Supporting Information

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

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Materials and Methods

Reagents and protein. The heterodimeric sGC N-terminal fragment from *Manduca sexta* (msGC-NT) was expressed and purified as described elsewhere.[1] Briefly, msGC α1 (residues 1-471) and β1 (1-401) fragment sequences were assembled into a single plasmid (pETDuet-1, Novagene) with a 6xHis tag fused onto the N-terminus of the α1 subunit, and expressed in *E. coli* strain BL21 (DE3) pLysS. Recombinant protein was purified as a heterodimer containing ferrous heme using metal affinity and size exclusion chromatography. Final purity was greater than 90%. Protein was then concentrated, buffer-exchanged into a buffer containing 50 mM K_2HPO_4 / KH_2PO_4 (pH 7.4), 100 mM KCl and 5% glycerol (protein buffer), and stored at -80 °C. For photolysis experiments, protein was diluted to 3-5 μM. YC-1 (Cayman Chemicals) and BAY 41-2272 (Alexis) were dissolved in dimethyl sulfoxide (DMSO) before adding to the protein sample. DMSO concentration did not exceed 1% in the final reaction mixtures. The NO donor 2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide (DEA/NO) was a gift from Dr Katrina Miranda and was dissolved in 10 mM NaOH.

Full-length *Manduca* sGC was expressed in a manner similar to that for msGC-NT, and partially purified using metal affinity chromatography, as previously described.[1]

Preparation of msGC-NT complexes. To make the msGC-NT-CO complex, msGC-NT samples were placed in a septum-capped photolysis cuvette and purged with a stream of CO gas over the top of the sample for 20 minutes. Formation of the CO complex was confirmed by UV-visible spectroscopy spectrum, with the msGC-NT-CO complex displaying a characteristic

Soret absorbance at 425 nm in the absence of activator or 423 nm in the presence of YC-1 or BAY 41-2272 (Figure S1). For experiments using non-saturating CO, the CO complex was prepared by mixing with a known volume of CO saturated protein buffer (assumed to be 1 mM) to yield the desired CO concentration. To make the msGC-NT-NO complex, protein buffer in a septum-covered cuvette was bubbled with argon for 10 min followed by addition of protein and 20-30 min additional purging with an argon stream above the solution. A 5-fold excess of DEA/NO stock solution was added to the protein solution followed by a 10 min period to allow for NO release.

Laser flash photolysis. Millisecond flash photolysis experiments were carried out with a nitrogen-pumped dye laser (LN 102, Photochemical Research Associates, Inc.) with a pulse width of 200 ps, using 2.5 mM BBQ ($\lambda_{emission}$ = 386 nm, Photochemical Research Associates, Inc.) in toluene / ethanol (50/50) as the dye. Samples were excited at 386 nm and kinetic transients were recorded, digitalized, and averaged on a Tektronix TDS 410A oscilloscope. Nanosecond flash photolysis experiments were conducted using an Edinburgh LP920 laser flash photolysis spectrometer, in combination with a Q-switched Continuum Surelite I-10 Nd:YAG laser and Continuum Surelite OPO. Briefly, a 532 nm laser pulse was focused onto the sample cell, and used to trigger the reactions. The pump energy per pulse was ~1.2 mJ. The samples were kept at 25 °C during the measurements. Full spectral scans were performed for both the millisecond and nanosecond photolysis experiments to ensure that amplitude loses were not due to the small shift in absorption maxima on binding YC-1 or BAY 41-2272. Monitoring wavelengths were

chosen as the maxima and minima in the difference spectra, which differ slightly from the Soret maxima of the individual species.

We also conducted nanosecond laser photolysis of the msGC-NT NO complex, but were unable to detect a signal. A previous study examining sGC from bovine lung indicated photolyzed NO recombined with the sGC heme extremely efficiently (97% geminate recombination) and extremely quickly (τ = 7.5 ps).[2] Most likely, NO rebinding in msGC-NT behaves similarly.

Data processing. Kinetic data were plotted using SigmaPlot and fitted to single or double exponential models with background, as appropriate. First order rate constants (k_{obs}) are reported as the mean and standard deviation of 3 or more measurements, while the second order rate constants are from the slope and least squares fitting error of k_{obs} vs. [CO], where k_{obs} is the average of 3 or more measurements (Figure S2). For comparison of spectra from different experiments, the data were normalized to the maximum signal after the laser pulse.

