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Binding of YC-1 or BAY 41-2272 to Soluble Guanylyl Cyclase Induces a Geminate Phase in CO Photolysis

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Abstract

Soluble guanylyl/guanylate cyclase (sGC), a heme-containing heterodimeric protein of ~150 kDa, is the primary receptor for nitric oxide, an endogenous molecule of immense physiological importance to animals. Recent studies have identified compounds such as YC-1 and BAY 41-2272 that stimulate sGC independently of NO binding, properties of importance for the treatment of endothelial dysfunction and other diseases linked to malfunctioning NO signaling pathways. We have developed a novel expression system for sGC from *Manduca sexta* (the tobacco hornworm) that retains the N-terminal two-thirds of both subunits, including heme, but is missing the catalytic domain. Here, we show that binding of compounds YC-1 or BAY 41-2272 to the truncated protein leads to a change in the heme pocket such that photolyzed CO cannot readily escape from the protein matrix. Geminate recombination of the trapped CO molecules with heme takes place with a measured rate of $6 \times 10^7 \text{ s}^{-1}$. These findings provide strong support for an allosteric regulatory model in which YC-1 and related compounds can alter the sGC heme pocket conformation to retain diatomic ligands and thus activate the enzyme alone or in synergy with either NO or CO.

Nitric oxide (NO) regulates numerous physiological processes in animals, including blood pressure, platelet aggregation, memory formation and tissue development.¹ Soluble guanylyl/guanylate cyclase (sGC), a heme-containing heterodimeric protein of ~150 kDa, is the primary NO receptor. The protein is composed of two subunits, α and β , that are evolutionarily related and have similar domain structures. The N-terminus of each subunit begins with a predicted H-NOX domain followed by a PAS domain, a coiled-coil region and a cyclase domain. A single catalytic site is formed at the interface of the two C-terminal catalytic domains and catalyzes the conversion of GTP to 3',5'-cyclic GMP (cGMP) and pyrophosphate (PPi). Ferrous (Fe^{II}) heme resides in the N-terminal H-NOX domain of the β subunit and is coordinated through His 105. NO binding to the distal side of the sGC heme leads to release of the proximal histidine, formation of a five-coordinate nitrosyl complex and an ~200 fold stimulation of cyclase activity. CO binding also stimulates cyclase activity, but by only ~5-fold in the absence of allosteric activators. However, on binding allosteric activators such as synthetic compound YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole) or its derivative BAY 41-2272, CO stimulates sGC catalysis to nearly the same extent as NO, but without cleavage of the proximal histidine bond.² Molecules that stimulate sGC have long been sought for the treatment of cardiovascular and related diseases. Organic nitrates and other NO releasing compounds are commonly used for this purpose but suffer from the nonspecific side reactions of NO and a tendency for tolerance to develop with prolonged usage. Compounds that activate sGC in a

NO-independent manner, such as YC-1 or BAY 41-2272, may therefore provide a substantial therapeutic advantage.³

Very little is known about how YC-1 functions or where it binds in the protein. To address this question, we developed a novel expression construct that contains the N-terminal two thirds of $\alpha 1/\beta 1$ sGC from the tobacco hornworm (*Manduca sexta*) and demonstrated that this protein retains the YC-1 binding site despite the absence of a cyclase domain.⁴ YC-1 binding leads to increased affinity for CO and decreased release rates for both CO and NO, suggesting that YC-1 binding somehow traps CO and NO in the heme binding pocket.⁴ Here, we demonstrate, through direct observation of a geminate recombination phase for CO photolysis, that binding of YC-1 or BAY 41-2272 alters the heme distal pocket.

We examined the transient kinetics of photolyzed CO using a truncated *Manduca* sGC containing residues $\alpha 1$ 1-471 plus an N-terminal His-tag, and $\beta 1$ 1-400 with a ferrous heme, together referred to as msGC-NT. In a typical experiment, 3 μM msGC-NT was present in a 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 5% glycerol. The solution was purged with CO gas for 20 minutes. Binding of CO to msGC-NT shifted the Soret absorption band maximum from 433 nm to 425 nm.⁴ Photolysis of CO resulted in initial loss of A_{425} amplitude, initial appearance of A_{433} , and complete recovery of both absorbances on the milliseconds time scale with the same kinetics (Figure 1, top panel). The rate of slow recovery was dependent on the CO concentration, indicating that rebinding was from CO free in solution. The bimolecular rate constant of $41.5 \pm 4.0 \text{ mM}^{-1} \text{ s}^{-1}$ was obtained for this slow process (Figure S1 in Supporting Information), a value similar to that previously determined from rapid mixing in a stopped-flow spectrophotometer ($28 \text{ mM}^{-1} \text{ s}^{-1}$).⁴

