Soluble guanylyl/guanylate cyclase (sGC), the primary biological receptor for nitric oxide, is required for proper development and health in all animals. We have expressed heterodimeric full-length and N-terminal fragments of Manduca sexta sGC in Escherichia coli, the first time this has been accomplished for any sGC, and have performed the first functional analyses of an insect sGC. Manduca sGC behaves much like its mammalian counterparts, displaying a 170-fold stimulation by NO and sensitivity to compound YC-1. YC-1 reduces the NO and CO off-counterparts, displaying a 170-fold stimulation by NO and sensitivity to nucleotides, YC-1, and changes in NO concentration up to 20968

The primary physiological role of sGC is required for proper development of insect cell expression systems for mammalian enzyme, but, as in mammals, insect sGC plays an important physiological role. In the Manduca sexta larva (tobacco hornworm), sGC is implicated in antennal lobe morphogenesis (31–33), and in the Manduca adult (hawkmoth), sGC is a key component for olfactory processing of odors (34–37). The development of insect cell expression systems for mammalian sGC proteins has facilitated mechanistic studies (38–40), but obtaining functional material in sufficient quantity remains a major difficulty in the field. Here we have developed Escherichia coli expression systems for both the full-length Manduca sGC heterodimer (msGC) and for two heterodimeric, N-terminal truncations (msGC-NT1 and msGC-NT2). Our analysis
provides the first mechanistic results for an insect-derived sGC. We demonstrate that Manduca sGC behaves much like its mammalian counterparts and that binding of YC-1 occurs within the N-terminal portion of the protein.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pCR®2.1-TOPO (Invitrogen) was used for cloning PCR products into the expression vector pETDuet1 (Novagen, Milwaukee, WI). PCR primers were obtained from Midland Certified Reagent Co. (Midland, TX). *E. coli* strain DH5α was the cloning host and *E. coli* strains BL21(DE3) pLysS and Rosetta(DE3) pLysS (Novagen) the expression hosts. 2-[(N,N-Diethylamino)-diazenolate-2-oxide (DEA/NO) was the kind gift of Dr. Katrina Miranda. YC-1 was obtained from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals were obtained in Sigma unless otherwise described.

Expression of Recombinant Manduca sGC in *E. coli*—Constructs for expression of full-length and truncated msGC α1/β1 (msGC-NT) in *E. coli* were prepared using the previously described clones obtained from a cDNA library of the Manduca prepupal abdominal nervous system (35). For the truncated constructs, DNA fragments corresponding to msGC α1 residues 1–471 (for msGC-NT1) or 49–471 (for msGC-NT2) and msGC β1 residues 1–400 (for both msGC-NT1 and -NT2) were cloned into vector pETDuet1, allowing for expression of both subunits from a single plasmid. The appropriate msGC α1 fragments were obtained from vector pET-28b-Mana1 by PCR amplification using primers 5′-gatccgctgatcagctttc-3′ (for msGC-NT1) or 5′-gatccggatccctcttttagtttt-3′ (for msGC-NT2) and 5′-ttctcagcagttaccagcttcc-3′. For msGC α1, the appropriate fragment was amplified from pET-17b-Manb1 with primers 5′-catgtgtagcggctgtctgg-3′ and 5′-taacctggtgctcagcttccttggtcc-3′. For msGC β1, the appropriate fragment was amplified from pET-17b-Manb1 with primers 5′-ctctcagcagttaccagcttcc-3′ and 5′-taacctggtgctcagcttccttggtcc-3′. For msGC β1 gene, the β1 gene was cut from pET-17b-Manb1 and ligated into pETDuet-1 using the Ndel and EcoRV restriction sites. The final constructs have a His6 tag fused onto the N terminus of the α1 subunit. All pETDuet-1-derived plasmids were verified by sequencing and transformed into BL21 (DE3) pLysS or Rosetta(DE3) pLysS competent cells for expression.

Mutations in the H-NOX domain were introduced by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the msGC-NT2 plasmid as the template. The primers used to generate the α1 L211A mutant were 5′-gacgcttgctcactttggtagtttagcctgtgaag-3′ and 5′-ctttcagcagttaccagcttcc-3′. For the α1 T223A mutant, primers were 5′-gctgccagcacttgctcactttggtagtttagcctgtgaag-3′ and 5′-ggctgtgctgctctgtgctcactttggtagtttagcctgtgaag-3′. The protein solution (700 μl) was used to determine the hemin concentration, assuming an extinction coefficient of 32 mmol l⁻¹ cm⁻¹. NO and CO dissociation constants were estimated by their ratio to the unliganded Soret band.

