NO and CO differentially activate soluble guanylyl cyclase via a heme pivot-bend mechanism

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Diatomic ligand discrimination by soluble guanylyl cyclase (sGC) is paramount to cardiovascular homeostasis and neuronal signaling. Nitric oxide (NO) stimulates sGC activity 200-fold compared with only four-fold by carbon monoxide (CO). The molecular details of ligand discrimination and differential response to NO and CO are not well understood. These ligands are sensed by the heme domain of sGC, which belongs to the heme nitric oxide oxygen (H-NOX) domain family, also evolutionarily conserved in prokaryotes. Here we report crystal structures of the free, NO-bound, and CO-bound H-NOX domains of a cyanobacterial homolog. These structures and complementary mutational analysis in sGC reveal a molecular ruler mechanism that allows sGC to favor NO over CO while excluding oxygen, concomitant to signaling that exploits differential heme pivoting and heme bending. The heme thereby serves as a flexing wedge, allowing the N-terminal subdomain of H-NOX to shift concurrent with the transition of the six- to five-coordinated NO-bound state upon sGC activation. This transition can be modulated by mutations at sGC residues 74 and 145 and corresponding residues in the cyanobacterial H-NOX homolog.

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Introduction

Sensing gaseous molecules such as nitric oxide (NO), carbon monoxide (CO), and oxygen is a key trait present in virtually all life forms and predominantly mediated by heme-based sensors (Rodgers, 1999). In animals, differential sensing of NO, CO, and oxygen is critical for a wide range of physiological processes such as vascular homeostasis, platelet aggregation, host defense, neuronal signaling, and stress responses (Waldman and Murad, 1987; MacMicking *et al.*, 1997; Ignarro, 2002; Lahiri *et al.*, 2006; Moncada and Higgs, 2006; Ryter *et al.*, 2006). Bacteria need this gas sensing capability

for, for example, aerotaxis, to combat the host immune response (Roberts *et al.*, 2004; D'autreaux *et al.*, 2005), to sense CO as the sole energy source (Ascenzi *et al.*, 2004), or to induce certain genes under anaerobic conditions (Bailey-Serres and Chang, 2005).

In animals, the major NO sensor is the soluble guanylyl cyclase receptor (sGC), which, when activated by NO, produces the second messenger cGMP (Garbers *et al.*, 1994; Koesling *et al.*, 2004; Padayatti *et al.*, 2004; Cary *et al.*, 2006). In addition to NO binding while excluding oxygen, there is now substantial evidence that CO can also regulate sGC, in particular in olfactory neurons in which heme oxygenase produces CO (Boehning and Snyder, 2003; Ryter *et al.*, 2006). The NO–cGMP signaling pathway is involved in cardiovascular and neurological processes, and recent efforts lead to the development of sGC activators for potentially treating hypertension, erectile dysfunction, congestive heart failure, and chronic renal disease (Behrends, 2003; Evgenov *et al.*, 2006). Despite this strong pharmaceutical interest, the molecular details of sGC activation are not well understood.

sGC's NO/CO sensing and catalytic abilities are contained within a single heterodimeric protein comprised of an α and β subunit. Four isoforms of these subunits are found, with $\alpha 1/\beta 1$ being the most ubiquitous heterodimer (Koesling *et al.*, 2004). The sGC subunits have three distinct regions: sGC $\beta 1$ containing an N-terminal NO sensing heme domain, a central dimerization region, and a C-terminal catalytic guanylyl cyclase domain (Figure 1A) (Russwurm and Koesling, 2004a; Pyriochou and Papapetropoulos, 2005). sGC $\alpha 1$ has a similar subunit arrangement, except its N-terminal domain does not contain a heme. The NO sensing domain belongs to the heme nitric oxide oxygen binding (H-NOX) domain family, which also includes bacterial members, some with ligand binding characteristics similar to sGC (Boon *et al.*, 2006).

