Dissociation of Nitric Oxide from Soluble Guanylate Cyclase and Heme-Nitric Oxide/Oxygen Binding Domain Constructs*

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Regulation of soluble guanylate cyclase (sGC), the primary NO receptor, is linked to NO binding to the prosthetic heme group. Recent studies have demonstrated that the degree and duration of sGC activation depend on the presence and ratio of purine nucleotides and on the presence of excess NO. We measured NO dissociation from full-length α1β1 sGC, and the constructs β1(1–194), β1(1–385), and β2(1–217), at 37 and 10 °C with and without the substrate analogue guanosine-5′-[(α,β-methylene)triphosphate (GMPCPP) or the activator 3-(5′-hydroxymethyl-3′-furyl)-1-benzylindazole (YC-1). NO dissociation from each construct was complex, requiring two exponentials to fit the data. Decreasing the temperature decreased the contribution of the faster exponential for all constructs. Inclusion of GMPCPP also dramatically accelerated NO dissociation from sGC at 10 °C. The presence of GMPCPP also dramatically accelerated NO dissociation from sGC at 10 °C. This acceleration is due to increases in the observed rate for each exponential and in the contribution of the faster exponential. Increases in the contribution of the faster exponential correlated with higher activation of sGC by NO. These data indicate that the sGC ferrous-nitrosyl complex adopts two 5-coordinate conformations, a lower activity “closed” form, which releases NO slowly, and a higher activity “open” form, which releases NO rapidly. The ratio of these two species affects the overall rate of NO dissociation. These results have implications for the function of sGC in vivo, where there is evidence for two NO-regulated activity states.

Soluble guanylate cyclase (sGC)4 is the best characterized physiological receptor for the gaseous signaling agent nitric oxide (NO) (1–5). In response to NO, sGC produces the second messenger cGMP, modulating physiological processes such as neurotransmission and vasodilation (6). The α1β1 sGC heterodimer is activated several hundredfold above the basal level by the binding of NO to the heme of the β1 H-NOX domain (7–9), a conserved domain of unique structure (10–12). However, it remains unclear how this binding event is translated into increased catalytic activity.

The mechanism by which sGC deactivation occurs has been a focus of much investigation (8, 13, 14). Initially thought to result from simple dissociation of NO from the heme, the deactivation process has turned out to be more complicated. In fact, regulation of sGC by NO has been shown to involve a complex interplay between binding of NO to the heme and to non-heme sites as well as allosteric regulation by GTP, also a substrate, and ATP, a reporter for the energy status of the cell (15). In integrating information from all these signals, sGC has been shown to have a remarkable attribute, the ability to exist in a stable low activity state or a transient high activity state, both containing NO bound to the heme (16, 17). Many questions concerning the existence and characteristics of these two sGC heme-NO species, and how they might be regulated by NO and nucleotides, remain to be addressed.

In addition to responding to cellular inputs, sGC can be activated by a class of small molecules exemplified by 3-(5′-hydroxymethyl-3′-furyl)-1-benzylindazole (YC-1). These molecules not only activate sGC in the absence of heme ligands but also synergize with the NO- and CO-bound forms of the enzyme to reach maximal activity. There has been much speculation about the binding site and mechanism of action of YC-1 (18–20); however, experimental results remain inconclusive. By using purified sGC and sGC heme domain constructs (Fig. 1) that possess heme characteristics similar to those of the full-length α1β1 enzyme (21, 22), we carried out spectroscopic and kinetic analyses of NO dissociation in the absence and presence of YC-1 and the substrate analogue guanosine-5′-[(α,β-methylene)triphosphate (GMPCPP), as well as activity studies with YC-1. Dissociation was found to exhibit two exponential phases, the relative contributions of which could be differentially affected by YC-1, GMPCPP, or changes in temperature.

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‡‡The abbreviations used are: sGC, soluble guanylate cyclase; NO, nitric oxide; β1(1–194), the first 194 amino acids of the β1 subunit of sGC; β1(1–385), the first 385 amino acids of the β1 subunit of sGC; β2(1–217), the first 217 amino acids of the β2 subunit of sGC; H-NOX, heme-nitric oxide/oxygen binding domain; YC-1, 3-(5′-hydroxymethyl-3′-furyl)-1-benzylindazole; GMPCPP, guanosine-5′-[(α,β-methylene)triphosphate; DEA/NO, diethylnitrosoamine (Z)-1-(N,N-diethy lamino)diazene-1-ium-1,2-diolate; DTT, dithiothreitol; Me2SO, dimethyl sulfoxide.
We propose a model for dissociation of NO from sGC involving the existence of two 5-coordinate sGC heme-NO species indistinguishable by electronic absorption spectroscopy but clearly different in their ability to release NO from the heme; this model is discussed in context of the current hypothesis for regulation of sGC by NO and nucleotides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers were obtained from Invitrogen. All restriction enzymes were from New England Biolabs. SF9 cells were obtained from the Department of Molecular and Cell Biology Tissue Culture Facility, University of California, Berkeley. Rat lung sGC α1 and β1 cDNAs were provided by Dr. Masaki Nakane, Abbott. H-NOX domain constructs from rat sGC β1 and β2 subunits (β1(1–194), β1(1–385), and β2(1–217)) were purified as described (21, 23).