**UV-visible Spectroscopy**—Spectra were recorded on a Cary Bio50 spectrophotometer at room temperature at a scan rate of up to 600 nm/min. In a typical NO binding experiment, a 1-ml sGC sample was deoxygenated in a septum-capped cuvette with an argon stream (30 min), while stirring with a stir bar, before DEA/NO was added using a gas-tight syringe. A DEA/NO stock solution was prepared fresh in 10 mM NaOH and quantified by its absorbance at 250 nm using the extinction coefficient ε₂₅₀ = 8000 M⁻¹ cm⁻¹, a value adjusted to account for incomplete release of NO (41). Complete degradation of DEA/NO was assumed to release two molecules of NO after 10 min. For CO binding, the sGC sample was purged with CO gas for 15–20 min before the spectrum was recorded.

**Heme Soret Extinction Coefficient**—Molar extinction coefficients were measured using the pyridine hemochromogen assay as described previously (42). The protein solution (700 μl) was mixed with 100% pyridine (300 μl), 5% KOH (20 μl), and crystals of dithionite. The peak absorbance at 556 nm minus that at 700 nm was used to determine the hemin concentration, assuming an extinction coefficient of 32 mmol l⁻¹ cm⁻¹. NO and CO complex Soret band and all Q-band extinction coefficients were estimated by their ratio to the unliganded Soret band.

**CO Dissociation Constants**—The msGC-NT samples were placed in a septum-capped cuvette with minimal head space at room temperature (22°C). Aliquots from CO-saturated protein buffer (50 mM KPO₄, pH 7.4, 100 mM KCl, and 5% glycerol), assumed to be 1 mM in CO, were added to the cuvette and stirred for 10 min, and the spectrum was measured. When present, nucleotide (0.5–1 mM) or YC-1 compound (50 μM) was added before CO binding. CO binding was measured by the shift in Soret band after accounting for dilution because of the
addition of CO. This shift was estimated as $A_{424} - A_{437}$ times the dilution factor, except in the presence of YC-1, where $A_{322}$ was used rather than $A_{424}$. Data were fitted to a single-site saturation ligand binding model using SigmaPlot (SPSS, Inc., Chicago). For the wild-type protein in the presence of YC-1, titration was also undertaken in a cuvette with a 10-cm path length, using an RSM-1000 spectrophotometer (OLIS, Inc., Bogart, GA), which yielded a value indistinguishable from that measured with the 1-cm cuvette.

Kinetics for Proximal Histidine Release—The rates for release of $\beta_1$ His-105 upon NO binding to msGC-NT were measured at 10 °C by mixing msGC-NT and NO in an RSM-1000 stopped-flow spectrophotometer (OLIS, Inc.). Samples of msGC-NT (0.7–2 $\mu$M) were prepared by first deoxygenating protein buffer through bubbling of argon gas for 10 min, followed by addition of protein and additional deoxygenation with an argon stream placed above the solution for ~30 min. The protein solution was then transferred to the instrument in a gas-tight syringe. NO solutions were prepared by addition of DEA/NO from a stock solution to argon-purged protein buffer in a gas-tight syringe and then connected to the stopped-flow device. Decomposition was allowed to proceed for 20 min at room temperature before transfer to the instrument, where the solution was allowed to equilibrate to the desired temperature (5 min). Absorbance changes ($A_{424}$) were fitted to single or double exponential equations using SigmaPlot; values reported are the average and standard deviation of 5–7 consecutive measurements. For experiments with nucleotides or YC-1, the compounds were pre-mixed with the protein sample.

Kinetics of NO Release—Rates for denitrosylation of msGC-NT-NO were estimated using a dithionite/CO trap, as described (7, 43). A slight excess of NO (from DEA/NO) was added to an anaerobic msGC-NT sample prepared as described above, and a spectrum was measured to ensure saturation. Nucleotide or YC-1 was pre-mixed with the protein sample before NO addition. The trapping solution was prepared by bubbling CO gas (10 min) into a freshly prepared dithionite solution ($\text{Na}_2\text{S}_2\text{O}_4$, 60 mM in protein buffer). Denitrosylation was initiated by mixing the trapping and protein solutions either in a stopped-flow device at 20 °C, or in a cuvette at room temperature (22 °C), and monitoring the change in absorbance ($A_{425} - A_{413}$ or $A_{424} - A_{412}$). Rate constants were obtained by fitting the relevant time interval to a single or double exponential equation, as appropriate. For the cuvette data, the first 100 s were discarded to remove the fast phase, which was better estimated in the stopped-flow experiment.

