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

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Supporting Information

ABSTRACT: Oxidation and loss of heme in soluble guanylyl/guanylate cyclase (sGC), the nitric oxide receptor, is thought to be a major contributor to cardiovascular disease and is the target of compounds BAY 58-2667 and HMR1766. Using spectroelectrochemical titration, we found a truncated sGC to be highly stable in the ferrous state (234 mV) and to bind ferrous heme tightly even in the presence of NO, despite the NO-induced release of the proximal histidine. In contrast, oxidized sGC readily loses ferric heme to myoglobin ($0.47 \pm 0.02\ h^{-1}$). Peroxynitrite, the presumed cellular oxidant, readily oxidizes sGC in 5 mM glutathione.

The biological production of nitric oxide (NO), a free radical molecule, is vital to the development and function of nearly all animals. NO is produced by NO synthase and regulates numerous physiological activities, including blood pressure, wound healing, and memory formation. The NO signal is propagated through the heme-containing enzyme soluble guanylyl cyclase (sGC), an ~150 kDa heterodimeric protein that may reside in the same cell as NO synthase, or in nearby cells. The two sGC subunits are gene duplications with each containing H-NOX, PAS, coiled-coil, and cyclase domains.[1] NO binds to ferrous heme in the β subunit, which leads to rupture of the proximal histidine bond, stimulation of cyclase activity, and conversion of GTP to cGMP. A variety of tissue-specific physiological responses can result, including smooth muscle relaxation and vasodilation. There are no structures of sGC, but several structures of bacterial H-NOX proteins have been determined and reveal a single-domain fold with a deep hydrophobic cleft for heme binding.[2−4]

sGC is prone to heme loss during isolation[5] and apparently also in the cell, particularly after oxidation, which can occur, for example, during inflammation or heart failure. Compounds BAY 58-2667 (cinaciguat) and HMR1766 (ataciguat) were designed to rescue apo-sGC by filling the heme pocket, leading to an sGC complex with high catalytic activity and a decreased rate of turnover in the cell.[6−11] Investigations into the stability of the sGC heme, its reduction potential, and its propensity for loss are needed but have been challenging to pursue, in part because of the difficulty in working with the protein.

We have produced recombinant forms of sGC from *Manduca sexta* (*Ms*), the tobacco hornworm, that include H-NOX, PAS, and coiled-coil domains of both α and β subunits, but not the cyclase domains, and display greater stability than the full-length protein does[12,13] (Figure S1 of the Supporting Information). These proteins are bacterially expressed with an intact ferrous heme, display CO and NO binding that is similar to that for the full-length protein, and respond to allosteric stimulators 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) and BAY 41-2272.[12,13] Here, we make use of previously described construct *Ms* sGC-NT1 (α 1−471, β 1−401) to measure the heme reduction potential and newly developed *Ms* sGC-NT13 (α 49−450, β 1−380) to measure heme loss kinetics. Construct *Ms* sGC-NT13 was trimmed to remove the first 48 residues from the α subunit, which are predicted to be disordered, and 21 residues from the C-termini of both α and β subunits, which are predicted to link the coiled-coil and cyclase domains, leading to a protein with increased levels of expression and stability. Ferrous, unliganded *Ms* sGC-NT13, as well as its complexes with CO and YC-1, is stable at room temperature on the time scale of days to weeks with no change in the Soret absorbance maximal position or intensity. The Soret absorbance maxima for unliganded *Ms* sGC-NT13 (433 nm), its complexes with CO (423 nm), CO/YC-1 (422 nm), and NO (400 nm), and *Ms* sGC-NT13 after oxidation (394 nm) correspond with values previously published for *Ms* sGC[12] and other sGC proteins.[14]

One of the remarkable properties of heme as a protein cofactor is the degree to which heme reduction potential and ligand specificity can be tuned by the protein for the job at hand. We determined the midpoint potential for *Ms* sGC-NT1 by spectroelectrochemical titration at three pH values (Figure 1 and Table 1). *Ms* sGC has an unusually high midpoint potential for a *b*-type heme, 234 mV at pH 7.4, which stabilizes the ferrous state. This value is relatively insensitive to small changes in pH, indicating the absence of ionizable groups near the heme, including heme-ligated water, that have pK_a values below 10. Binding of compound YC-1, which stimulates sGC and blocks escape of both CO and NO from the heme pocket,[12,13] leads to a small (22 mV) increase in the measured reduction potential (Figure 1 and Table 1), consistent with an increase in the level of heme pocket desolvation upon YC-1 binding.