**Rat sGC Construction**—Rat sGC β1 cDNA was inserted between the NotI and XbaI sites of the plasmid pFastBac1 (Invitrogen) to generate pFastBac1/sGCβ1. PCR was used to insert an in-frame C-terminal RGSH$_4$ tag in front of the stop codon of the α1 cDNA. The forward primer was 5’-TGGCGG-CCGCAAGGAGGAAACAC-3’, and the reverse primer was 5’-CGTCTAGATTTATGCGTGGCTGATGAGATC-TCTATCTACCTCCTGATGTGGCTGTGTT-3’. PCR products were sequenced to confirm the presence of the desired changes (University of Michigan Biomedical Research Core Facilities). The H$_2$SGC α1 gene was inserted between the NotI and XbaI sites of pFastBac1 to generate the construct pFastBac1/sGCa1. The Bac-to-Bac baculovirus expression system (Invitrogen) was used to generate recombinant baculoviruses from pFastBac1/sGCa1 and pFastBac1/sGCβ1 according to the manufacturer’s protocol. High titer stocks of recombinant baculoviruses were prepared by standard methods. Optimization of the amount of each virus used for protein production was carried out as described previously (23).

**Cell Culture and Production of Recombinant sGC**—SF9 cells were cultured in Ex-Cell 420 insect serum-free medium (JRH Biosciences) supplemented with 10% fetal calf serum (HyClone) and 1% antibiotic/antimycotic (Invitrogen) at 28 °C. Cultures were grown in 2800-ml Fernbach flasks with shaking at 135 rpm. Cells were subcultured between 0.7 × 10$^6$ and 5 × 10$^6$ cells/ml. Cell density and viability were determined by trypsin blue exclusion using a hemocytometer. For protein expression, 1-liter cultures of SF9 cells at a density of 1.5–2 × 10$^6$ cells/ml in 2800-ml Fernbach flasks were infected with H$_2$α1 and β1 recombinant viruses. Cells were harvested 3 days post-infection by centrifugation, and the pellet was stored at −80 °C.

**Purification of Recombinant sGC**—All manipulations were carried out at 4 °C. Frozen cell pellets from 5-liter expression cultures were thawed on ice and resuspended in buffer A (50 mM KH$_2$PO$_4$, pH 8.0, 200 mM NaCl, 5 mM B-mercaptoethanol, 1 mM imidazole, 1 mM Pefabloc (Pentapharm), 1 mM benzamidine, 5% glycerol) plus Complete EDTA-free protease inhibitor mixture (Roche Applied Science). Resuspended cells were broken with a Bead Beater (BioSpec Products) using 0.1-mm diameter glass beads, and the lysate was centrifuged at 200,000 × g for 2 h. The supernatant was applied to a 2.5-ml column of nickel-nitrioltriacetic acid-agarose (Qiagen) equilibrated with buffer A at a flow rate of 1 ml/min using a BioLogic LP (Bio-Rad). The column was washed with buffer A until the A$_{280}$ was stable, and then an aliquot of buffer A (25 ml) was brought to 1.2 M NaCl and applied to the column. The column was washed with 50 ml of 12.5 mM imidazole in buffer A and eluted with 25 ml of 125 mM imidazole in buffer A, collecting 2-ml fractions during the elution. Fractions containing sGC (identified by yellow color) were pooled, concentrated to 1–1.5 ml in a Vivasin-20 50K filter (Vivascience), and exchanged into buffer B (25 mM triethanolamine, pH 7.4, 25 mM NaCl, 5 mM dithiothreitol) on a PD-10 column (Amersham Biosciences). The sample was diluted to 7 ml with buffer B and applied to a 2-ml prepacked POROS HQ2 anion-exchange column (Applied Biosystems) at 2 ml/min using a BioLogic Duo Flow (Bio-Rad). The column was washed with 5 ml of buffer B and developed with a 35-ml 120–285 mM gradient of NaCl in buffer B, collecting 1-ml fractions. Fractions containing purified sGC (exhibiting an A$_{280}$/A$_{431}$ < 1.1) were pooled, concentrated in a Vivasin-6 50K filter, drop-frozen in liquid N$_2$, and stored in liquid N$_2$.

Protein purity was assessed by SDS-PAGE using pre-cast 10% Tris-glycine gels (Invitrogen) and was routinely greater than 95%. Protein concentrations were determined using the Bradford microassay (Bio-Rad) or calculated from the A$_{431}$ using an extinction coefficient of 148,000 M$^{-1}$ cm$^{-1}$ (8).