NO binds to the heme of sGC and forms a transient six-coordinated NO-bound state that progresses, upon heme-His bond breakage, to a five-coordinated NO-bound activated state (Pyriochou and Papapetropoulos, 2005; Cary *et al.*, 2006). Whether this final step involves a second NO molecule and whether the transient six-coordinated NO-bound state is physiologically important as a partially active tonic signaling state is contentious (Cary *et al.*, 2006; Roy and Garthwaite, 2006). The conversion of the six- to five-coordinated state of sGC can be positively regulated by the substrate GTP, whereas it is inhibited by ATP (Ruiz-Stewart *et al.*, 2004; Russwurm and Koesling, 2004b; Cary *et al.*, 2005). Similarly, NO stimulation of sGC can be sensitized by YC-1 (Koesling *et al.*, 2005; Cary *et al.*, 2006). These molecules are thought to bind to the cyclase domain, suggesting that there is crosstalk between both ends of the sGC protein (Lamothe *et al.*, 2004; Chang *et al.*, 2005; Yazawa *et al.*, 2006).

In contrast to NO, CO only reaches the six-coordinated CO-bound state and has a much lower affinity and weak

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stimulatory properties for sGC compared with NO (Stone and Marletta, 1994; Martin *et al*, 2006). As the activity of CO-bound sGC is also drastically enhanced by YC-1 to NO activated levels (Friebe and Koesling, 1998; Stone and Marletta, 1998), it is speculated that one or more endogenous compounds mimicking YC-1 might exist to account for this discrepancy in CO-mediated sGC signaling/activation (Boehning and Snyder, 2003). The synergistically CO/YC-1-activated sGC is predominantly in the six-coordinated state (Friebe and Koesling, 1998; Stone and Marletta, 1998).

The differential regulation of sGC by NO and CO in terms of affinity, activity, and coordination states demonstrates an intriguing complexity of sGC. Although some insights have been gained from structures of the adenylyl cyclase catalytic domain (~30% sequence identical) (Tesmer *et al*, 1997; Zhang *et al*, 1997; Tews *et al*, 2005) and oxygen-bound H-NOX domain (18% sequence identical) from *Thermoanaerobacter tengcongensis* (*Tt* H-NOX) (Nioche *et al*, 2004; Pellicena *et al*, 2004), the molecular details of NO/CO binding to H-NOX and sGC activation are poorly understood. H-NOX domains are observed in a number of proteins found in bacteria and animals as either stand-alone proteins or fused to other domains (Figures 1A and B) (Iyer *et al*, 2003). Remarkably, the stand-alone H-NOX domains in *Nostoc* cyanobacteria are adjacent to genes containing domains that are homologous to the central dimerization of sGC (Ohmori *et al*, 2001), including a putative PAS domain (Iyer *et al*, 2003) and a coiled-coil region (Figure 1A). It was therefore suggested that the stand-alone H-NOX domains could work in concert with these proteins transcribed from the adjacent genes (Iyer *et al*, 2003). The H-NOX domain of *Nostoc punctiforme* (*Np* H-NOX) has similar ligand binding properties as sGC (Boon *et al*, 2006), and the homologous H-NOX domains from *Nostoc* cyanobacteria are therefore good models for sGC. Furthermore, phylogenetic analysis even suggested that the bacterial H-NOX domains were probably transferred to animals, resulting in the mammalian NO-responsive sGC (Iyer *et al*, 2003). These bacterial H-NOX domains were therefore speculated to act as sensors for gaseous molecules such as NO (or CO) (Iyer *et al*, 2003), probably as part of a bacterial chemotaxis system owing to H-NOX's close proximity to genes involved in the two-component system (Figure 1A). This could perhaps allow these cyanobacteria to possibly respond to the presence of (toxic) NO, which can be produced by for example other bacteria and plants.