Addition of YC-1 to the protein led to a 2 nm blue shift in the A_{425} Soret maximum (Figure S2) and a loss of photolysis amplitude in the milliseconds time scale (Figure 1, bottom panel). Possible explanations for the loss of signal amplitude include a change in photolytic quantum yield or the appearance of a geminate phase such that some of the photolyzed CO molecule was unable to escape from the heme pocket before recapture by the heme. To investigate these possibilities, we undertook laser photolysis on the nanosecond time scale, which is typical for CO geminate recombination in heme proteins (Figure 2). In the absence of an allosteric activator, a single slow phase is observed for recovery of the photolyzed species. In the presence of YC-1 (50 μM) or BAY 41-2272 (5 μM), a new, faster phase appears (Figure 2, top panel) with a characteristic lifetime of ~ 20 ns that behaves as a single exponential, suggesting a single site mechanism (Figure 2, bottom panel). The extent of photolysis (i.e. the yield of free Fe(II)) is similar in the presence or absence of YC-1; however, the faster phase (1) comprises $\sim 50\%$ of the total amplitude. Both YC-1 and BAY 41-2272 gave rise to a fast phase with similar rate constants (Table 1). Moreover, for both compounds, the measured fast phase rate constants are independent of CO concentration (Table 1), indicating that this fast decay is due to geminate recombination. We also examined full-length *Manduca* sGC and found that CO stimulates as well as NO in the presence of YC-1 or BAY 41-2272, but not in their absence (Figure S3), behaving much like its mammalian counterparts.²

Taken together, these data strongly indicate that YC-1 or BAY 41-2272 induce a change in sGC conformation that blocks escape of CO from the distal heme pocket and facilitates catalysis. Electrostatic effects, such as those that govern dioxygen binding to myoglobin, are unlikely to influence NO and CO escape due to the apolar nature of the Fe-NO and Fe-CO complexes.⁵ However, a change in protein conformation can have a substantial effect on NO and CO escape as demonstrated by the nitrophorins, proteins that make use of a change in protein conformation for transporting NO.⁶

It remains unknown where YC-1 and related compounds bind to the protein, and whether their binding modes are similar to that of an endogenous compound. Several binding sites have been proposed, including in the catalytic domain,⁷ the $\alpha 1$ subunit,⁸ or within the heme pocket.⁹ Our present and previous results⁴ confirm that activator binding occurs somewhere in the N-terminal two-thirds of the protein; however, our results do not rule out a second binding site in the catalytic domain. A recent study indicates that both YC-1 and BAY 41-2272 induce a change in the EPR and IR spectra of five-coordinate nitrosyl heme for the full-length rat protein but not for a truncated version of the protein containing only the $\beta 1$ heme domain, suggesting binding does not occur in the heme domain.¹⁰ We have proposed that the YC-1 binding site may be in the $\alpha 1$ H-NOX domain, which our modeling studies indicate may have a similar fold to that of the $\beta 1$ H-NOX domain but without heme.⁴ Support for this model also comes from photoaffinity labeling using a modified BAY compound containing an azido group (BAY-9491), which was found covalently linked to $\alpha 1$ Cys 238,⁸ a residue near in sequence to the putative $\alpha 1$ H-NOX domain.⁴