**Dissociation of NO from the Heme of β1(1–194), β1(1–385), β2(1–217), and sGC**—The dissociation of NO from the heme of each H-NOX domain construct and sGC was measured at 37 and 10 °C using the CO/dithionite trapping method described previously (16, 24). The trapping solution was prepared as follows: a solution of sodium dithionite (Na$_2$S$_2$O$_4$) in 50 mM HEPES, pH 7.4, 50 mM NaCl was prepared in a Teflon-sealed...
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Reacti-Vial (Pierce) using an anaerobic chamber (Coy Laboratory Products). The solution was removed from the anaerobic chamber and saturated with CO by bubbling the gas through the solution for 10 min. Protein-NO complexes were formed by incubation with excess DEA/NO (in 10 mM NaOH) at 25 °C in 50 mM HEPES, pH 7.4, 50 mM NaCl for 10 min. Complete conversion to the nitrosyl species was verified by following the shift in the Soret maximum from 431 to 399 nm. Stock solutions of YC-1 were made in Me2SO. When present, YC-1 concentrations ranged from 0.96 to 96 μM, and the final concentration of Me2SO was 1%. Experiments with GMPCPP contained 10–1000 μM nucleotide (added before DEA/NO addition) and included 5 mM MgCl2, which alone had no effect on NO dissociation. Proteins were placed in a septum-sealed anaerobic cuvette and deoxygenated using an oxygen-scavenged gas train. A small amount of DEA/NO (∼3 eq) was added just before deoxygenation to maintain the nitrosyl species (any remainder was subsequently destroyed by the large excess of Na2S2O4 in the trapping solution). The head space of the anaerobic cuvette was replaced with CO, and the cuvette and trap solutions were equilibrated at assay temperature for 1 min. The reaction was initiated by addition of CO/dithionite solution to the anaerobic cuvette with a Hamilton gas-tight syringe and mixing. The final concentration of Na2S2O4 in the reaction mixture was 30 mM. Final protein concentrations were 1.9–2.5 μM for β1(1–194), β1(1–385), and β2(1–217), and 0.88–2.5 μM for sGC. Data collection was initiated ~10 s after trap addition. The reaction was monitored by electronic absorption spectroscopy using a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller. Data were collected over the range of 380–450 nm at 909 nm/min with a 1.5-nm data point interval. Spectra were recorded every 18 s for 5 min, every 1 min for 10 min, and every 2 min thereafter for a total of 3 h, or until the reaction was complete. A buffer base line was subtracted from each spectrum, and spectra were corrected for base-line drift by normalization to an isosbestic point at ~410 nm. For data obtained in the absence of YC-1 or GMPCPP, difference spectra were obtained by subtraction of the time 0 spectrum from all subsequent spectra. To obtain difference spectra for data acquired in the presence of YC-1 or GMPCPP, a time 0 spectrum from a reaction carried out in the absence of either compound and containing an identical amount of protein was subtracted from all subsequent spectra, and all time points were offset by an amount corresponding to the mixing time for the experiment. Values for the change in absorbance at 423 nm (ΔA423: β1(1–194) and β1(1–385)) or 424 nm (ΔA424: sGC and β2(1–217)) were extracted from the difference spectra and plotted versus time to obtain dissociation time courses for each experiment. Dissociation time courses were obtained in duplicate or triplicate, and each experiment was repeated 2–5 times over several days. Generally, because of the relative difficulty in obtaining large amounts of purified sGC, ΔA424 values for full-length sGC, which are proportional to the experimental protein concentrations, were smaller than for the heme domain constructs.

YC-1 Activation of the sGC-NO Complex—End point assays were performed in triplicate at 10 and 37 °C as described previously (25). Stock solutions of DEA/NO (10 mM) were prepared in NaOH (10 mM). Stock solutions of YC-1 (15 mM) were prepared in Me2SO. Assay mixtures contained 0.2 μg of sGC in 50 mM HEPES, pH 7.4, 2 mM Dithiothreitol, and 150 μM YC-1 where indicated. sGC was incubated with DEA/NO (100 μM) for 10 min at 25 °C and equilibrated at assay temperature for 1 min. Assays were initiated by addition of GTP and MgCl2 to 1 and 3 mM, respectively. Final assay volumes were 100 μl and contained 2% Me2SO, which did not affect enzyme activity. Reactions were quenched after 3 min by the addition of 400 μl of 125 mM Zn(CH3CO2)2 and 500 μl of 125 mM Na2CO3. cGMP quantification was carried out using a cGMP enzyme immunoassay kit, format B (Biomol), per the manufacturer’s instructions. Each experiment was repeated four times to ensure reproducibility.

Data Analysis and Statistics—Curve fitting, data analysis, and figure generation were carried out using Kaleidagraph (Synergy Software). The data from each dissociation experiment were fit to single or double exponentials as shown in Equations 2 and 3 under “Results” to obtain observed rate constants. To determine whether a single exponential or two exponentials best fit the data, the residuals from each fit were compared. Additionally, for each set of dissociation data, the fit to a single exponential was compared with the fit to two exponentials using the F test. A two-exponential fit was considered better than a one-exponential fit if p < 0.0001. For dissociation experiments, rates are expressed as means ± S.D. For activity assays, significant differences between the means were determined using the two-tailed t test; the significance level used was 0.05.

RESULTS

Kinetic Considerations; Developing a Model for NO Dissociation from sGC—The binding of NO to the sGC heme has been shown to proceed through initial formation of a 6-coordinate intermediate, followed by rupture of the iron-histidine bond to form the final 5-coordinate ferrous nitrosyl complex (7, 26, 27). The simplest mechanism for dissociation of NO from the 5-coordinate sGC heme-NO complex would be the reverse of NO binding as follows: rebinding of the proximal histidine ligand to form a 6-coordinate heme-NO intermediate followed by dissociation of NO to form a 5-coordinate histidyl-ligated heme, as shown in Scheme 1.

\[
\text{heme-NO}_5 \rightleftharpoons \text{heme-NO}_6 \rightleftharpoons \text{heme}_5 + \text{NO} \]

SCHEME 1

The values of \( k_{\text{on}} \) and \( k_{\text{off}} \) are the rate constants for binding and dissociation, respectively, of the proximal histidine ligand; \( k_{\text{on}} \) is the rate constant for binding of NO to the heme, and \( k_{\text{off}} \) is the rate constant for the dissociation of NO from the 6-coordinate heme-NO complex. In this study, we employed an NO trap, consisting of a sodium dithionite (Na2S2O4) solution saturated with CO (COsat), to obtain rates for dissociation uncomplicated by NO rebinding. This system has been used previously to determine the NO dissociation rates for a number of heme proteins (16, 24, 28) and functions by destroying dissociated NO through reaction with dithionite and by preventing NO
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rebinding by blocking the open heme coordination site with CO. At the concentrations of dithionite and CO used in these experiments, the reaction of NO with dithionite and the binding of CO to the vacated heme coordination site are not rate-limiting (Refs. 24 and 29 and data not shown). Thus, under our NO-trapping conditions, the above reaction scheme simplifies to Scheme 2,

\[
\begin{align*}
\text{heme-NO}^{5C} & \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{heme-NO}^{6C} \\
& \xrightarrow{k_{\text{off}}} \text{heme-CO}
\end{align*}
\]