Kinetics of CO Binding and Release—CO binding rates were measured for a series of CO concentrations (0.05–0.5 mM) in a stopped-flow spectrophotometer by monitoring absorbance change ($A_{424} - A_{412}$) and fitting $k_{obs}$ versus [CO], which displayed the expected linear dependence for a monophasic process. Protein- and CO-containing solutions were prepared as described above. The second-order rate constants reported are for the slope and error from the linear fit.

CO release rates from msGC-NT-CO were measured by replacing released CO with excess NO upon rapid mixing in the stopped-flow device. One syringe contained msGC-NT-CO, and the second contained protein buffer saturated with NO from DEA/NO (~2 mM). All reactions were performed at 20 °C, and YC-1, where included, was pre-mixed with the protein sample.

Guanosyl Cyclase Enzymatic Assay—The cGMP producing activity of msGC in cell lysates and metal-affinity column elution fractions was measured using an enzyme immunoassay kit (Cayman Chemical Co.), following the manufacturer’s instructions. In a typical assay, 10 $\mu$L of reaction buffer (0.5 mM HEPEs, pH 7.5, 30 mM GTP, 60 mM MgCl$_2$, 20 mM dithiothreitol) was added to protein sample for a total reaction volume of 100 $\mu$L. The mixture was incubated at room temperature for 10 min and then quenched with 200 $\mu$L of 250 mM zinc acetate and 200 $\mu$L of 250 mM sodium carbonate. For experiments measuring NO-activated enzyme activity, protein samples were pre-mixed with DEA/NO before initiating catalysis by addition of reaction buffer.

Molecular Modeling of N-terminal msGC—Sequences of the $\alpha_1$ and $\beta_1$ subunits of msGC were submitted to the Robetta structure prediction server for domain analysis using Ginzu (44). Three domains were predicted for each subunit as follows: an N-terminal domain predicted to be an H-NOX domain (20–22) (pdb-blast confidence 30 and 36 for the $\alpha_1$ and $\beta_1$ subunits, respectively), a largely helical middle domain, and a C-terminal cyclase domain with homology to the catalytic domain of adenylate cyclase (pdb-blast confidence 52 and 47). A 25-residue sequence between the second and third domains in each subunit was identified as a possible coiled-coil region. The 50 N-terminal residues of the $\alpha_1$ subunit, which have no counterpart in the $\beta_1$ subunit, were predicted to be disordered. Ginzu did not predict the PAS domains.

After domain analysis, the sequence of the $\alpha_1$ N-terminal domain (minus the 50 N-terminal residues predicted to be disordered) was submitted to the 3D-Jury Meta Server for structure prediction and initial model building (45). A template structure file was not specified. C-α models and alignments of $\alpha_1$ H-NOX with known H-NOX structures (Protein Data Bank entries 1XBN (21) and 2009 (20)) were returned from several servers. All had very significant 3D-Jury scores (100–108). Full-atom models were built from these alignments using Modeiler (46). The resulting models were nearly identical, varying only in the placement of a short insertion near the vacant heme cavity. The final model was minimized using NAMD (47).

RESULTS

Expression and Purification of Heterodimeric N-terminal Fragments of Manduca sGC—Studies on NO signaling have been limited by difficulties in obtaining sufficient quantities of intact sGC. To overcome this shortcoming, we pursued functional domains of sGC from M. sexta. Sequence analyses and homology modeling (described below) suggested boundaries for the C-terminal cyclase domains and N-terminal H-NOX domains of both subunits. Two constructs were prepared, msGC-NT1 ($\alpha_{1-471}/\beta_{1-490}$), which lacks the cyclase domains and is expressed from a single plasmid, and msGC-NT2 ($\alpha_{49-471}/\beta_{1-490}$), which is identical to msGC-NT1 except that it also lacks the putatively disordered N-terminal region of the $\alpha_1$ subunit (Fig. 1A). Both constructs produced soluble heterodimeric protein in an E. coli expression system, and both...
displayed identical functional behaviors, suggesting the α1 subunit N-terminal His tag did not affect activity; however, msGC-NT2 was the more stable of the two. Co-expression of both subunits and addition of heme precursor δ-aminolevulinate was required to produce soluble recombinant protein; expression of individual subunits or expression without δ-aminolevulinate led only to insoluble inclusion bodies.

