Experimental Procedures.” Ferrous sGC (14.1–16.9 μM) was placed in one half-cell of the dialyzer. GMP-CPP (25–500 μM) was placed either in the same half-cell or in the opposite half-cell. The dialysis chamber was shaken for 16 h at 4 °C. The dissociation constants were obtained as described under “Experimental Procedures.” The buffer used was 40 mM Hepes, pH 7.5, containing 5% (v/v) ethylene glycol, 4% (v/v) DMSO, 2.65 mM DTT, and 50 mM NaCl, supplemented with 3.5 mM of MnCl₂, CaCl₂, or MgCl₂. The best-fit curves obtained by nonlinear regression analyses in the presence of Mn²⁺ (○), Ca²⁺ (▲), or Mg²⁺ (■) were illustrated as solid lines. In the inset, data obtained in the presence of Mn²⁺ were plotted on Scatchard coordinate (●), and inhibition of the GMP-CPP binding by 144 μM AMP-PNP was demonstrated by the Scatchard plot (▲). In the latter experiment, AMP-PNP was included in both half-cells of the dialysis chamber.

tested, only GMP-CPP was stable, but the other analogues, including guanosine-5′-β,γ-imido triphosphate and dGTP, were metabolized to cGMP during dialysis. Other nucleotides including 2′-deoxy-3′-GMP (uncompetitive inhibitor) and cGMP exhibited no detectable affinity for the ferrous enzyme even in the presence of an excess amount of pyrophosphate.

Saturation plots for GMP-CPP binding in the presence of metal cofactors such as Mn²⁺ are illustrated in Fig. 1. The binding of Mn²⁺–GMP-CPP is not univalent. Nonlinear regression analysis, based on the two-site model without constraints, indicates the presence of two classes of binding sites; there are a high affinity site with a Ka = 5.5 μM and a Bmax = 0.90, and a low affinity site with a Keq = 188 μM and a Bmax = 1.12. A Scatchard plot for the binding of Mn²⁺–GMP-CPP becomes curved at elevated concentrations of the nucleotide, representing the presence of the high and low affinity sites for the binding of this nucleotide (Fig. 1, inset). Ca²⁺–GMP-CPP also occupied both sites, but the saturation curve appeared to be univalent (Fig. 1). Although the result means that the affinity of the high affinity site was reduced to a level comparable with that of the low affinity site when Ca²⁺ was included, we were unable to estimate the minor difference by nonlinear curve fitting based on the two-site model. Accordingly, the Ca²⁺–GMP-CPP binding was analyzed by the one-site model. The result yielded the Bmax = 2.07 and Kd = 109 μM (Fig. 1). However, Mg²⁺–GMP-CPP gave no reliable data because of the extremely low affinity: Kd > 400 μM (Fig. 1). For this reason, we have selected Mn²⁺ and Ca²⁺ as metal cofactors in the present analyses, although both metals may not be physiologically important.

The presence of two classes of binding sites is not due to degradation of the enzyme during dialysis, because the subunit structure and the optical absorption spectrum of the enzyme are significantly unaffected before and after dialysis (supplemental Fig. S1). Moreover, 75–90% of the enzyme activity was recovered after dialysis. A non-univalent saturation as shown in Fig. 1 generally represents either two independent classes of binding site, with high and low affinities for the ligand, or a negatively cooperative interaction between binding sites. The latter possibility is less likely because 2′-dADP binds independently to the two sites as described below. Therefore we propose that the enzyme has both high and low affinity binding sites for the nucleotide. Hereafter, we designate the two binding sites as the “high affinity site” and the “low affinity site” accordingly. The presence of nonequivalent binding site was not limited to nucleotide with guanine base, because binding of AMP-CPP displayed high and low affinity sites (Fig. 2A).

To examine the function of the individual nucleotide-binding sites, we searched for nucleotides that selectively bind to either the high or the low affinity site. Of the nucleotides tested, 2′-dADP inhibited the binding of GMP-CPP, i.e. the addition of 0.5 mM 2′-dADP reduced the amount of the enzyme-bound GMP-CPP from 23.5 to 5.8 μM at 4 °C (data not shown). This result suggests that 2′-dADP occupies the same sites as GMP-CPP or possibly adjacent sites that overlap with those for GMP-CPP. Although 2′-dADP binds unselectively to both sites, it is nevertheless useful for analyzing the function of individual binding sites (see below). By contrast, AMP-PNP reduced the Bmax for the binding of GMP-CPP to 0.97 from 2.02 at 4 °C (▲ in inset of Fig. 1), suggesting that AMP-PNP is tightly and selectively associated with the high affinity site.