**SCHEME 2**

where \(k_{\text{off}}\) is the rate constant for the dissociation of NO from the 6-coordinate heme-NO complex to form the CO complex, which is irreversible because of the CO\textsubscript{sat}/dithionite trap.

Assuming a steady-state equilibrium between heme-NO\textsubscript{5C} and heme-NO\textsubscript{6C}, Equation 1 can be derived for the observed reaction rate after mixing (derivation of the first-order rate constant \(k_{\text{obs}}\) for mechanisms similar to that described in Scheme 2 has been discussed in detail (29–33)),

\[
k_{\text{obs}} = \frac{k_{\text{on}} k_{\text{off}}}{k_{\text{on}} + k_{\text{hr}} + k_{\text{off}}}
\]

(Eq. 1)

A single exponential increase in the concentration of heme-CO is expected when starting from a uniform population of either heme-NO\textsubscript{5C} or heme-NO\textsubscript{6C}, as described by Equation 2,

\[
\Delta A_t = \Delta A_1 (1 - e^{-k_1 t}) + \Delta A_2 (1 - e^{-k_2 t})
\]

(Eq. 2)

where \(\Delta A_1\) is the change in signal amplitude at time \(t\); \(\Delta A_1\) is the total change in signal amplitude, and \(k_1\) is the observed reaction rate constant. Importantly, when starting from a mixture of heme-NO\textsubscript{5C} and heme-NO\textsubscript{6C}, if \(k_{\text{off}}\) is faster than \(k_{\text{hr}}\), a two-exponential increase as described by Equation 3 is predicted,

\[
\Delta A_t = \Delta A_1 (1 - e^{-k_1 t}) + \Delta A_2 (1 - e^{-k_2 t})
\]

(Eq. 3)

where \(\Delta A_1\) is the change in signal amplitude at time \(t\); \(\Delta A_1\) and \(\Delta A_2\) are the contributions of each exponential process to the total change in signal amplitude, and \(k_1\) and \(k_2\) are the observed rate constants for each process. That is exactly what is observed for dissociation of NO from several prokaryotic H-NOX domains, for which the heme-NO complexes have been demonstrated to exist as an equilibrium mixture of 5- and 6-coordinate states (29). However, previous studies using electronic absorption and resonance Raman spectroscopy have demonstrated that for sGC and the sGC H-NOX domain constructs studied in this work, the heme-NO complex is exclusively 5-coordinate (21, 34, 35). From these observations, it can be inferred that there is no appreciable amount of heme-NO\textsubscript{6C} in a solution of NO-bound sGC, relegating heme-NO\textsubscript{5C} to the status of a transient intermediate in the dissociation reaction pathway. Thus, in order to accommodate any observed two-exponential dissociation of NO, Scheme 2 must be expanded to include an additional 5-coordinate heme-NO species, as shown in Scheme 3,

\[
\begin{align*}
\text{heme-NO}^{5C} & \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{heme-NO}^{6C} \\
& \xrightarrow{k_{\text{off}}} \text{heme-CO}
\end{align*}
\]

**SCHEME 3**

where heme-NO\textsuperscript{5C} and heme-NO\textsuperscript{6C} are two 5-coordinate heme-NO species that are spectroscopically identical but kinetically distinct. In this mechanism, the 5-coordinate heme-NO complex of sGC exists as an equilibrium mixture of 5-coordinate heme-NO species (heme-NO\textsuperscript{5C} and heme-NO\textsuperscript{6C}), with slow interconversion between the two forms compared with NO dissociation. A two-exponential NO dissociation time course would be observed if, upon mixing with the NO trap, NO dissociated from the heme-NO\textsuperscript{5C} fraction with a \(k_{\text{obs}}\), according to the reaction in Scheme 2, with the remainder of the dissociation reaction proceeding from the heme-NO\textsuperscript{6C} fraction, as indicated in Scheme 3, and dependent on the rate of interconversion between 5-coordinate species (\(k_{\text{off}} - k_{\text{off}}\)). Thus, the observation of two exponentials versus one in the dissociation of NO from a sGC H-NOX domain would indicate that NO dissociates from a mixture of 5-coordinate heme-NO species, with \(\Delta A_1\) and \(\Delta A_2\) proportional to the amount of each species at the start of the dissociation reaction.