Purification using the α1 subunit N-terminal His tag was complicated by a tendency of the protein to lose heme. This tendency was exacerbated by the imidazole in the elution buffer, because imidazole can coordinate to heme and facilitate its removal. Heme loss has long been known to occur in sGC during isolation. To circumvent this difficulty, we developed a purification procedure where sGC was released from the nickel-affinity column by the addition of EDTA, which chelates the nickel and releases the protein from the column. Rapid purification over nickel-affinity and gel filtration columns yielded ~1 mg of >90% pure protein/liter of cell culture (Fig. 2A). The protein behaved as a heterodimer by size-exclusion chromatography and typically displayed an $A_{433}/A_{280}$ ratio of 1.2. Best stability for the protein was achieved under anaerobic conditions and bound to CO, where little change was observed over several days. Soret band degradation is seen with time in the presence of the reductant dithiothreitol but not in the presence of tris(2-carboxyethyl)phosphine.

**Truncated Manduca sGC Displays Typical Electronic Spectra**—Truncated *Manduca* sGC heterodimers display Soret and Q-band absorption maxima that are typical for sGC proteins (Fig. 2B). The isolated protein has a broad Soret maximum centered at 433 nm, consistent with a high spin ferrous heme center. Binding of CO, which requires ferrous heme, shifts the Soret maximum to 425 nm and sharpens both the Soret and Q-bands, consistent with a low spin six-coordinate heme. The high purity of the protein allowed us to determine molar extinction coefficients for these bands, using the pyridine hemochromogen assay (Table 1). The resulting values were in good agreement with those reported for globins, nitrophorins, and full-length and β1 truncated mammalian sGC proteins (42, 48).

**YC-1 Binding Increases the CO Affinity of msGC-NT**—Small molecule effectors of sGC catalytic activity, including ATP, GTP, and the compound YC-1, have been described, but the binding site(s) for these molecules and their mechanisms of action remain unclear. The interface of the α1/β1 catalytic domains in sGC contains both a catalytic site and a second pseudo-symmetric site that has been suggested to be a regulatory binding site much like the forskolin-binding site in the related protein, adenylyl cyclase (10, 29, 49, 50). In contrast, cross-linking studies with the YC-1 related molecule BAY 41-2272 indicated binding was near α1 residues Cys-238 and Cys-243, well away from the cyclase domain (30).

To address this issue, we determined the effect of YC-1, ATP, and GTP on CO binding to msGC-NT1 and msGC-NT2, neither of which contains the sGC catalytic domain. CO binding to the full-length protein has been shown previously to increase dramatically in the presence of YC-1, leading to strong stimulation of sGC activity. CO binding to msGC-NT1 (not shown) and msGC-NT2 (Fig. 3) was monitored spectroscopically. Binding was similarly modest for both proteins in the absence of YC-1 and increased dramatically upon addition of YC-1 (Fig. 3 and Table 2). Addition of ATP and GTP (Table 2) or cGMP (not shown) had little effect on CO binding. We conclude that the major YC-1-binding site lies in the N-terminal two-thirds of the protein, away from the catalytic domain.
sGC from Manduca sexta

FIGURE 3. Effect of YC-1 on CO binding to msGC-NT2. A, difference spectra for CO addition in the presence and absence of YC-1. Spectra were measured at room temperature for 1.5 μM protein in buffer containing 50 mM KPO₄, pH 7.4, 100 mM KCl, 5% glycerol, and 50 μM YC-1 and were corrected for dilution by addition of CO-saturated buffer and for base-line drift (monitored at 700 nm). B, fitting of difference spectra to a single-site saturation model to obtain the CO dissociation constants for CO, μM.

TABLE 2

| Ligand* | WT | αL211A | αY223A |
|---------|-----|---------|---------|
| ATP     | 77 ± 7 | 45 ± 3 | 46 ± 5 |
| GTP     | 55 ± 4 | ND     | ND     |
| YC-1    | 1.7 ± 0.1 | 3.5 ± 0.2 | 3.1 ± 0.3 |

* Ligand concentrations are as follows: ATP, 1 mM; GTP, 0.5 mM; YC-1, 50 μM.

- The wild-type (WT) value in the presence of YC-1 was measured in a 10-cm cuvette with a protein concentration of 0.15 mg/mL at 22 °C.