Although NO binds to both ferri- and ferroheme centers, ferroheme provides at least two functional advantages for sGC. First, binding of NO to ferrous heme is extremely tight, exhibiting dissociation constants in the picomolar to femtomolar range. This allows very low NO concentrations to initiate the signaling cascade and prevents the higher NO concentrations that give rise to nitrosative stress. Recent estimates for typical NO concentrations of importance for signaling in vivo are between 0.1 and 5 nM.[15] Second, the trans effect of NO binding is

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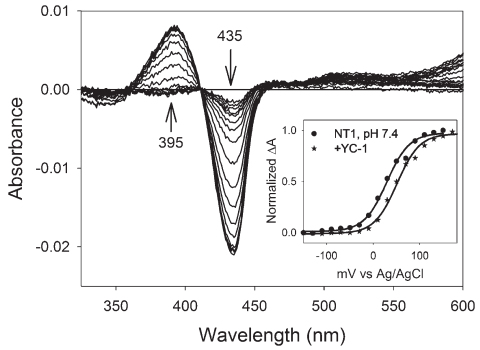

Figure 1. Spectroelectrochemical titration of *Ms* sGC-NT1. Applied voltages were from −150 to 170 mV vs Ag/AgCl in 20 mV increments (add 205 mV for potential vs NHE). The inset shows Nernst plots with or without YC-1. Measurements were taken in protein buffer containing 50 mM potassium phosphate (pH 7.4), 100 mM KCl, and 5% glycerol.

Table 1. *Ms* sGC-NT1 Midpoint Potentials (millivolts)

	pH 7.0	pH 7.4	pH 8.0
without YC-1	241 ± 2	234 ± 3	228 ± 2
with YC-1[a]	ND[b]	256 ± 2	ND[b]

[a] At 50 μM. [b] Not determined.

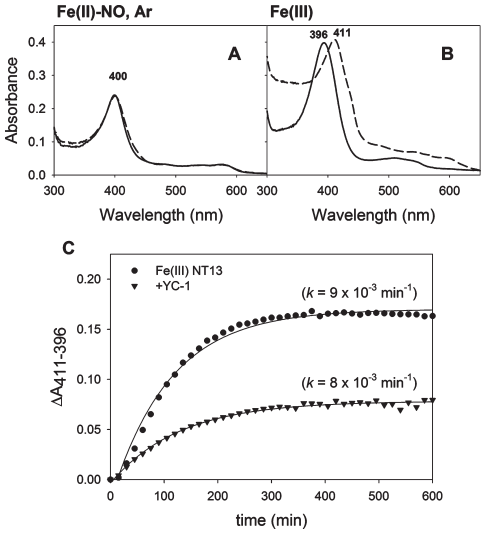

Figure 2. Heme loss measurements. All samples contained 2 μM *Ms* sGC-NT13 and 20 μM ApoMb H64Y/V68F. (A) *Ms* sGC-NT13−NO complex (ferrous) formed with 10-fold excess of DEA/NO after 0 (—) and 15 h (---) under saturating argon. No change in the heme Soret band is observed, indicating no loss of heme to ApoMb. (B) Ferric *Ms* sGC-NT13 (—) loses heme to ApoMb, forming metmyoglobin (---). (C) Absorbance change ($\Delta A_{411-396}$) plotted vs time for the loss of *Ms* sGC (Soret maximum at 396 nm) and subsequent formation of metmyoglobin (Soret maximum at 411 nm), with or without YC-1. Rates were determined by a fit to a three-parameter single exponential.

particularly prominent for ferroheme,[16] allowing for proximal histidine release and the propagation of an NO-dependent conformational change from the heme pocket to the catalytic center.

The measured reduction potential of *Ms* sGC-NT1 is approximately 200 mV more positive than that of myoglobin[17] and 67 mV more positive than that of the oxygen-binding *Tt* H-NOX protein,[18] both of which function as ferroheme proteins. The ferriheme nitrophorins from *Rhodnius prolixus*, which transport NO from the insect saliva to a victim during blood feeding, maintain a midpoint potential of approximately −300 mV,[19] more than half a volt more negative than that of sGC. The ferriheme allows the nitrophorins to readily bind, transport, and release NO. How heme proteins acquire an appropriate midpoint potential is incompletely understood but likely involves electrostatic stabilization, the influence of coordinating ligands, and heme geometry. The *Rhodnius* nitrophorins achieve their negative reduction potential in part through judicious placement of negatively charged side chains inside the protein, which stabilizes the positively charged ferric heme,[20] and through heme ruffling distortion, which mixes porphyrin and metal orbitals to stabilize Fe(III) and disfavor heme reduction.[21,22] Myoglobin has a partially polar heme pocket and a relatively planar heme, even when bound to NO.[23] The H-NOX proteins, however, have very hydrophobic heme pockets,[2−4] which favor neutral ferrous heme, but are also highly distorted, which should disfavor ferrous heme but may not, on the basis of the results of site-directed mutagenesis of *Tt* H-NOX.[18,24] In those experiments, mutations leading to a decreased level of heme distortion also lead to decreased reduction potentials at the pH examined. However, the mutations alter the pK_a of the water ligand axial to the ferric heme,[24] suggesting the reduction potential may have substantial pH dependence. A homology model of the *Ms* sGC β H-NOX domain[12] suggests that sGC has an even more hydrophobic pocket than *Tt* H-NOX (Figure S2 of the Supporting Information), which is consistent with the more positive midpoint potential presented here and its invariance with pH (Table 1).