To gain molecular insights into the NO and CO activation mechanism of sGC, we targeted the homologous H-NOX domain of *Nostoc* sp (*Ns* H-NOX) for crystallographic studies, as the sGC H-NOX domain has been refractory to crystallization. Here we describe the crystal structures of the free, NO, and CO liganded *Ns* H-NOX domains at 2.1, 2.6, and 2.5 Å resolution, respectively. Our structures, together with structure-guided mutational and biophysical characterization, provide evidence that the NO activation mechanism in sGC involves a stepwise heme pivot to facilitate an ~20° rotational shift of the N-terminal helical region in H-NOX, causing the heme to bend in the activated five-coordinated state. CO signals differ from NO by having a larger heme pivot shift without proceeding to the heme-bend five-coordinated state.

Results and discussion

Structure of *Ns* H-NOX heme domain

We determined the structure of *Ns* H-NOX heme domain at 2.1 Å resolution using single wavelength anomalous diffraction (SAD) (Table I). There are two *Ns* H-NOX molecules in the asymmetric unit and each of the two similar molecules (r.m.s.d. of 0.40 Å for 182 C α atoms between molecules A and B) contains one heme moiety sandwiched between a small α -helical subdomain at its distal side and a larger mixed- α/β subdomain at its proximal side (Figure 2A). The proximal side of *Ns* H-NOX provides the conserved histidine (H105), which is the fifth ligand for a five-coordinated heme iron (NE2-Fe distance is 2.2 Å in both molecules). The *Ns* H-NOX absorption spectra is consistent with a five-coordinated iron (Figure 2B, left-hand panel), and is very similar to the spectrum of free sGC yet different from that of *Tt* H-NOX, as the latter binds oxygen under aerobic conditions (Karow *et al*, 2004; Pellicena *et al*, 2004). Correspondingly, *Ns* H-NOX shares a much higher sequence identity with sGC (33% identity) than *Tt* H-NOX with sGC (18% identity) (Figure 1B). This similarity extends to the heme pocket, in which 17 of 27 residues are identical between *Ns* H-NOX and sGC, compared with only eight of 27 between *Tt* H-NOX and sGC. The heme interactions are predominantly hydrophobic, except for the proximal heme ligand H105, and interactions with both propionate groups of the heme involving the main-chain nitrogen of Y2 and the conserved Y134-X-S136-X-R138 sequence (Figure 2C). The latter sequence was found to be key for heme binding in sGC (Schmidt *et al*, 2004, 2005) and *Tt* H-NOX (Nioche *et al*, 2004; Pellicena *et al*, 2004). In addition, the *Ns* H-NOX structure reveals an aromatic residue, W74 (F74 in sGC), oriented perpendicular to the heme at its distal face with the W74 NE1 atom located 3.3 Å from the heme NB atom (Figure 2C). Such an aromatic residue is absent in *Tt* H-NOX, which contains an N at this position. A possible explanation for *Ns* H-NOX's lack of oxygen binding is that it does not have Y140 found in *Tt* H-NOX (M in *Ns* H-NOX), which interacts with the bound oxygen (Nioche *et al*, 2004; Pellicena *et al*, 2004). Oxygen binding to heme model compounds, such as Fe(II)PP(1-Melm), is much weaker compared with NO and CO binding (Martin *et al*, 2006), and heme proteins therefore require additional polar/electrostatic interactions for oxygen to bind (Jain and Chan, 2003). The absence of such polar interactions provides an explanation for *Ns* H-NOX's and sGC's lack of oxygen binding.

Although similar in fold, a striking difference between *Tt* and *Ns* H-NOX is the relative orientation of the N-terminal subdomain, in particular helices α A- α C although some of the other nearby helices have shifted somewhat as well. Excluding α A- α C from the superposition lowers the r.m.s.d. from 2.5 to 1.6 Å and reveals an 18° difference in the orientation of this N-terminal helical region (Figure 2D). This N-terminal shift in oxygen-bound *Tt* H-NOX molecule A causes a strong heme distortion, as indicated by the saddling and ruffling distortions, both around -1 Å (Pellicena *et al*, 2004). The heme in *Ns* H-NOX is considerably less bent, with only moderate ruffling (~-0.35 Å) and dome (~0.5 Å) distortions. One of the four refined oxygen-bound *Tt* H-NOX molecules (monoclinic molecule B), however, contains an intermediate N-terminal shift of 11° with respect to *Ns* H-NOX, resulting in a moderate heme distortion

(Figure 2D) (Pellicena *et al*, 2004). The H-NOX domain can thus adopt a range of N-terminal subdomain shifts and concomitant heme distortions.