Binding of AMP-PNP and 2′-dADP—In contrast to the binding of GMP-CPP and AMP-CPP (Figs. 1 and 2A), AMP-PNP occupies solely a single binding site with high affinity both in the presence of Mn²⁺ and Ca²⁺ (Fig. 2B). This result together with findings described above (Fig. 1, inset, ▲) suggests that AMP-PNP binds exclusively to the high affinity site. This site would not be a target for YC-1, because the high affinity site constitutes catalytic site as described below. As expected, YC-1 has no significant effect on the AMP-PNP binding, suggesting that the binding site of YC-1 is distinct from the high affinity site (Fig. 2C).

It seems likely that the high affinity site participates in catalysis. Indeed, we confirmed that the high affinity site serves as the catalytic site by kinetic analyses. The initial velocity of the cyclase reaction in the presence of various amounts of AMP-PNP was plotted against the concentration of GTP. Analysis by Lineweaver-Burk plot shows that AMP-PNP displays competitive inhibition with a single intersection point on the ordinate, indicating that GTP and AMP-PNP occupy the same site (Fig. 3A). A secondary plot, in which the slopes were plotted versus the concentration of AMP-PNP, was linear (Fig. 3A, inset). The analysis of data obtained by the secondary plot gave inhibition constants (Kf) of 15.0 μM at 27 °C (Fig. 3A, inset) and...
5.1 μM at 10 °C (data not shown). The $K_i$ at 10 °C essentially agreed with the $K_d$ for AMP-PNP binding under comparable conditions (1.5 μM at 4 °C; Fig. 2B). Taking these results into consideration, we propose that the high affinity site corresponds to the catalytic site of the enzyme.

The Lineweaver-Burk plot for inhibition by 2'-dADP gave the intersection point above the abscissa and to the left of the ordinate (Fig. 3B). This inhibition pattern agrees with that of a mixed type inhibition, a mixture of competitive and noncompetitive inhibition (39). Thus, binding of 2'-dADP to the substrate site excludes the substrate (i.e., GTP), whereas binding to the noncompetitive site has no effect on GTP binding, although the resulting complex is catalytically inactive. The secondary plot that is concave upwards with increasing concentrations of 2'-dADP (Fig. 3B, inset) suggests that 2'-dADP binds to at least two sites (39).

Saturation plots of the 2'-dADP binding determined in the presence of Mn$^{2+}$ at 4 and 25 °C and of Ca$^{2+}$ at 4 °C were univalent with $B_{\text{max}}$ and $K_d$ (Fig. 4). These results represent the two binding sites of 2'-dADP with equivalent affinities irrespective of the metal used as a cofactor. The $K_d$ of 38.4 μM in the presence of Mn$^{2+}$ at 25 °C essentially agreed with $K_i$ determined kinetically under comparable conditions (∼30 μM at 27 °C), as shown in the inset of Fig. 3B. Taken together, these findings indicate that 2'-dADP binds to the high and low affinity sites with equivalent affinities and also suggest that the noncompetitive inhibitory site corresponds to the low affinity site.

Effect of YC-1 on the Binding of 2'-dADP—In contrast to the effect on binding of AMP-PNP (Fig. 2C), YC-1 impairs the affinity of 2'-dADP without changing the $B_{\text{max}}$ for 2'-dADP (i.e., $B_{\text{max}} = 1.95$) at 25 °C (Fig. 4B). Such a mode of inhibition implies that YC-1 competes for the same sites as 2'-dADP.
FIGURE 4. Equilibrium binding between 2'-dADP and ferrous sGC. A, bindings were measured in the presence of 3.5 mM Mn^{2+} (○) or Ca^{2+} (●). The ferrous sGC (12.3–18.0 μM) in one half-cell was incubated with 15–500 μM 2'-dADP in the opposite half-cell for 16 h at 4 °C. B, the ferrous sGC (14.2–18.4 μM) in one half-cell was incubated with 20–550 μM 2'-dADP with (●) or without (○) 250 μM YC-1 in the presence of 3.5 mM Mn^{2+} for 7 h at 25 °C. YC-1 was added to both half-cells. The B_max and K_d values are given in the figure. The buffer used was 40 mM Hepes, pH 7.5, containing 5% (v/v) ethylene glycol, 4% (v/v) DMF, 6.25 mM DTT, and 50 mM NaCl.

