Supporting Information

Oxidation and Loss of Heme in Soluble Guanylyl Cyclase from *Manduca Sexta*

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Materials and Methods

Protein purification. All chemicals were obtained from Sigma-Aldrich unless otherwise described. The heterodimeric N-terminal fragment from *Manduca sexta* sGC (*Ms* sGC-NT1) was expressed and purified as described elsewhere (*1*). Briefly, *Ms* sGC α1 (residues 1-471) and β1 (1-401) fragment sequences were assembled into a single plasmid (pETDuet-1, Novagen) with a 6xHis-tag fused on the N-terminus of the α1 subunit, and expressed in *E. coli* strain BL21 (DE3) pLysS. *Ms* sGC-NT2, which expresses α 49-471 and β 1-401 has also been reported previously (*1*), and was used as the starting point for construct *Ms* sGC-NT13. NT13 was prepared by inserting stop codons into the *Ms* sGC-NT2 plasmid at positions α451 and β381 using the QuikChange Lightening site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the *Ms* sGC-NT2 plasmid as template and primer 5′-caaggaacgagagaagtaagtcagcctgctgcatttaatattcc-3′ for αN451-Stop and primer 5′-

ctggaattggaaaaacagaagtaggacaggcttctttactcag-3′ for βT381-Stop. Recombinant protein was purified as a heterodimer containing ferrous heme using Ni-affinity and size exclusion chromatography. Final purity was greater than 90% for both NT1 and NT13. Protein was concentrated in a final buffer containing 50 mM potassium phosphate (pH 7.4), 100 mM KCl and 5% glycerol, and stored at -80°C. All protein used in the electrochemical study was from a batch of the same NT1 preparation and all protein used in the heme kinetics study was from a batch of the same NT13 preparation. Apo-myoglobin H64Y/V68F (ApoMb) was prepared as described by Hargrove and coworkers (*2*) from a plasmid provided by Dr. John Olson.

Heme Dissociation from Manduca sexta sGC. Rates of heme dissociation were measured by following the shift in the Soret absorbance of *Ms* sGC-NT13 to ApoMb H64Y/V68F as described by Hargrove and coworkers with minor changes (*2*). Full spectra from 700-280 nm were taken at 15 minute intervals for 15 hours on a Cary UV-vis spectrophotometer with cooling to 20°C using a 1-cm path-length screw-cap cuvette sealed with a silicone septum. Maximal absorbance changes were plotted versus time and fit to a 3-parameter single exponential using SigmaPlot (SPSS, Inc., Chicago). Each sample contained 2.0 μM *Ms* sGC-NT13 and 20 μM ApoMb in a 50 mM sodium phosphate buffer (pH 7.4) with 300 mM NaCl, and 0.45 M sucrose. The oxidized NT13 complex was formed by adding 50 μM ferricyanide at the start of the reaction. The NO-complex was formed by adding 10 μM 2-(*N*,*N*-Diethylamino)-diazenolate-2-oxide (DEA/NO), a kind gift of Dr. Katrina Miranda, and allowing to equilibrate at room temperature for 5 minutes before the first spectroscopic measurement. YC-1 (Cayman Chemicals, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO) before adding to the protein sample. DMSO concentration did not exceed 0.5% in the final sample mixtures for measurement. Peroxynitrite was obtained from Calbiochem.

Mediator Preparation. Mediator stock solution is a mixture of 16 different mediators at 130 mM each, buffered with 100 mM sodium phosphate (pH 7.5). Included mediators are 2-methyl-1,4-naphthalenedione, N,N-Diethyl-2-methyl-1,4-phenylenediamine, neutral red, 1,2-naphthoquinone, 1,1'-dimethylferrocene, tetrachlorobenzoquinone, benzyl viologen, N,N-Dimethyl-1,4-phenylenediamine, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulfonate, methyl viologen, tetramethyl-p-phenylenediamine, 2,6-dichlorophenolindophenol, trimethylhydroquinone, hexaammineruthenium chloride. To achieve different pH for reduction potential measurement, protein buffers were prepared with the same ionic strength (50 mM

potassium phosphate, 100 mM KCl) at various pH (pH 7.0, 7.4, 8.0). The pH of the mediator solutions was adjusted accordingly for each specific measurement. To prepare a sample for measurement, 42 μl of mediator solution was mixed with an aliquot of protein stock and protein buffer to bring to a total volume of 137 μl with final protein and mediator concentrations 6 μM and 40 mM respectively.