NO and CO binding to H-NOX

In order to investigate the structural effects of NO and CO binding, we measured electronic absorption spectra of

purified *Ns* H-NOX. Our results indicate that the NO:*Ns* H-NOX complex is predominantly a six-coordinated NO-bound heme species, with a small amount of five-coordinated NO-bound heme, as indicated by the small shoulder at 400 nm near the main 416 nm peak (Figure 2B, left-hand panel). This is similar to previous spectroscopic results for *Np* H-NOX (~60% sequence identical to *Ns* H-NOX) (Boon *et al*, 2006).

Table 1 Data collection, phasing, and refinement statistics for *Ns* H-NOX

	Native	Se peak	NO complex	CO complex
<i>Data collection</i>				
Wavelength (Å)	1.1000	0.9789	0.9793	1.0719
Space group	P2 ₁ 3	P2 ₁ 3	P2 ₁ 3	P2 ₁ 3
Cell dimension (Å)	$a = b = c = 123.42$ $\alpha = \beta = \gamma = 90$	$a = b = c = 123.76$ $\alpha = \beta = \gamma = 90$	$a = b = c = 123.56$ $\alpha = \beta = \gamma = 90$	$a = b = c = 123.63$ $\alpha = \beta = \gamma = 90$
Resolution (Å)	2.1	2.9	2.6	2.5
Total observations	153 963	148 837	52 349	99 121
Unique observations	36 257	14 307	18 960	21 865
$I/\sigma I$	18.4 (1.7)	14.8 (4.5)	12.0 (2.0)	29.3 (3.7)
Redundancy	4.2 (3.9)	10.40 (10.55)	2.8 (2.8)	4.5 (4.5)
Completeness (%)	98.9 (98.1)	100.0 (100.0)	96.6 (99.0)	99.1 (99.6)
R_{sym} (%)	6.7 (43.9)	9.1 (42.3)	9.3 (60.6)	5.8 (47.5)
<i>SAD phasing</i>				
No. of sites		10		
Figure of merit		0.70		
<i>Refinement</i>				
Resolution (Å)	25–2.1		40–2.6	39–2.5
No. of protein atoms	2866		2866	2866
No. of hetero atoms	86		90	90
No. of waters	229		64	122
R_{factor}	18.5		19.2	18.7
R_{free}	22.7		23.6	22.8
R.m.s.d. bond lengths (Å)	0.009		0.011	0.009
R.m.s.d. bond angles (deg)	1.22		1.33	1.12
<i>Average B-factors (Å²)</i>				
Protein	45.1		52.8	51.0
Heme moieties	32.0		38.0	38.6
NO or CO ligands	—		49.1	45.8
Solvent	53.9		48.4	53.4
<i>Ramachandran plot statistics residues in</i>				
Most favored regions	93.7		93.0	94.0
Additional allowed regions	6.3		7.0	6.0
In generously allowed regions	0		0	0
Disallowed regions	0		0	0

As expected, CO binding leads to a six-coordinated state of *Ns* H-NOX (Figure 2B, left panel). In order to obtain NO/CO complex structures, we soaked *Ns* H-NOX crystals in solutions containing NO or CO. The resulting crystal structures of *Ns* H-NOX at 2.6 and 2.5 Å resolution, respectively, show strong difference density peaks corresponding to NO or CO in both molecules positioned at the distal face of the heme (Figures 3A and B). Both ligands are situated in a hydrophobic pocket, lined by V5, F70, W74, M144, and L148. In NO-bound H-NOX, the nitrogen atom of NO forms a 1.8 Å bond with Fe and the Fe–N–O angle is 143 and 157° for molecules A and B, respectively. These angles are more perpendicular (163 and 168°) for the CO-bound complex.