**Dissociation of NO from sGC and sGC H-NOX Domain Constructs at 37 °C—**In the presence of the NO trap (CO\textsubscript{sat}/dithionite), dissociation of NO from the heme of sGC and sGC H-NOX domain constructs resulted in an increase in absorbance at 423–424 nm because of formation of the heme-CO complex. A representative set of data for dissociation at 37 °C from sGC, \(\beta_1(1–194), \beta_1(1–385),\) and \(\beta_2(1–217)\) is shown in Fig. 2. The corresponding plots of \(\Delta A_{423}\) for each construct. For each dissociation time course, the fits to both single and double exponentials (Equations 2 and 3) are shown. The residuals for each fit are plotted above each time course. In each case, examination of the residuals suggested that a two-exponential fit provided a better model for the data than a single exponential fit. A comparison of the one- and two-exponential fits using the \(F\) test supported the two-exponential fit as the better model in each case (\(p < 0.0001\)). The two rate constants obtained for each construct (a faster constant \(k_1\) and a slower constant \(k_2\), averaged from 2–4 experiments per construct) and the amplitudes of each corresponding phase (as a percent of the calculated total) are shown in Table 1. The observed data are consistent with a model where dissociation proceeds from an initial equilibrium mixture of two 5-coordinate heme-NO complexes, as outlined in Scheme 3. Accordingly, we propose that \(k_1\) corresponds to dissociation of NO from the heme-NO\textsuperscript{5C} conformation at a rate equal to \(k_{\text{obs}}\), whereas \(k_2\) represents the observed rate of reaction, corresponding to \(k_{\text{off}} - k_{\text{off}}\), that is limited by the slower conversion from heme-NO\textsuperscript{5C} to heme-NO\textsuperscript{6C}.

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To further investigate the possibility that the 5-coordinate heme-NO complex of sGC can exist in two conformations, we examined the effect of the small molecule activator YC-1 (36) on NO dissociation from sGC, β1(1–194), β1(1–385), and β2(1–217) at 37 °C (Fig. 3 and Table 1). YC-1 caused no significant change in the amplitudes or rate constants for NO dissociation from β1(1–194) or β1(1–385). However, for dissociation of NO from full-length sGC in the presence of YC-1, k1 increased ∼15-fold, k2 increased roughly 5-fold, and ΔA1, the fractional amplitude due to k1, modestly increased from 35 to 66% (Fig. 3C). The increase in both rate constants and the doubling of ΔA1 indicate that NO dissociation from sGC is significantly accelerated by YC-1. A similar effect was observed for β2(1–217) as follows: k1 doubled, k2 tripled, and ΔA1 increased from 40 to 66% of the total amplitude change (Fig. 3D). Thus, at 37 °C, YC-1 appears to accelerate the dissociation of NO from sGC and β2(1–217), but not from β1(1–194) or β1(1–385), by increasing k1, k2, and the fraction of sGC-NO with the fast dissociation rate.

Dissociation of NO from sGC and sGC H-NOX Domain Constructs at 10 °C—The temperature dependence of the observed rate constants and amplitudes for each exponential in the NO dissociation time course was examined at 10 °C in the absence and presence of YC-1. Plots of NO dissociation time courses at 10 °C for each construct are shown in Fig. 4, and the derived rate constants and amplitudes are shown in Table 2. In the absence of YC-1, the values of the observed rate constant k1 for β1(1–194) and β1(1–385) are similar to those obtained at 37 °C, whereas those of full-length sGC and β2(1–217) are slightly increased. However, ΔA1, the fraction of the calculated total amplitude described by k1, is markedly diminished for all constructs, dropping from 25, 29, 35, and 41% at 37 °C to 6, 7, 5, and 15% at 10 °C for β1(1–194), β1(1–385), sGC, and β2(1–217), respectively (compare Tables 1 and 2). Furthermore, although the slower rate constant k2 for each construct appears to double for every 10 °C increase in temperature (exhibiting an Arrhenius temperature dependence), the faster rate constant k1 changes very little from 10 to 37 °C.

The presence of YC-1 had negligible effect on the dissociation of NO from β1(1–194) and β1(1–385) at 10 °C, with little change in k1, k2, ΔA1, or ΔA2 (Fig. 4, A and B). Furthermore, the acceleration of NO dissociation from β2(1–217) by YC-1 at 37 °C was not detected at 10 °C; k1, k2, ΔA1, and ΔA2 were unaffected by the presence of YC-1 (Fig. 4D). In contrast, the acceleration of the dissociation of NO from sGC by YC-1 was even more pronounced at 10 °C than at 37 °C (Fig. 4C). Although the presence of YC-1 resulted in a moderate increase in both k1 and k2, ΔA1 increased from 5 to 73% of the calculated total amplitude at 10 °C, a 15-fold change, compared with an ∼2-fold change for sGC at 37 °C. Thus, at 10 °C, although YC-1 did not significantly alter the dissociation of NO from β1(1–194), β1(1–385), or β2(1–217), it drastically accelerated the dissociation of NO from sGC, primarily by increasing ΔA1, the fraction of the calculated total amplitude described by k1.

The Effect of YC-1 on the Exponentials Observed in Dissociation of NO from sGC—To gain more information about the YC-1-induced acceleration of NO dissociation from sGC, we carried out dissociation reactions at 10 °C in the presence of increasing amounts of YC-1. As shown in Fig. 5A, ΔA1 increased as the concentration of YC-1 was increased. ΔA1 values for each curve were plotted against log[YC-1] and fit to a concentration dependence function (Equation 4), yielding an approximate EC50 value of 4 μM for the ability of YC-1 to increase ΔA1 at 10 °C (Fig. 5B).

\[ \Delta A_1 = \min + \frac{(\max - \min)}{1 + 10^{\log EC_{50} - x}} \]  

(Eq. 4)

Together, these observations suggest that YC-1 shifts the equilibrium ratio of 5-coordinate sGC-NO species from heme-NO5c toward heme-NO5c (as shown in Scheme 3), leading to
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FIGURE 3. Time courses for dissociation of NO from sGC and H-NOX domain constructs at 37 °C. Data were extracted from difference spectra and plotted along with single (dotted line) and double (solid line) exponential fits to the time courses. The residuals from each single (open circles) and double (closed circles) exponential fits are shown above each plot. The time courses for dissociation of NO from 2.2 μM β1(1–194), 2 μM β1(1–385), 0.88 μM sGC, and 1.9 μM β2(1–217) in the absence (top panels) or presence (bottom panels) of 96 μM YC-1 are shown in A–D, respectively. The time courses shown are representative of dissociation experiments repeated 2–5 times in duplicate or triplicate for each construct. Differences in ΔA values between constructs reflect different experimental protein concentrations. These data illustrate that time courses for NO dissociation are biphasic on this time scale and that the presence of YC-1 accelerates NO dissociation from sGC and β2(1–217).