The resulting NO release behavior and associated rate constants are shown in Fig. 5 and Table 4. By itself, msGC-NT1 displayed two prominent phases by stopped-flow analysis, a very fast phase with rate constant of 0.1 s⁻¹ (56% of the total amplitude) and a slower phase with rate constant of 0.0066 s⁻¹ (44%). Analysis in a cuvette, after allowing for decay of the fast phase, was best fit with a single exponential, yielding a more robust value for the second phase of 0.0015 s⁻¹. This value is ~5-fold smaller than that reported for the first phase of the rat protein (55) and ~3-fold larger than that of the single phase reported for the bovine protein (54). Addition of GTP or ATP had very little effect on msGC-NT1 rate measurements, whereas GTP or YC-1 greatly accelerated NO release for the full-length mammalian proteins (7, 54). To our surprise, addition of YC-1 to msGC-NT1 had a profound effect on NO release; the fast phase was completely eliminated leaving only the second phase in place. This result is clearly shown in plots of the absorbance change associated with CO formation; in the stopped-flow experiment, msGC-NT1-CO formation was markedly delayed when YC-1 was present (Fig. 5A), whereas in the cuvette experiment, the amplitude for the absorbance change because of msGC-NT1-CO formation was much greater in the presence of YC-1, because the loss of NO was not diminished by the fast phase, which was not observable in the cuvette experiment.

YC-1 Decreases Rates for CO Release from msGC-NT—We also examined CO binding and release to further characterize changes in the protein. CO release from msGC-NT1-CO was measured in a stopped-flow device by replacement with NO, which binds more quickly and more tightly to the protein. CO release was faster than NO release; nonetheless, the release rate was decreased dramatically (by ~10-fold) by the addition of YC-1, much like with NO release (Table 5). In contrast to NO, however, CO had only one detectable release phase.

CO binding, unlike NO binding, was sufficiently slow for measurement in the stopped-flow device. The rate constants for CO binding were similar in the presence and absence of YC-1 (Table 5). The $k_{off}/k_{on}$ ratios were similar to the measured values for $k_d$ (71 versus 77 μM in the absence of YC-1 and 8
partially purified protein displayed a basal activity of 6.3 nmol of cGMP mg\(^{-1}\) min\(^{-1}\) and a maximal activity of 1058 nmol of mg\(^{-1}\) min\(^{-1}\), values that are 6–12-fold smaller overall than those reported for mammalian sGC proteins (7, 8, 29). The lower value we obtained is most likely because of having a mixture of full-length and \(\alpha_1\)-degraded material in the preparation, leading to overestimation of total intact protein from the Soret band absorption. However, the possibility that the intact Manduca protein is inherently less active then its mammalian counterparts cannot be ruled out.

NO stimulation of msGC was similar to that of the mammalian proteins. Typically, NO stimulates mammalian sGC by 100–200-fold; in our study, NO stimulated the recombinant Manduca sGC by 135-fold alone and by 175-fold in the presence of YC-1 (Fig. 6). Importantly, YC-1 alone was also a potent stimulator of catalytic activity (Fig. 6). For maximal activity, msGC required both NO and YC-1, as has been generally reported for the mammalian proteins (29, 58–60), although one group has reported that only CO and YC-1, not NO and YC-1, are synergistic (56).

Mammalian sGC is inhibited by ATP, suggesting that the nucleotide is an allosteric regulator of the protein (7, 10, 61). When we examined inhibition of msGC, we found that 1 mM ATP, a physiologically relevant concentration (62), inhibited the enzyme by \(\sim70\%
of the presence of stoichiometric NO concentrations (Fig. 6). Overall, msGC behaves much like its mammalian counterparts, indicating our results with msGC-NT1 and msGC-NT2 are generally applicable for the entire sGC family.

**Modeling sGC Functional Domains**—The foregoing data confirm that YC-1 binding occurs in the N-terminal two-thirds of msGC. We reasoned that the regulatory effect of YC-1 might occur through binding to the \(\alpha_1\) subunit in the region homologous to the \(\beta_1\) heme-binding domain, which has recently been recognized to have evolved from an ancient prokaryotic heme-protein called H-NOX (20–22). We examined the domain structure of \(\alpha_1\), searching for known folds using the Ginzu protocol as implemented in the Robetta server (see “Experimental Procedures” for details). This led to the prediction that \(\alpha_1\) has an H-NOX fold between residues 51 and 247. A homology model of residues 61–234 based on Protein Data Bank entries 1XBN (21) and 2O09 (20) yielded a satisfactory fold with a score similar to that of the template structure 1XBN (Fig. 7).

Based on this result, we hypothesized that a small molecule binding pocket might reside in the site equivalent to the heme-binding site in the H-NOX domain and that conserved residues
in this region, such as α1 Leu-211 and Tyr-223 (Fig. 7), might be of importance in YC-1 binding. Therefore, we produced the msGC-NT2 mutants α1 L211A and Y223A. Both proteins were expressed in soluble form; however, heme loading was reduced in both mutant proteins, suggesting that a direct contact between α1 and heme might have been lost. YC-1 stimulation of CO binding was unaltered for the heme-intact portion of both mutated proteins (Table 2), and the precise location of YC-1 binding therefore remains unknown.