FIGURE 5. Effects of 2'-dADP, AMP-PNP, and BAY 41-2272 on the YC-1 binding. A, the ferrous sGC (15.1–18.5 μM) in one half-cell was incubated with 25–250 μM YC-1 in the opposite half-cell, in the presence of 3.5 mM Mn^{2+} for 8 h at 25 °C. B, sGC with 100 μM YC-1 in one half-cell was incubated with indicated amounts of 2'-dADP or AMP-PNP in the opposite half-cell for 8 h at 25 °C. For the effects of BAY 41-2272, sGC in one half-cell was incubated with 200 μM YC-1 in the opposite half-cell. In this experiment, both half-cells included 50 μM BAY 41-2272. The concentration of the enzyme used was 16.8 μM for AMP-PNP inhibition, 15.6 μM for 2'-dADP inhibition, and 13.2 μM for BAY 41-2272 inhibition. The buffer used in the experiments to assess the effects of 2'-dADP and AMP-PNP was the same as that described in the legend to Fig. 1. For the experiment in the presence of BAY 41-2272, the concentration of DMF included in the buffer was increased to 7% (v/v). The determination of YC-1 was performed according to the method described previously (31).

is assumed that YC-1 binds solely to the low affinity site, the resultant saturation curve should be a biphasic curve consisted of high and low affinity components. However, we were unable to experimentally obtain a biphasic curve, because YC-1 reduces the binding affinity of 2'-dADP by only about 1.5-fold (Fig. 4B). We thus tested whether 2'-dADP was able to inhibit the binding of YC-1.

We have previously reported the presence of a single binding site for YC-1 with K_d = 124 μM at 27 °C in the absence of metal cofactor (31). In the present work, we examined the effect of metal on the binding of YC-1. As shown in Fig. 5A, 1.18 molecule of YC-1 was found to bind to the ferrous sGC with a K_d of 51.9 μM in the presence of Mn^{2+} at 25 °C. This indicated that the addition of Mn^{2+} increased the affinity of YC-1 by about 2.5-fold without changing the B_max. No significant effects of AMP-PNP on the YC-1 binding (Fig. 5B, left columns) confirm again that the binding site of YC-1 is distinct from the high affinity site. The addition of 2'-dADP inhibited the binding of YC-1, but the inhibitory effect was incomplete even in the presence of an excess amount of 2'-dADP (Fig. 5B). Although partial inhibition by 2'-dADP renders likely the possibility of YC-1 binding to the low affinity site, this result is inadequate to conclude the binding of YC-1 to the low affinity site. To solve the issue, we have used BAY 41-2272 instead of YC-1, because BAY 41-2272 has been reported to tightly bind to sGC and to occupy the same site as YC-1 (30, 33). Indeed, BAY 41-2272 effectively prevented the binding of YC-1 to sGC (Fig. 5B), confirming a previous report (30).

Effect of BAY 41-2272 on the Binding of GMP-CPP and 2'-dADP—In Fig. 6, the binding of GMP-CPP or 2'-dADP is compared in the presence and the absence of BAY 41-2272. BAY 41-2272 apparently abolished the low affinity component of the GMP-CPP binding, as indicated by a decrease in the B_max to 1.08 (Fig. 6A). The affinity for the binding of GMP-CPP to one site in the presence of BAY 41-2272 essentially agreed with that for the high affinity site (8.2 versus 5.5 μM; compare Fig. 6A with Fig. 1). Likewise, the binding of BAY 41-2272 prevented 2'-dADP from binding to either of the two sites (Fig. 6B). The K_d (44.4 μM) of 2'-dADP for a single site is comparable with that for 2'-dADP binding in the absence of BAY 41-2272 (38.4 μM) (Fig. 4B). These results are consistent with a notion that the low affinity site is a target for BAY 41-2272.