To assess rates of heme loss in *Ms* sGC, we adapted the approach of Hargrove et al. using an H64Y/V68F myoglobin variant that has a distinct absorbance spectrum and a unique green color.[25] Addition of hemin to the apoprotein led to a ferric (met) myoglobin with Soret and α/β band absorbance maxima of 411 and 600 nm, respectively (Figure S3A of the Supporting Information). Excess YC-1 had no effect on the uptake of hemin by apomyoglobin (ApoMb). Addition of ferrous hemin led to a ferrous myoglobin with Soret and α/β band absorbance maxima of 428 and 560 nm, respectively.

We investigated the transfer of heme from *Ms* sGC-NT13 to a 10-fold excess of ApoMb under a variety of conditions. No loss of heme was detected from ferrous *Ms* sGC, its CO complex, or its CO/YC-1 complex over a 15 h period (Figure S3 of the Supporting Information), suggesting that ferrous heme does not escape from *Ms* sGC in this time period or binding is substantially tighter to ferrous *Ms* sGC than to ApoMb. Interestingly, the ferrous sGC−NO complex, which is five-coordinate after cleavage of the proximal histidine bond, retains high heme affinity and does not lose heme to ApoMb (Figure 2A). Under anaerobic conditions, the sGC−NO complex is completely stable in the presence and absence of ApoMb but will slowly lose NO under aerobic conditions, presumably through NO release and reaction with dioxygen (Figure S4 of the Supporting Information). Addition of YC-1, which slows NO release, completely stabilizes the sGC−NO complex over 15 h.

In contrast, oxidation of *Ms* sGC-NT13 and addition of ApoMb lead to the loss of absorbance at 396 nm and an increase in absorbance at 411 nm (Figure 2B), indicating the transfer

of heme from *Ms* sGC to ApoMb. Transfer is relatively rapid at 20 °C and occurs with a first-order rate constant of 0.55 ± 0.02 h^{-1} (Figure 2C), which is 20−80 times faster than the loss of heme from native sperm whale metmyoglobin at 37 °C, depending on pH conditions (0.007 h^{-1} at pH 7.0 and 0.03 h^{-1} at pH 8.0[25]). A strong increase in absorbance at 280 nm in addition to a large drift in the baseline signal over the course of the experiment results from precipitation of apo-*Ms* sGC. In the presence of YC-1, the rate of ferriheme loss and the propensity for precipitation are unchanged (Figure 2C).

The formation of peroxynitrite ($ONOO^-$) from the reaction of NO and superoxide (O_2^-) is thought to occur in vivo during inflammation and to directly oxidize sGC heme.[8,26] To investigate this possibility, we added ∼80 μM peroxynitrite to 5 μM *Ms* sGC-NT13 while monitoring the sGC spectra. Conversion of the ferrous heme to ferric heme was rapid and complete (Figure S5A of the Supporting Information) and completely reversible with sodium dithionite, a strong reductant. Addition of 5 mM reduced glutathione, the major reductant in the cytosol and present at a concentration of 1−10 mM, was able to only partially re-reduce oxidized sGC. Addition of ∼160 μM peroxynitrite to 5 μM sGC in a buffer containing 5 mM GSH still yielded a small percentage of oxidized sGC, which remained unchanged after 1 h (Figure S5B of the Supporting Information). Taken together, these data suggest that peroxynitrite can lead to oxidized sGC under cellular conditions.

In summary, *Ms* sGC is highly stabilized toward the ferrous state and, when ferrous, is highly resistant to heme loss in the absence and presence of NO. Heme oxidation leads to a highly unstable protein that readily loses heme ($t_{1/2}$ = 76 min). Peroxynitrite rapidly oxidizes the ferrous heme and can occur even in the presence of 5 mM glutathione. These data are consistent with the proposed mechanism for compounds BAY 58-2667 and HMR1766 in overcoming the loss of sGC activity during oxidative stress in the cell, which is thought to occur by filling the apo-sGC heme pocket and rescuing the protein, leading to an activated and stabilized sGC molecule.[6,8,9] Our data also highlight the stability of the ferrous heme complex even in the absence of a proximal histidine bond, which occurs in the Fe(II)−NO complex. Loss of Fe(III) heme in sGC appears to be driven by the unfavorable energetics of burying the positively charged heme center (formally +1) in a highly nonpolar heme pocket.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods and Tables S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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