**DISCUSSION**

We have expressed functional heterodimeric full-length and truncated sGC from the tobacco hornworm (*M. sexta*) in *E. coli*, the first time this has been accomplished for sGC from any species. Both express as soluble, heterodimeric proteins with intact ferrous heme. The partially purified full-length protein
shows behavior similar to its mammalian counterparts, including stimulation by NO and compound YC-1, and inhibition by ATP (Fig. 6). The specific activity of this material is 6–12-fold lower than that of its mammalian counterparts, most likely because of the presence of proteolytically degraded material in the preparation that retains heme but not catalytic activity. Truncated msGC, which contains the N-terminal two-thirds of both the α1 and β1 subunits but not the catalytic domains (Fig. 1), binds YC-1, which decreases release rates for both NO (Table 4) and CO (Table 5) and increases the binding affinity for CO (Table 2) and presumably NO (the NO dissociation constant is too small to be readily measured). In contrast, ATP, GTP, and cGMP have little effect on NO and CO binding to msGC-NT. Taken together, these results indicate that a specific binding site for YC-1 and related compounds lies away from the catalytic domain and is distinct from the allosteric site proposed for GTP, ATP, cGMP, and pyrophosphate (PP). Below, we explore the implications of these results.

YC-1 Binding Alters the Heme Pocket—In the absence of YC-1, NO release from msGC-NT is biphasic, whereas in the presence of YC-1, release is monophasic because of loss of the fast phase (Table 4). Similarly, CO release from msGC-NT is 10-fold faster in the absence of YC-1 than in its presence, but only a single phase is observed (Table 5). YC-1 binding also leads to an ~2-nm blue shift in the msGC-NT-CO heme Soret band (not shown), as reported previously for the full-length bovine protein (57), indicating a change in the heme pocket takes place. A Soret band shift is not observed in the absence of CO or in the presence of NO. Additionally, photolysis of CO from msGC-NT-CO in the presence of YC-1, but not in its absence, displays a large germinate recombination phase, where the photolyzed CO becomes trapped in the protein matrix and rapidly recombines with heme rather than escaping into bulk solvent. Taken together, these data suggest that YC-1 binding closes the msGC-NT heme pocket such that access to solvent is reduced.

An alternative possibility is that YC-1 binding changes electrostatic stabilization of the sGC NO and CO complexes. Elegant studies on myoglobin by Olson and co-workers (63, 64) indicate that dioxygen release is particularly sensitive to changes in distal pocket hydrogen bonding, and furthermore, discrimination between dioxygen and CO in myoglobin depends heavily on electrostatic stabilization of heme-bound dioxygen. However, their studies indicate that electrostatic effects have little influence on the release rates of NO and CO because of the apolar nature of the FeNO and FeCO moieties (63, 64). Thus, our results fit better with a model invoking steric trapping of the molecules.

One of two mechanisms is likely to give rise to a blocked heme pocket. First, direct blockage of the distal pocket by YC-1 would be consistent with the kinetic data and has been suggested previously (65), but it is inconsistent with the limited effects on the heme absorption spectra. Alternatively, and more likely in our view, is an allosteric model wherein YC-1 binding leads to a change in protein conformation that closes the distal pocket. That such a mechanism is possible despite the propensity for NO and CO to diffuse away is illustrated by the nitrophorins from the kissing bug (Rhodnius prolixus), which are used for NO transport during blood feeding (66). Binding of NO to the Rhodnius nitrophorin heme leads to desolvation of the distal pocket, due in part to the hydrophobic nature of the NO molecule, and to a large change in loop conformation (67), which hinders escape of NO from the heme (68–70). A similar mechanism can be envisioned for sGC.

Functional Interaction between α1 and β1 H-NOX Domains—The location of the YC-1-binding site in the N-terminal two-thirds of sGC is still unknown. To aid in the discovery of the binding site, we undertook molecular modeling, which led us to conclude that the α1 N-terminal region contains an H-NOX domain that is much like that for β1, but lacking bound heme (Figs. 1 and 7). Although the α1 domain is less conserved than the rest of the protein, a few conserved residues are present, including Phe-157, Leu-211, and Tyr-223. In the model, these residues lie in the region analogous to the β1 heme-binding pocket (Fig. 7) and are near α1 Cys-238 and Cys-243 in the human sGC sequence, which can be modified through photoaffinity labeling with a YC-1 analogue (30).