Binding of both YC-1^{2,9,11} and nucleotide¹² to full-length sGC affects the sGC heme pocket, making their individual contributions to function difficult to resolve. For example, binding of GTP and YC-1 to full-length bovine sGC leads to three CO association rates, a slower bimolecular phase ($90 \text{ mM}^{-1}\text{s}^{-1}$), a faster bimolecular phase ($97 \mu\text{M}^{-1}\text{s}^{-1}$) and a geminate recombination phase (not characterized), while in the absence of YC-1, only the slowest phase is seen.⁹ In contrast, msGC-NT displays only a slow bimolecular phase in the absence of YC-1 ($41.5 \text{ mM}^{-1}\text{s}^{-1}$), and both the slow phase and a second geminate recombination phase in the presence of YC-1 that is ~ 1000 times faster than the bovine bimolecular fast phase. Importantly, msGC-NT, which does not contain the cyclase domain, does not respond to cGMP, GTP or ATP, indicating that the YC-1 binding site is distinct from that for nucleotide.⁴ We conclude from this that both YC-1 and nucleotides induce a change in the heme pocket of the fulllength protein, but do so independently of one another, resulting in multiple ligand binding effects.^{11,12} By isolating the YC-1 binding site in msGC-NT through removal of the cyclase domain, we provide a powerful new tool for investigating NO-independent stimulation of sGC and the discovery of improved compounds for the treatment of cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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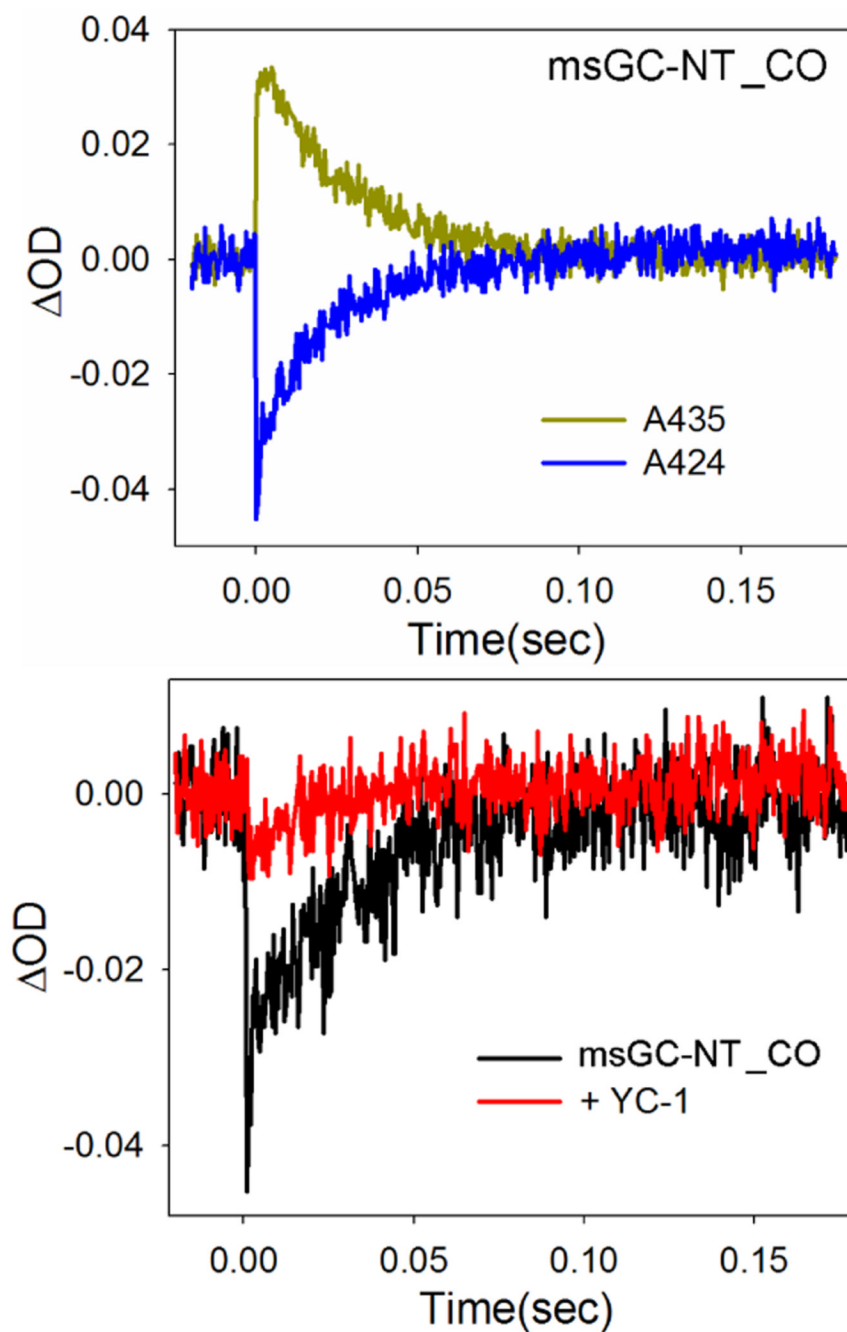


Figure 1.

Effect of YC-1 on photolysis of CO from msGC-NT. Top panel: Photolysis by a 386 nm nitrogen dye laser pulse leads to bleaching of the A_{425} band, appearance of the A_{433} band, and a complete recovery exhibiting a single phase. Monitoring was at 424 and 435 nm, respectively. Bottom panel: Addition of YC-1 leads to the loss of signal amplitude (A_{422}) on the milliseconds time scale.