We have tried but failed to obtain a reliable binding constant for BAY 41-2272 by equilibrium dialysis because of high affinity. A kinetic analysis revealed that one molecule of BAY 41-2272 was required for maximal enzyme activation and tightly associated with sGC (supplemental Fig. S2).
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**FIGURE 6.** Effects of BAY 41-2272 on the binding of GMP-CPP and 2′-dADP. A, the ferrous sGC (12.0–15.9 μM) in one half-cell was incubated with 20–460 μM GMP-CPP in the opposite half-cell in the presence of 3.5 mM Mn²⁺ for 16.5 h at 4 °C. B, sGC (13.5–18.4 μM) in one half-cell was incubated with 24–600 μM 2′-dADP in the opposite half-cell in the presence of 3.5 mM Mn²⁺ for 8 h at 25 °C. In these experiments, both half-cells included 40 μM BAY 41-2272. The dotted lines denote the best fit curves analyzed by nonlinear regression for the binding of GMP-CPP and 2′-dADP in the absence of BAY 41-2272 that were shown in Figs. 1 and 4, respectively. The buffer used was 40 mM Hepes, pH 7.5, containing 5% (v/v) DMF, 5% (v/v) ethylene glycol, 7% (v/v) DTT, and 6.25 mM DTT.

**FIGURE 7.** Fourier transform infrared spectra of the CO complex of sGC in the presence of YC-1, BAY 41-2272 and nucleotide. Fourier transform infrared spectra (traces a–g) were measured at 27 °C using F2Ca cell with 2 cm⁻¹ resolution. Trace a, the CO complex of sGC. Trace b, the CO complex in the presence of 330 μM YC-1 and 3 mM Ca²⁺. Trace c, in the presence of 330 μM YC-1, 1 mM AMP-PNP and 3 mM Ca²⁺. Trace d, in the presence of 1 mM AMP-PNP and 3 mM Ca²⁺. Trace e, in the presence of 330 μM YC-1 and 5 mM Mn²⁺. Trace f, in the presence of 330 μM YC-1, 1.8 mM 2′-dADP and 5 mM Mn²⁺. Trace g, in the presence of 1.8 mM 2′-dADP and 5 mM Mn²⁺. In these experiments, the concentration of sGC was 158 μM as heme. In trace h, sGC-CO complex of 174 μM was reacted with 145 μM BAY 41-2272 at 25 °C, and the spectra were collected at 2 cm⁻¹ resolution. The buffer was 50 mM Hepes, pH 7.5, containing 5% (v/v) DMF, 5% (v/v) ethylene glycol, 2.5 mM DTT, and 50 mM NaCl.

**YC-1 Binding Analyses by Infrared C-O Stretch Measurements**—We have found that the C-O stretch at 1987 cm⁻¹ down-shifted to 1972 cm⁻¹ with producing a shoulder at 1965 cm⁻¹ when YC-1 bound to the CO complex of sGC (31). Such a YC-1-induced change does not depend on the metal ion used as a cofactor (compare trace b with trace e in Fig. 7). The species with 1972- and 1987-cm⁻¹ bands had been assigned to the six-coordinate CO-heme with and without YC-1, respectively. The species with the 1965-cm⁻¹ band was identified as the five-coordinate CO-heme (31). Being consistent with a recent report (33), BAY 41-2272 produced nearly identical CO-heme species with YC-1, in which 1970.6- and 1965-cm⁻¹ bands were assigned as six- and five-coordinate CO-heme species, respectively (Fig. 7, trace h). The result indicates that BAY 41-2272 yields nearly identical CO-heme species with YC-1, although the spectral pattern differs because of enhanced production of the five-coordinate CO-heme in the presence of BAY 41-2272.