Electrochemical Measurements. *Ms* sGC-NT1 electrochemical reduction measurements in the presence or absence of 50 mM YC-1 were carried out using the same instrumentation and the same reference electrode (Ag/AgCl, $E°$ = -205 mV vs. SHE) as described previously (*3-5*). After setting the potential at certain value E_{app} and waiting until the optical spectrum did not change (typically 5-10 minutes), the spectrum was recorded, followed by setting a new potential E_{app} (20 mV step) and repeating the process. The sample temperature was maintained between 7-10 °C during the experiments by dry ice dispersion from the top of the cuvette chamber. The data are analyzed in terms of the Nernst equation, which describes the effect of applied potential on the ratio of the concentrations of oxidized (ferric, Fe^{III}) and reduced (ferrous, Fe^{II}) forms of the enzyme and the standard reduction potential, E:

$$E_{app} = E° + \left(\frac{RT}{nF}\right)\ln\left(\frac{[Ox]}{[Red]}\right)$$

where E_{app} is the applied potential, $E°$ is the reduction potential determined from these data, and [Ox] and [Red] are the concentrations of the protein in the ferric and ferrous states, respectively. Concentrations of the ferric and ferrous protein are proportional to the absorbance peaks at 392 nm and 433 nm in the optical spectra according to Beer's law. For best signal output, the maximal absorbance difference between two wavelengths (395 nm and 435 nm) of an individual spectrum was chosen to fit into the following equation to give the reduction potential:

$$\Delta A = y0 + \frac{a}{1 + e^{\left(\frac{E_{app} - (E° - 205) \times nF}{1000 \times RT}\right)}}$$

where $\Delta A = A_{395} - A_{435}$, E_{app} and $E°$ are in units of mV, n = 1, T = 281.65 K. Data were plotted and fit using SigmaPlot (SPSS, Inc., Chicago).

References

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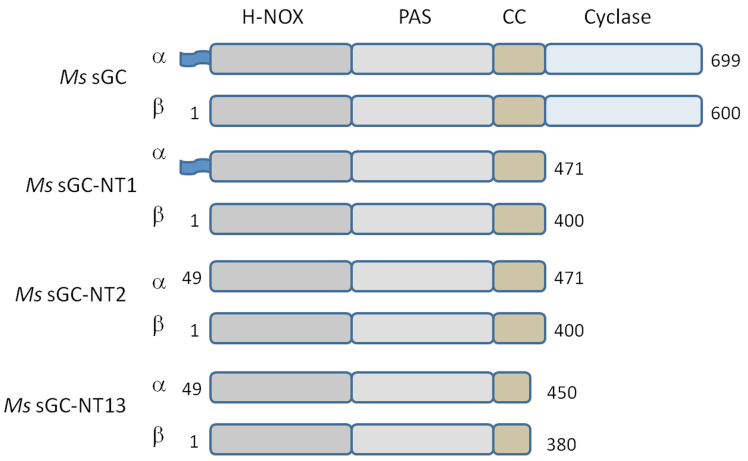

Figure S1. Domains of *Ms* sGC and boundaries for constructs used.