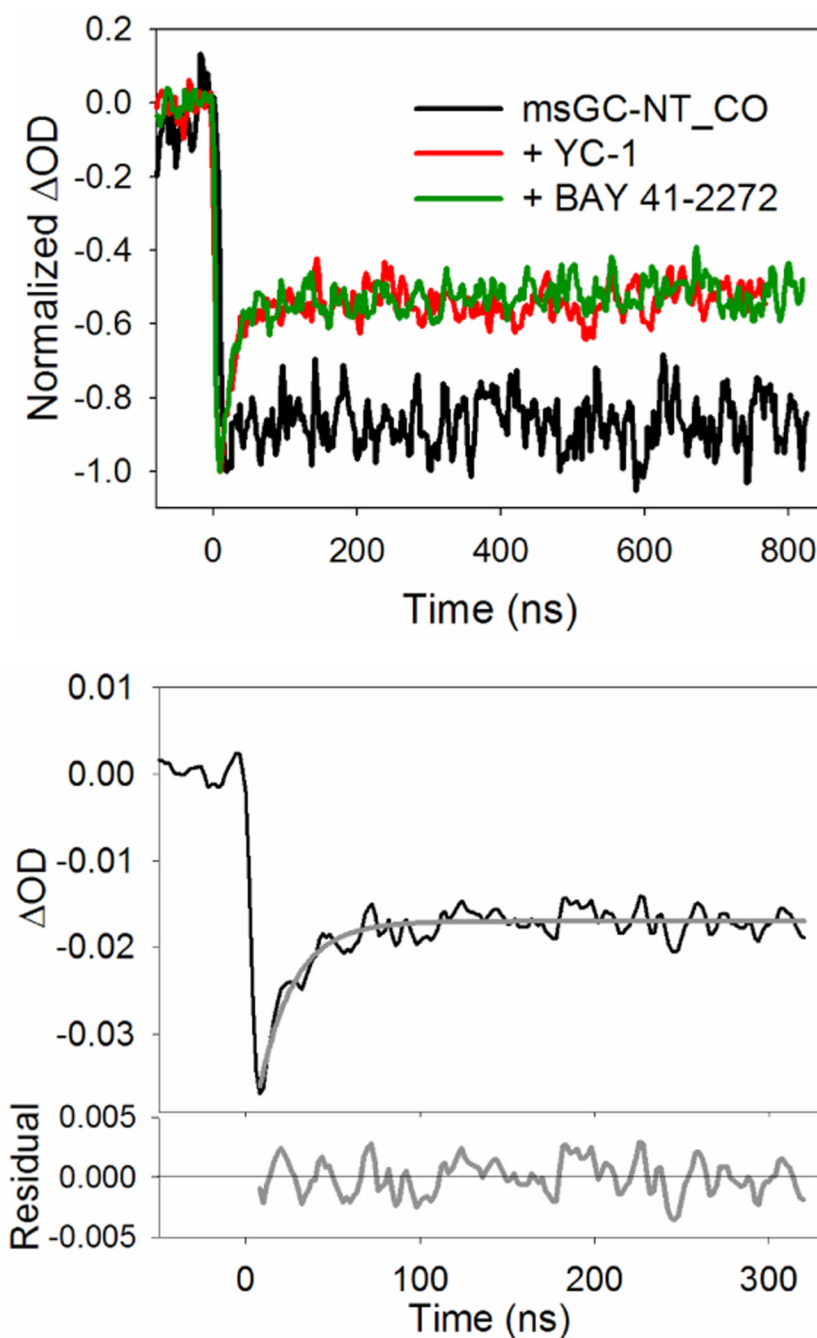


Figure 2.

Nanosecond laser photolysis of CO from msGC-NT. Top panel: Photolysis by a 532 nm Nd:YAG laser pulse in the presence of YC-1 or BAY 41-2272 yields fast and slow phases (red and green lines, monitored at 422 nm); in the absence of activator, only a single slow phase is evident (black line, monitored at 424 nm). Bottom panel: Single exponential fitting of the faster phase for the YC-1 bound sample.

Table 1

Rate constants for CO geminate rebinding to msGC-NT

Compound	[CO] ^a	$k_{\text{gem}}, \text{s}^{-1} \times 10^7$
YC-1	Saturated	5.8 ± 0.8
	10%	6.7 ± 1.2
BAY 41-2272	Saturated	5.9 ± 0.9
	10%	7.0 ± 1.0

^aSaturated CO is of ~1 mM; 10% CO is of ~0.1 mM.