The YC-1-dependent changes to the infrared absorption pattern were helpful to analyze the YC-1-binding site as follows. The C-O stretching mode of the YC-1-bound form slightly changed by a successive addition of AMP-PNP (Fig. 7, trace e). The minor change implies that the AMP-PNP binding may increase slightly the affinity of YC-1, but the exact reasons remain unclear. We confirmed that the addition of AMP-PNP alone did not produce the 1972-cm⁻¹ C-O stretch (Fig. 7, trace d), and this spectrum essentially agreed with that in the absence of effectors (Fig. 7, trace a). In contrast, the addition of an excess amount of 2′-dADP in the presence of YC-1 markedly diminished the intensity of the 1972-cm⁻¹ band, which was accompanied by a concomitant increase in the intensity of the 1987-cm⁻¹ band (Fig. 7, trace f). The addition of 2′-dADP alone (Fig. 7, trace g) did not generate the 1972-cm⁻¹ band, as observed for the addition of AMP-PNP alone (Fig. 7, trace d). The mutual exclusion between YC-1 and 2′-dADP is consistent with our model that YC-1 exclusively binds to the low affinity site.

**EPR Analyses of YC-1 and Nucleotide Effects on NO-Heme**—The binding of YC-1 to the NO complex of sGC has been shown to cause a geometrical distortion of the five-coordinate NO-heme, which is characterized by the formation of a rhombic EPR signal (31). In the present study, the effect of nucleotide on
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FIGURE 8. EPR spectra of the $^{14}$N$^{16}$O complex of sGC. Trace $a$, EPR spectrum of the $^{14}$N$^{16}$O complex of sGC. Trace $b$, in the presence of 0.7 mM 2'-dADP. Trace $c$, in the presence of 0.7 mM 2'-dADP and 300 μM YC-1. Trace $d$, in the presence of 0.19 mM AMP-PNP. Trace $e$, in the presence of 0.19 mM AMP-PNP and 300 μM YC-1. Trace $f$, in the presence of 300 μM YC-1. In all of the experiments, the mixtures contained 3.3 mM Ca$^{2+}$. The buffer used was 40 mM triethanolamine-HCl, pH 7.5, containing 50 mM NaCl, 4.5 mM DTT, 4% (v/v) DMF, and 5% (v/v) ethylene glycol. EPR spectra were obtained at 77 K and 100 kHz field modulation with 0.5 millitesla width. The microwave power was 5 milliwatt, and 10 scans were averaged. The enzyme concentration was 32 μM as heme.

the EPR signal of the enzymic NO-heme was examined using Ca$^{2+}$ instead of Mn$^{2+}$ as a cofactor to avoid overlapping with the EPR signals of Mn$^{2+}$. The five-coordinate enzymic NO-heme exhibited an axial symmetric EPR signal characterized with $g_{z} = 2.068$ and $g_{x} = 2.009$ (Fig. 8, trace $a$), whereas the binding of YC-1 converted the axial EPR signal to a rhombic symmetric signal with $g_{x} = 2.103$, $g_{y} = 2.033$, and $g_{z} = 2.009$ (Fig. 8, trace $f$). Ca$^{2+}$-dADP or Ca$^{2+}$-AMP-PNP did not induce this conversion (Fig. 8, traces $b$ and $d$). Similarly, neither Mg$^{2+}$-dADP nor Mg$^{2+}$-AMP-PNP cause the conversion (data not shown). Hence the conversion is YC-1-specific and can be used as an indicator for the formation of the YC-1-bound form.

The addition of YC-1 to the 2'-dADP-bound enzyme produced only a small amount of YC-1 complex, as indicated by a slight decrease in signal intensity at $g_{z} = 2.068$ (Fig. 8, trace $c$). However, the addition of YC-1 fully converted the AMP-PNP-bound NO enzyme to a NO-heme species with a rhombic EPR signal (Fig. 8, compare trace $d$ with trace $e$). These results imply that YC-1 barely associates with the 2'-dADP-enzyme complex but readily binds to the AMP-PNP-enzyme complex even in the presence of a large excess of AMP-PNP (about 35-fold excess over the $K_{d}$ value at 4°C). These EPR findings imply that 2'-dADP inhibits the binding of YC-1 at the low affinity site.