TABLE 1
Observed rate constants and fractional amplitudes for NO dissociation from full-length sGC and H-NOX domain constructs at 37 °C

| Protein  | \(k_1\) (s\(^{-1}\)) | \(k_2\) (s\(^{-1}\)) | ΔA\(_1\)/ΔA\(_2\) (%) |
|----------|----------------------|----------------------|---------------------|
| ~YC-1    |                      |                      |                    |
| β1(1–194)| 0.0041 ± 0.0003      | 0.0009 ± 0.0001      | 25.75 (±1)          |
| β1(1–385)| 0.0087 ± 0.0017      | 0.0023 ± 0.0002      | 29.71 (±6)          |
| sGC      | 0.0038 ± 0.0018      | 0.0012 ± 0.0005      | 35.65 (±22)         |
| β2(1–217)| 0.0069 ± 0.0009      | 0.0011 ± 0.0003      | 41.39 (±3)          |
| +YC-1    |                      |                      |                    |
| β1(1–194)| 0.0040 ± 0.0001      | 0.0007 ± 0.0001      | 30.70 (±2)          |
| β1(1–385)| 0.0109 ± 0.0035      | 0.0024 ± 0.0007      | 44.56 (±12)         |
| sGC      | 0.0063 ± 0.0054      | 0.0065 ± 0.0002      | 66.34 (±3)          |
| β2(1–217)| 0.0122 ± 0.0014      | 0.0036 ± 0.0010      | 66.34 (±12)         |

an acceleration of NO dissociation. YC-1 has been shown to potentiate the NO stimulation of sGC (37); to examine whether the YC-1-induced changes in the fraction of the calculated total amplitude described by \(k_1\) have any implications for enzyme activation, the basal and NO-stimulated activities of sGC in the absence and presence of YC-1 were measured at 37 °C and 10 °C (Fig. 5C). As expected, in each case sGC activity was lower at 10 °C than at 37 °C. However, the fold-activation by NO was much lower at 10 °C (1.5-fold) than at 37 °C (107-fold), qualitatively mirroring the much smaller ΔA\(_1\) at 10 °C (5%) versus 37 °C (35%) (Fig. 5D). Furthermore, YC-1 potentiated the NO-stimulated activity of sGC to a much greater extent at 10 °C (7.1-fold over NO alone) than at 37 °C (1.4-fold over NO alone), which correlates well with the increase in ΔA\(_1\) caused by YC-1 at 10 °C (5 to 73%) versus 37 °C (35 to 66%) (Fig. 5E). These results are consistent with the observation that YC-1 increases ΔA\(_1\), the fraction of NO dissociation from sGC that occurs via \(k_1\), and suggest that the heme-NO5C form of the enzyme exhibits higher activity than does the heme-NO\(_{5C}\) form.

The Effect of Substrate on the Exponentials Observed in NO Dissociation from sGC—The presence of substrate GTP has been reported to greatly accelerate the dissociation of NO from sGC (16, 38). However, the presence of two phases in the NO dissociation time course was not examined. To further investigate the effect of substrate on the dissociation of NO from the sGC heme, experiments were carried out with increasing concentrations of GMPCPP, a noncyclizable GTP analogue, at 10 °C (Fig. 6). In the presence of 1 mM GMPCPP (or GTP, data not shown; the noncyclizable analogue was used to ensure that the nucleotide concentration remained constant throughout the course of the experiment), NO dissociation was extremely rapid, as reported (16). Furthermore, time courses for NO dissociation in the presence of GMPCPP exhibited two exponential phases. The data were fit to Equation 3 to obtain values of 0.178 ± 0.009 s\(^{-1}\) for \(k_1\), 0.00154 ± 0.00011 s\(^{-1}\) for \(k_2\), and a ΔA\(_1\) of 83% (Table 3). Compared with data obtained in the
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absence of GMPCPP at 10 °C, $k_1$ increased 22-fold; $k_2$ increased 8-fold; and $\Delta A_{1,1}$, the fractional amplitude attributed to $k_1$, increased 6-fold, from 13 to 83% (Table 3). As shown in Fig. 6, the increase in $\Delta A_{1,1}$ was found to be dependent on the concentration of GMPCPP, and fitting the data to Equation 4 yielded an $EC_{50}$ of 9 μM for the ability of GMPCPP to increase $\Delta A_{1,1}$ at 10 °C. The observed substrate-mediated increase in dissociation of NO from sGC appears to occur by a mechanism similar to that of YC-1, namely an increase in the fraction of the reaction that occurs via the fast phase. Although YC-1 and GMPCPP have similar potency (EC$_{50}$ values of 4 and 9 μM, respectively) and efficacy (Δ$A_1$, values of 84 and 75%, respectively), GMPCPP increases $k_1$ and $k_2$ to a greater extent than YC-1 (Table 3), leading to faster overall NO dissociation. GMPCPP or GTP caused no significant change in the amplitudes or rates for NO dissociation from β1(1–194), β1(1–385), or β2(1–217) (data not shown), indicating that the acceleration of NO dissociation by GTP for the full-length enzyme must be mediated by domains or interactions not present in the constructs used here.