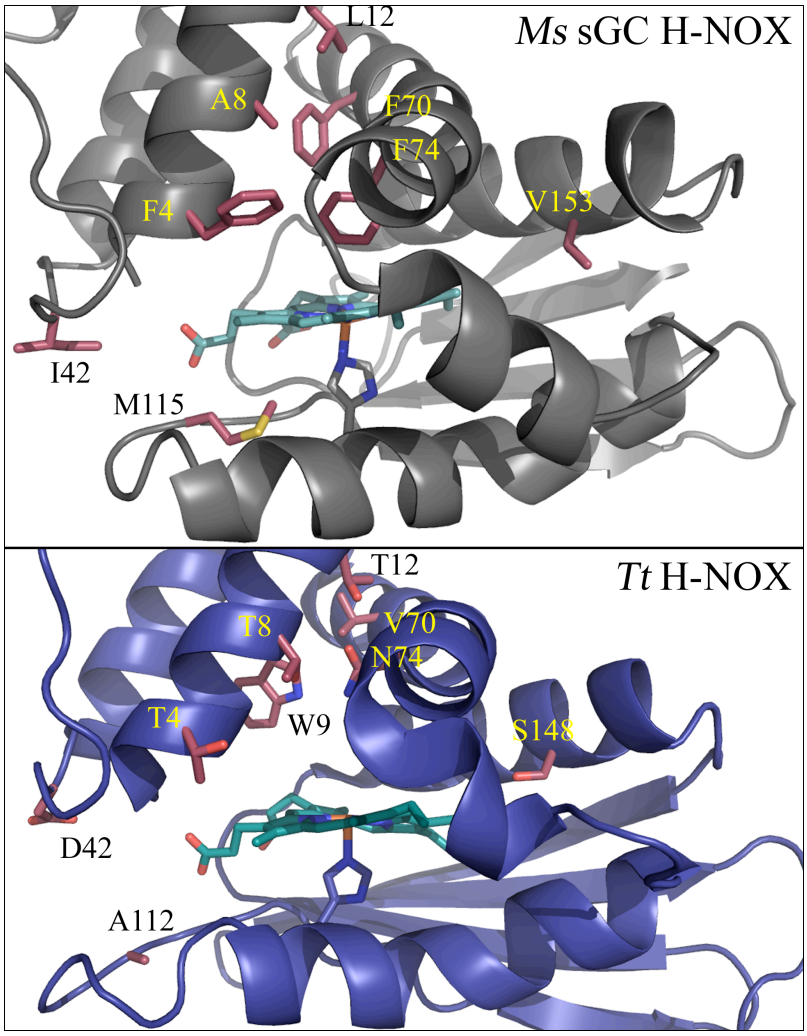

Figure S2. Comparison of the *Ms* sGC β H-NOX domain model with the structure of *Tt* H-NOX (PDB ID 1U4H). The non-conserved residue side chains displayed in the *Ms* sGC model (F4, A8, L9, L12, I42, F70, F74, M115, V153) are significantly more hydrophobic than those found in the same positions in the *Tt* H-NOX structure (T4, T8, W9, T12, D42, V70, N74, A112, S148).

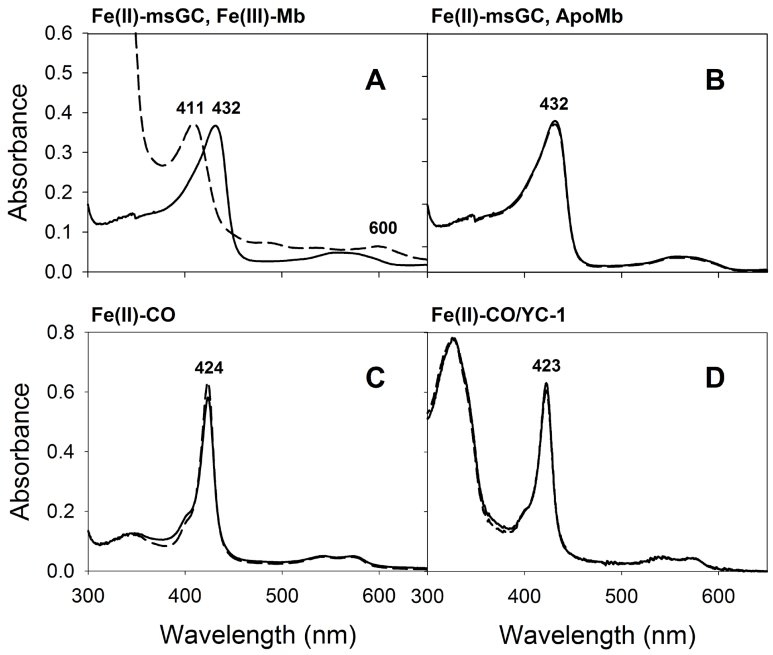

Figure S3. Initial and final spectra of ferrous *Ms* sGC complexes mixed with ApoMb. **A.** Overlay of the UV/visible spectrum of 2 μM *Ms* sGC-NT13 (solid line) with that for 20 μM ApoMb mixed with 2 μM hemin (ferric) and 50 μM YC-1 (dashed line). Addition of dithionite yields myoglobin with Soret and α/β maxima of 428 and 560 nm (not shown). **B.** *Ms* sGC-NT13 (2 μM) in the presence of 10-fold excess ApoMb at 0 (solid line) and 15 hr (dashed line). The spectra are perfectly overlaid, indicating that no sGC heme is lost. In separate experiments, the spectra for *Ms* sGC-NT13 is unchanged on the order of weeks at room temperature (not shown). **C.** As in B but under saturating CO. **D.** As in B but under saturating CO plus 50 μM YC-1.