DISCUSSION

Although current biochemical investigations of sGC provide precise information concerning the activation of the NO receptor by NO, most of these studies have focused on the reaction of the heme group with NO (12, 13, 17, 18, 40). However, the role of the catalytic domains in the activation of the enzyme by NO should not be ignored. Recent reports point out that the activation of the enzyme by NO requires the existence of nucleotide (41, 42). We therefore examined the function of the nucleotide-binding sites by equilibrium dialysis. The results described herein strongly suggest that the high affinity site serves as catalytic site, and the low affinity site is a target for YC-1 and BAY 41-2272 and also is a noncompetitive inhibitory site for 2'-dADP. BAY 41-2272, which shared an analogous core structure to YC-1, fully inhibited 2'-dADP binding to the low affinity site, whereas the inhibition by YC-1 was incomplete. The difference could be because of the bulkiness of substituent introduced to the core structure, although we have no direct evidence.

Based on the homology modeling of sGC, the GTP-binding site contains two Asp residues (αAsp$^{985}$ and αAsp$^{929}$) that are critical for metal coordination and hence for binding the phosphate moiety of the nucleotide (27, 29). The pseudosymmetrical site contains a Gly residue at the position corresponding to αAsp$^{985}$ within the GTP-binding site (27, 29). Therefore, it is feasible that the replacement of the Asp residue by a Gly results in destabilization of the interaction between the phosphate moieties of nucleotide. The consideration allows us to deduce that the pseudosymmetrical site serves as nucleotide-binding site only in the higher concentration range. Such property of the pseudosymmetrical site is compatible with that of the low affinity site. The following findings by Chang et al. (29) further support the hypothesis.

Despite low affinity for nucleotide, Chang et al. (29) pointed out the significance of the pseudosymmetrical site for the regulation of sGC activity. The binding of noncompetitive inhibitor ATPγS to a noncompetitive site allosterically altered the inhibitory pattern of NO-sGC activity, in which ATPγS induced the positively cooperative inhibitory phase at high concentrations (submillimolar). This inhibitory mechanism essentially agrees with that of 2'-dADP as described in this study, although the mechanism appears to differ because of the contribution of cooperative interaction between the catalytic site and the noncompetitive site. This positively cooperative inhibitory effect may result from binding of ATPγS to the pseudosymmetrical site, because the positive cooperativity imposed by ATPγS was lost by mutation of a single residue (βAsp$^{977}$) within the pseudosymmetrical site. This result, together with our present findings, strongly suggests that the low affinity site is the pseudosymmetrical site that acts as a regulatory site.

More significant is the finding that the pseudosymmetrical site appears to be an ATP sensor site of sGC. Ruiz-Stewart et al. (43) proposed an attractive model in which the ATP sensor site acts as regulatory site for the cyclase activity in response to an elevated concentration of ATP (i.e. relative to the intracellular [ATP]) of ~3
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mm). Similar kinetic results have since been reported (44). As described, we also found that an ATP analogue, AMP-CPP has affinity for the low affinity site (Fig. 2A). Intriguingly, the characteristics of this sensor site resemble those of the pseudosymmetric site described in this paper, particularly in terms of the unexpectedly low affinity for nucleotide.

The YC-1-dependent conversion of the enzymic NO-heme, with an axial EPR signal, to a rhombic EPR signal was a clear indication for reciprocal communication of signal imposed on the C-terminal domain to the heme-binding domain (Fig. 8). Similar conversion of EPR signal has been also noted in the presence of Ca²⁺-GTP (31) and Ca²⁺-GMP-CPP (data not shown). Therefore, the binding of GTP analogue or YC-1 to the pseudosymmetric site appears to be required for the reciprocal communication. Russwurm and Koesling (41) emphasized the significance of the reciprocal communication in the stimulation of the enzymic heme species. Cary et al. also reported the role of GTP and ATP for the formation of the active NO-heme enzyme (42). Our observations imply that the NO-heme species with a rhombic EPR signal is a possible candidate for the active NO-heme species, but further biophysical studies are required to verify the identity of the active NO-heme species.

In conclusion, we have used a variety of techniques to survey the function of the low affinity site of sGC. Our present results strongly suggest that this site is the pseudosymmetric site and constitutes the binding site for allosteric activators. If a compound with YC-1-like sensitizing properties for sGC is in physiological conditions, our results suggest that the endogenous compound is likely to be related to a guanine or an adenosine nucleotide.

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