We reasoned that ligand binding in the α1 H-NOX pocket might have a regulatory effect much like that of heme binding in the β1 H-NOX domain, because of the symmetric domain structure of the protein, and therefore the α1 H-NOX pocket might represent the YC-1-binding site. We generated α1 mutant proteins L211A and Y223A to test this possibility. Neither mutation affected YC-1 binding (Table 2), but to our surprise, both mutant proteins lost heme affinity and could not be isolated with a fully intact heme pocket, suggesting that the α1 and β1 H-NOX domains contact one another and possibly even share heme binding. Interestingly, the recombinant rat β1 H-NOX domain exists as a homodimer in solution (71), consistent with our conclusion that the α1/β1 dimer shares an H-NOX interface. The binding site for YC-1, however, remains unknown.

Relation to Full-length Mammalian sGC—Marletta and co-workers (7, 55) and Russwurm and Koesling (8) have demonstrated that NO stimulation of mammalian sGC is increased in the presence of GTP (Marletta and co-workers) or Mg2+/cGMP/PPi (Russwurm and Koesling), presumably through conformational linkage of the cyclase and H-NOX domains. Additionally, Marletta and co-workers (7) and Sharma and co-workers (54) have reported that the off rates for NO increase in the presence of GTP. For msGC-NT, we saw no such effect with GTP (Table 4) or cGMP (not shown), indicating functional binding for these ligands most likely occurs only in the cyclase domain.

Mammalian sGC has been shown to have both high and low output forms that display spectrally identical five-coordinate Fe-NO complexes (7, 8, 55). Russwurm and Koesling (8) have argued that the high and low output forms of sGC arise from two different Fe-NO binding geometries, one with NO bound to the distal side of the heme (high output) and one to the proximal side (low output), displacing β1 His-105, in a geometry reminiscent of that for nitrosylated cytochrome c’ (72). Their data indicate that formation of the low output form only

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3 X. Hu, C. Feng, and W. R. Montfort, unpublished results.
occurs in the absence of substrates or products. Marletta and co-workers (7, 55) have argued that high and low output forms of sGC represent two different conformations of the protein, differing in both catalytic rate and NO release rates and arising from a second NO-binding site that influences the rate of β1 His-105 release (52). In our hands, decay of the msGC-NT six-coordinate intermediate was quite fast (kτ = 12.8 s⁻¹, see Table 3) and independent of NO concentration at moderate NO levels (Fig. 4), consistent with formation of the high output form of full-length sGC without binding of a second NO molecule or influence from nucleotide binding. We conclude that binding of a regulatory nucleotide or a second NO molecule, if they occur for msGC, must therefore take place in the C-terminal catalytic domain.

It should also be noted that, curiously, the opposite effect of YC-1 binding on NO release was recently found by Marletta and co-workers (55) for the full-length rat protein. In that study, two phases were also described for NO release, but YC-1, like GTP, increased the proportion of the faster phase and, to a lesser extent, increased the rate constants themselves. The authors proposed a model quite similar to ours, but opposite in direction; they suggest that GTP and YC-1 bind to the equivalent site in the catalytic domain, leading to an open conformation with faster release kinetics. In contrast, Koesling and co-workers (49, 58), by monitoring cGMP production in the presence of an NO scavenger, inferred decreased NO off rates for full-length bovine lung sGC when bound to YC-1, much as we see with msGC-NT, and Kharitinnov et al. (57) reported that CO binds 10-fold more tightly to YC-1-ligated bovine lung sGC, again as we found for msGC-NT. Nonetheless, that a second YC-1-binding site exists in the cyclase domain is suggested by mutations to the catalytic domain that alter the response to the ligand (29, 49). Clearly, additional studies are required to resolve this complicated issue.