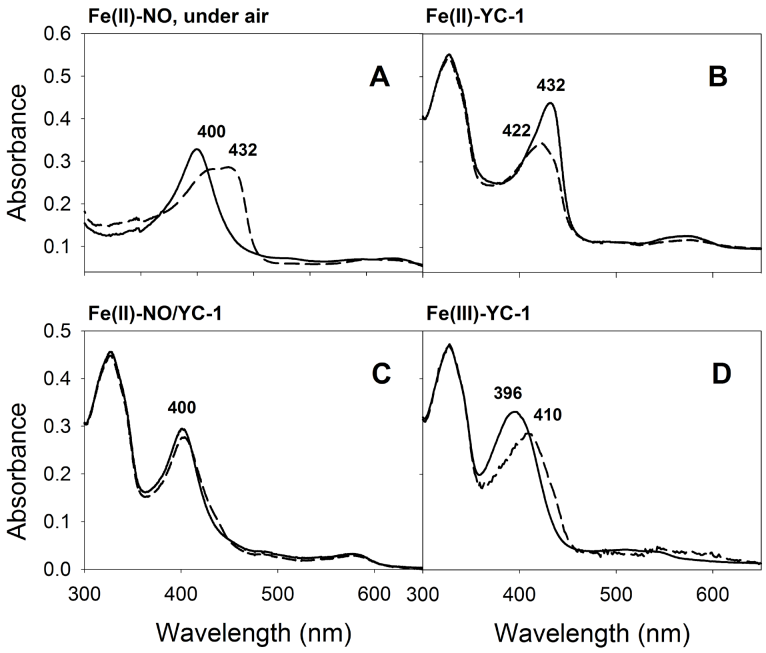

Figure S4**.** Initial and final spectra of ferrous NO and ferric *Ms* sGC complexes mixed with ApoMb. **A.** *Ms* sGC-NT13-NO-complex (2 μM) formed with 10-fold excess of DEA/NO, in the presence of 10-fold excess ApoMb at 0 (solid line) and 15 hr (dashed line) under air shows loss of the 400 nm peak and re-formation of the original reduced heme at 432 nm. **B.** As in A without NO and with 50 μM YC-1. The ferrous sGC Soret decays over 15 hr to an as-yet undefined species. **C.** As in A plus 50 μM YC-1. The NO-complex remains intact and stable in the presence of YC-1. **D.** As in B plus ferricyanide. Oxidation of sGC heme causes release of heme to ApoMb in the presence of excess YC-1.

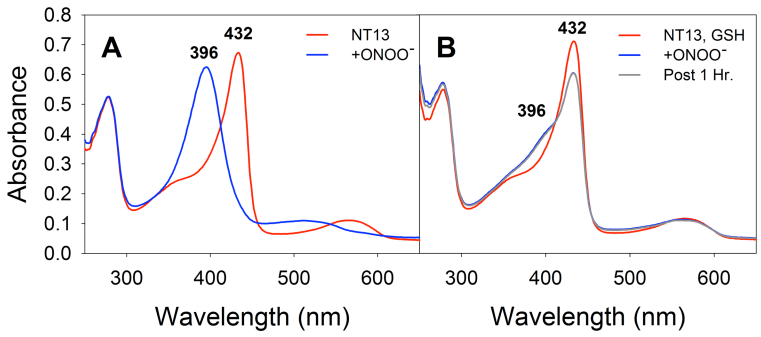

Fig. S5. Peroxynitrite treated sGC. **A.** 5 μM *Ms* sGC-NT13 (red) plus ~80 μM peroxynitrite immediately yields the ferric protein (blue). **B.** 5 μM *Ms* sGC-NT13 in 5 mM GSH (red) plus ~160 μM peroxynitrite yields a mixture of ferric and ferrous protein (blue), which remains unchanged after 1 hr (gray).