DISCUSSION

Over the years, the interaction of NO with sGC has been investigated with a variety of methods, leading to several models for the regulation of activation and deactivation of sGC by NO. Deactivation of the enzyme has been linked to dissociation of NO from the heme, and to gain insight into this process, NO dissociation has been studied in some detail (8, 16, 24, 38). The simplest model for the dissociation of NO from the sGC heme involves the rebinding of the proximal histidine to form a 6-coordinate heme-NO complex and subsequent dissociation of NO to form the 5-coordinate histidyl-ligated enzyme. Because studies employing electronic absorption spectroscopy have suggested that the sGC heme-NO complex is exclusively 5-coordinate (8, 35, 39), the model predicts that NO dissociation should occur as a single exponential process (Equation 2) with
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FIGURE 5. Effect of YC-1 on NO dissociation and the activity of the sGC-NO complex. A, time courses for NO dissociation from 1.3 μM sGC in the presence of increasing concentrations of YC-1 (0–96 μM) at 10 °C. The time courses shown are the average of duplicate dissociation experiments for each concentration of YC-1. B, mean ΔA1 values ± S.D. from the two-exponential fits from duplicate time courses were plotted as a function of the log of the concentration of YC-1. The data were fit to a concentration dependence equation (Equation 4) to obtain an EC50 of 9 μM for YC-1. C, effect of temperature on activation of sGC by NO in the absence and presence of YC-1. The specific activity of sGC (0.2 J9262) was determined at 37 °C (white bars) and 10 °C (dark bars). Assays were conducted in triplicate, and the data shown here are representative of four independent experiments. D, fold-activation of sGC by NO at 37 °C (white bars) and 10 °C (dark bars) obtained from the data in C. E, fold-activation of the sGC-NO complex by YC-1 at 37 °C (white bars) and 10 °C (dark bars) obtained from the data in C. Fold-activation of sGC by NO and potentiation of NO activation of sGC by YC-1 are correlated with larger ΔA1 values for NO dissociation. (** indicates significant differences: two-tailed t test, p < 0.05.)

FIGURE 6. Effect of GMPCPP on NO dissociation from the sGC heme. A, time courses for NO dissociation from 1.3 μM sGC in the presence of increasing concentrations of the substrate analogue GMPCPP (0–1000 μM) at 10 °C. The time courses shown are the average of duplicate dissociation experiments for each concentration of GMPCPP. B, mean ΔA1 values ± S.D. from the two-exponential fits from duplicate time courses were plotted as a function of the log of the concentration of GMPCPP. The data were fit to a concentration dependence equation (Equation 4) to obtain an EC50 of 4 μM for the increase in ΔA1 caused by YC-1. C, effect of temperature on activation of sGC by NO in the absence and presence of YC-1. The specific activity of sGC (0.2 μg) in the presence and absence of DEA/NO (100 μM) and YC-1 (150 μM) was determined at 37 °C (white bars) and 10 °C (dark bars). Assays were conducted in triplicate, and the data shown here are representative of four independent experiments. D, fold-activation of sGC by NO at 37 °C (white bars) and 10 °C (dark bars) obtained from the data in C. E, fold-activation of the sGC-NO complex by YC-1 at 37 °C (white bars) and 10 °C (dark bars) obtained from the data in C. Fold-activation of sGC by NO and potentiation of NO activation of sGC by YC-1 are correlated with larger ΔA1 values for NO dissociation. (** indicates significant differences: two-tailed t test, p < 0.05.)

TABLE 3
Comparison of observed rate constants and fractional amplitudes for NO dissociation from sGC in the absence and presence of YC-1 or GMPCPP at 10 °C

| Compound | k1 (s⁻¹) | k2 (s⁻¹) | ΔA1; ΔA2 (%) |
|----------|----------|----------|--------------|
| NO       | 0.0018 ± 0.0009 | 0.0018 ± 0.0001 | 13.87 ± 2.1 |
| NO/YC-1  | 0.0309 ± 0.0009 | 0.0080 ± 0.0004 | 75.25 ± 2.1 |
| NO/GMPCPP| 0.178 ± 0.009 | 0.00154 ± 0.00011 | 83.17 ± 2.1 |

Values are from averages of duplicate NO dissociation experiments containing 1.3 μM sGC-NO and 96 μM YC-1 or 1 mM GMPCPP.
study of the sGC heme-NO complex, it was suggested that upon formation the 5-coordinate sGC heme-NO species may undergo a conformational change to clamp down on the heme-NO complex, making NO dissociation more difficult (41). Dissociation from such a “clamped” complex might require a conformational change in sGC to switch the 5-coordinate heme-NO complex from closed back to open to facilitate NO dissociation. EPR spectroscopy has demonstrated that YC-1 and GTP cause similar changes in the EPR spectrum of the sGC heme-NO complex, indicating that both molecules bind to the ferrous nitrosyl protein and induce comparable changes in the heme environment (42). The data from these studies independently demonstrate that the heme-NO complex of sGC can exist in multiple 5-coordinate conformations that are differentially affected by the presence of YC-1 or GTP, consistent with the conclusions drawn in this work.