A Two-state Model for sGC Activation—In conclusion, we have demonstrated that msGC behaves much like its mammalian counterparts, and that YC-1 binding occurs in the N-terminal two-thirds of the protein and leads to a change in heme pocket conformation that is consistent with distal pocket closure. The simplest model for activation that emerges from these data is one in which the protein moves between two predominant conformations, “high output” and “low output,” such that multiple effector molecules, binding in multiple locations, can all shift the protein between the two states. In this respect, effector molecules for sGC act much like O₂; CO₂ and biphosphoglycerate act on hemoglobin, where each molecule affects the equilibrium between R and T states but do so through binding, coordinating, or reacting at distinct sites on the protein. As with hemoglobin, the ever-expanding list of molecules suggested to influence sGC, including non-heme NO, CO, GTP, ATP, Mg²⁺/cGMP/PP₁, YC-1, phosphorylation, nitrosylation, and protein partners, may all feed into a single conformational change pathway, with the sGC protein integrating all such signals to produce a final level of activity. In this model, release of β1 His-105 is unnecessary for sGC to achieve its high output conformation, but it is nonetheless favored when NO, with its strong trans effect, binds to the proximal heme site.
37. Collmann, C., Carlsson, M. A., Hansson, B. S., and Nighorn, A. (2004) J. Neurosci. 24, 6070–6077
38. Friebe, A., Wedel, B., Harteneck, C., Foerster, J., Schultz, G., and Koesling, D. (1997) Biochemistry 36, 1194–1198
39. Brandish, P. E., Buechler, W., and Marletta, M. A. (1998) Biochemistry 37, 16898–16907
40. Lee, Y.-C., Martin, E., and Murad, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10763–10768
41. Maragos, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Bove, A. A., Isaac, L., Hrable, J. A., and Keeler, L. K. (1991) J. Med. Chem. 34, 3242–3247
42. Maes, E. M., Roberts, S. A., Weichsel, A., and Montfort, W. R. (2005) Biochemistry 44, 12690–12699
43. Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997) Biochemistry 36, 6814–6818
44. Kim, D. E., Chivian, D., Malmstrom, L., and Baker, D. (2005) Proteins 61, Suppl. 7, 193–200
45. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L., and Schulten, K. (2005) J. Comput. Chem. 26, 1781–1802
46. Karow, D. S., Pan, D., Davis, J. H., Behrends, S., Mathies, R. A., and Marletta, M. A. (2005) Biochemistry 44, 16266–16274
47. Zhao, Y., Brandish, P. E., Ballou, D. P., and Marletta, M. A. (1999) Biochemistry 38, 15253–15257
48. Roy, B., and Garthwaite, J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 12185–12190
49. Zhao, Y., Ballou, D. P., and Marletta, M. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14753–14758
50. Ballou, D. P., Zhao, Y., Brandish, P. E., and Marletta, M. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12097–12101
51. Kharitonov, V. G., Russwurm, M., Magde, D., Sharma, V. S., and Koesling, D. (1997) Biochem. Biophys. Res. Commun. 239, 284–286
52. Winger, J. A., Derbyshire, E. R., and Marletta, M. A. (2007) J. Biol. Chem. 282, 897–907
53. Stone, J. R., and Marletta, M. A. (1998) Chem. Biol. 5, 255–261
54. Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1999) Biochemistry 38, 10699–10706
55. Friebe, A., and Koesling, D. (1998) Mol. Pharmacol. 53, 123–127
56. Hoener, M., Becker, E. M., Apeler, H., Sirichoke, T., Schroder, H., Gerzer, R., and Stasch, J. P. (1999) J. Mol. Med. 77, 14–23
57. Martin, E., Lee, Y. C., and Murad, F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12938–12942
58. Ruiz-Stewart, I., Tiyyagura, S. R., Lin, J. E., Kazerounian, S., Pitari, G. M., Schulz, S., Martin, E., Murad, F., and Waldman, S. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 37–42
59. Traut, T. W. (1994) Mol. Cell. Biochem. 140, 1–22
60. Olson, J. S., and Phillips, G. N., Jr. (1997) J. Biol. Inorg. Chem. 2, 544–552
61. Phillips, G. N., Jr., Teodoro, M. L., Li, T., Smith, B., and Olson, J. S. (1999) J. Phys. Chem. B 103, 8817–8829
62. Kondrashov, D. A., and Montfort, W. R. (2007) Mol. Cell. Biochem. 298, 189–191
63. Ribeiro, J. M. C., Hazzard, J. M. H., Nussenzveig, R. H., Champagne, D. E., and Walker, F. A. (1993) Science 260, 539–541
64. Weichsel, A., Andersen, J. F., Roberts, S. A., and Montfort, W. R. (2000) Nat. Struct. Biol. 7, 551–554
65. Maes, E. M., Weichsel, A., Andersen, J. F., Shepley, D., and Montfort, W. R. (2004) J. Biol. Chem. 279, 24907–24911
66. Andersen, J. F., Ding, X. D., Balfour, C., Shokhieva, T. K., Champagne, D. E., Walker, F. A., and Montfort, W. R. (2000) J. Biol. Chem. 275, 10118–10131
67. Kundrashov, D. A., and Montfort, W. R. (2007) J. Phys. Chem. B 111, 9244–9252
68. Zhao, Y., and Marletta, M. A. (1997) Biochemistry 36, 15959–15964
69. Lawson, D. M., Stevenson, C. E. M., Andrew, C. R., and Eady, R. R. (2000) EMBO J. 19, 5661–5671