Guanylyl Cyclase Enzymatic Assay. The cGMP producing activity of partially purified full length msGC was measured using an enzyme immunoassay kit (Cayman Chemical Co.), following the manufacturer's instructions, as previously described.[1] In a typical assay, 10 µl of reaction buffer (0.5 M 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 µl. Product was produced for a 10 min period at 23 °C and then quenched with 200 µl of 250 mM zinc acetate and 200 µl of 250 mM sodium carbonate. For experiments measuring NO- or CO-activated enzyme activity, protein

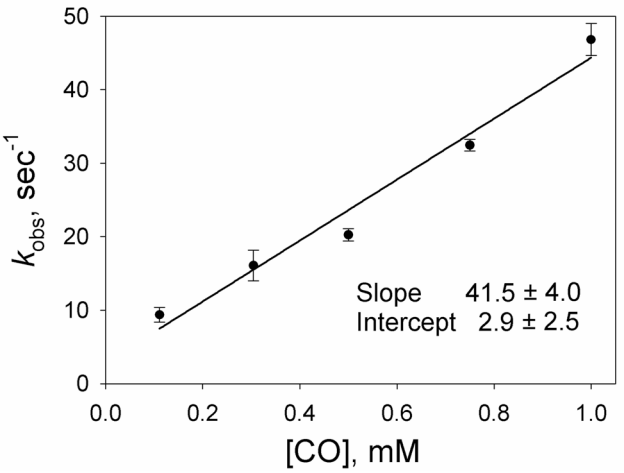
samples were pre-mixed with DEA/NO or CO before initiating catalysis by addition of reaction buffer.

References

1. Hu, X.; Murata, L.; Weichsel, A.; Brailey, J.; Roberts, S. A.; Nighorn, A.; Montfort, W. R. *J. Biol. Chem.* **2008**, *283*, 20968-20977.

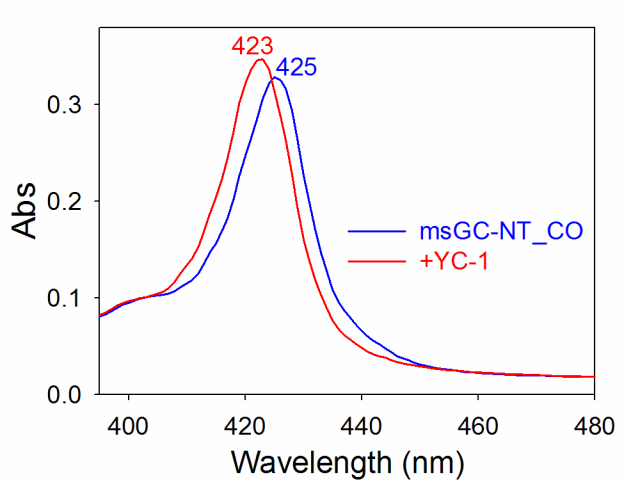
2. Négrerie, M.; Bouzhir, L.; Martin, J. L.; Liebl, U. *J. Biol. Chem.* **2001**, *276*, 46815-46821.

Figure S1 (Hu et al.)



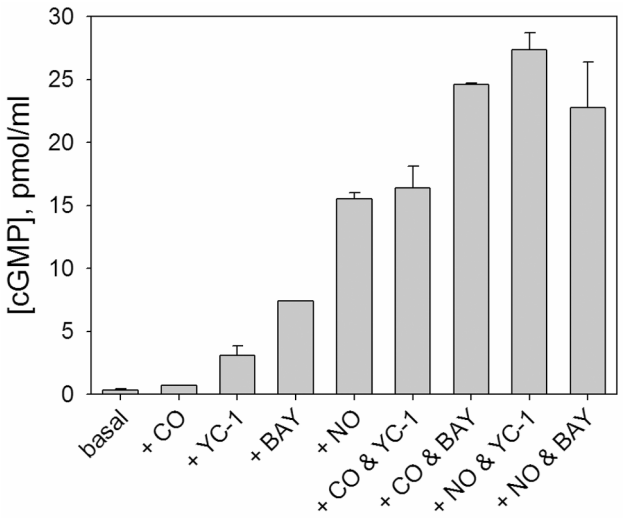
Dependence of k_{obs} on CO concentration for the slower photolysis phase. k_{obs} values are from the single-exponential fitting of ΔA_{424} (average of three or more measurements). Differing CO concentrations were achieved by dilution of a saturated CO solution assumed to be 1 mM.

Figure S2 (Hu et al.)



UV-visible spectra of the msGC-NT-CO Soret band ± YC-1. Note the 2 nm shift in, and sharpening of, the band in the presence of YC-1. BAY 41-2272 (not shown) induces a similar change in the spectrum.

Figure S3 (Hu et al.)



CO stimulation of guanylyl cyclase activity. Production of cGMP by full-length msGC was measured using an immunoassay. The concentration of msGC was ~1 μM as estimated by Soret-band absorption. Where included, the concentrations of CO, DEA/NO, YC-1 and Bay 41-2272 were 100 μM, 20 μM, 50 μM and 5 μM, respectively. Values shown are the average of two separate measurements. Error bars indicate the range of measured values.