The Mechanism of Action of YC-1 and the Effect of GTP—YC-1 weakly activates unliganded sGC (4–8-fold) and synergistically activates CO-bound sGC to levels similar to those caused by NO (36). Significantly, YC-1 has also been reported to potentiate activation of sGC by NO (36, 37). In one study it was found that the deactivation rate of sGC-NO was markedly reduced by the presence of YC-1. The authors proposed that YC-1 potentiation of NO-stimulated sGC activity is because of decreased dissociation of NO from the sGC heme in the presence of YC-1, based on the assumption that deactivation of NO-stimulated sGC is a proxy for NO dissociation (37). However, recent studies have shown that deactivation does not always correlate with dissociation (16). Moreover, YC-1 accelerates overall NO dissociation from full-length sGC (Table 1), an effect magnified by lowering the temperature from 37 to 10 °C (Table 2). Thus, the acceleration of NO dissociation by YC-1 reported here indicates that the potentiation of NO-stimulated activity by YC-1 cannot be a result of a reduction in the rate of NO dissociation.

The acceleration of NO dissociation by YC-1 is reminiscent of that caused by the presence of GTP (16, 38) and suggests that YC-1 and GTP might both accelerate NO dissociation through a similar mechanism. Indeed, we found that NO dissociation in the presence of the substrate analogue GMPCPP exhibits two exponentials, and the large increase in NO dissociation caused by GMPCPP, like that caused by YC-1, is because of an increase in $k_1$, $k_2$, and $\Delta A_1$ (Fig. 6). Together, these data imply that GTP and YC-1 may affect NO dissociation from sGC via the same kinds of conformational changes, perhaps involving overlapping binding sites. Like YC-1, the presence of GTP has no effect on NO dissociation from sGC via the same kinds of conformational changes, perhaps involving overlapping binding sites. Like YC-1, the presence of GTP has no effect on NO dissociation from sGC via the same kinds of conformational changes, perhaps involving overlapping binding sites. Like YC-1, the presence of GTP has no effect on NO dissociation from sGC via the same kinds of conformational changes, perhaps involving overlapping binding sites. Like YC-1, the presence of GTP has no effect on NO dissociation from sGC via the same kinds of conformational changes, perhaps involving overlapping binding sites.
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reported to increase the activity of NO-stimulated sGC (16, 17, 37), we predicted that the fraction of open sGC-NO species present would correlate with the degree of potentiation of enzyme activity by YC-1. This turned out to be the case; conditions that decreased \( \Delta A_1 \), such as lowering the temperature from 37 to 10 °C, resulted in diminished fold-activation by NO (a measure of relative NO stimulation) (Fig. 5D). Similarly, conditions that increased the fractional amplitude due to \( k_s \), such as the inclusion of YC-1, increased fold-activation by NO. Significantly, the potentiation of NO-stimulated activity by YC-1 was greatest at 10 °C (7.1-fold versus 1.4-fold at 37 °C) (Fig. 5E). Thus it is likely that the potentiation of NO activation of sGC by YC-1 is because of an ability to shift the equilibrium between the different 5-coordinate sGC-NO species from one with low activity to one with high activity.

Physiological Significance of Two Conformations for the 5-Coordinate sGC Heme-NO Complex—The existence of two species of sGC heme-NO has important implications for sGC function, especially in light of recent findings tying spectrally similar sGC heme-NO species with different levels of activation and NO-binding behavior in the presence of allosteric nucleotide modulators to two physiologically important NO signaling modes, tonic and acute (16, 17). It was shown that incubation with GTP prior to addition of NO, or with excess NO, is required for maximal activation of the sGC heme-NO complex, an effect that is blocked by ATP. Similarly, preincubation of sGC with GTP was found to accelerate NO dissociation from the heme, an effect that is also blocked by ATP. In the presence of both ATP and GTP, the sGC heme-NO complex was demonstrated to exist as a low activity species, and NO in excess of the heme was needed for full activity. Importantly, YC-1 also converted the low activity sGC heme-NO complex to a fully active species. The results reported in this work provide mechanistic details explaining the effects of GTP and of YC-1 on activation of sGC by NO (Fig. 7B); binding of these molecules causes a conformational change from a low activity species that binds NO tightly to a high activity species with a weaker affinity for NO. Furthermore, this work indicates that the effects of GTP and YC-1 require the presence of the sGC catalytic domains, suggesting a role for these domains in regulating the conformational transition from open to closed and providing support for proposed allosteric binding sites for nucleotides and YC-1 on sGC (19, 43, 44).

In this work we also studied NO dissociation from the H-NOX domain of the \( \beta_2 \) isoform of sGC. We found that the \( \beta_2 \) H-NOX domain is similar to the \( \beta_1 \) H-NOX domain in that it also exhibits a two-exponential NO dissociation time course with similar rates and amplitudes for each exponential. However, unlike for the \( \beta_1 \) H-NOX domain constructs, NO dissociation from \( \beta_2(1–217) \) was significantly accelerated by YC-1, a feature more like sGC. On the other hand, the acceleration of NO dissociation from \( \beta_2(1–217) \) by YC-1 was reduced by lowering the temperature, which is the opposite of what was observed for full-length sGC. Together, these observations indicate that the \( \beta_2 \) isoform of sGC, the H-NOX domain of which is 43% identical to the \( \beta_1 \) H-NOX domain (21), has different NO-binding characteristics. These differences might reflect the more specialized physiological localization and function of the \( \beta_2 \) isoform, which is expressed primarily in the kidney (45) and has been proposed to function as a homodimer (46).

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