These observed angle differences conform to the known angular preferences for NO and CO heme interactions (Kachalova *et al*, 1999; Carlsson *et al*, 2005; Copeland *et al*, 2006).

Conformational changes upon NO and CO binding to H-NOX

Unlike the five-coordinated NO-bound state, the six-coordinated state of sGC has low activity, and major conformational changes, compared with unbound H-NOX, are therefore not expected. Nevertheless, superposition of the free, NO-bound, and CO-bound *Ns* H-NOX structures reveals a striking heme

Figure 1 Structure and domain organization of H-NOX and H-NOXA-containing proteins. **(A)** Schematic diagram of the H-NOX and H-NOXA domains present in proteins from animals and *Nostoc* cyanobacteria. The sGC subunits contain additional coiled-coil (CC) and GC domains. The purple interrupted line indicates the domain that was targeted for crystallographic studies. The PAS-like domain is termed H-NOXA, as it is often associated with H-NOX and was previously named H-NOBA (Iyer *et al*, 2003) before the discovery that these heme domains could also bind oxygen (Pellicena *et al*, 2004). The proteins transcribed adjacent to the H-NOX gene in cyanobacteria are the signal transduction histidine kinases (STHK) and the two-component hybrid sensor and regulator (2-CHSR). **(B)** Structure-based sequence alignment of HNOX domains. Residues in the heme cavity are labeled in orange or red, the latter if the residue is fully conserved. Additional conserved residues in the hydrophobic core are shaded yellow. Key residues for the transition from six- to five-coordinated NO bound are labeled with a '#' (W74 and M144). The three N-terminal helical rods are red. Residues identical to rat sGCβ1 are in bold. Conserved glycine residues are shaded green. The GenBank identifiers for the selected sequences are *Nostoc* sp, GI:17229770; *N. punctiforme*, GI:23129606; *Legionella pneumophila*1, GI:52841290; *L. pneumophila*2, GI:52629778; *Vibrio Cholerae*, GI:15601476; *T. tengcongensis*, GI:20807169; *Clostridium acetobutylicum*, GI:15896488; *Rattus_sGCβ1*, GI:28564567; *Rattus_sGCβ2*, GI:21956635; *Caenorhabditis elegans_GCY35*, GI:52782806; *Drosophila melanogaster_sGCβ1*, GI:7302016.

Figure 2 Crystal structure of unliganded *Ns* H-NOX. (A) Schematic diagram of the structure of *Ns* H-NOX. The three N-terminal helices α A– α C (red), the heme (blue), and H105 (green) are highlighted. The heme propionate groups attached to pyrrole rings A and D are labeled P_A and P_D , respectively. Figure generated using MOLSCRIPT and Raster3D (Kraulis, 1991; Merritt and Bacon, 1997). (B) Electronic absorption spectra of wt and mutants of *Ns* H-NOX. The spectra before (solid line) and after adding 200 μ M SNAP (dotted line) as the NO donor are shown. The five-coordinate free heme signature peak is near 430 nm, the six-coordinated NO-bound heme signature peak is near 416 nm, and the five-coordinated NO-bound heme is near 400 nm. For the wt *Ns* H-NOX, a spectrum in the presence of CO was also measured (interrupted line), yielding a peak near 423 nm, indicating the presence of a six-coordinated CO-bound state. (C) Stereo figure showing electron density for the heme group in the free *Ns* H-NOX structure. The main chain is depicted in coil representation, with side chains in close proximity to the heme in stick representation; residues from the N-terminal residues 1–60 are shown in red. The omit $|F_o| - |F_c|$ density is in blue (2.1 Å resolution, contoured at 3σ). Figure generated using BOBSCRIPT (Esnouf, 1999). (D) Superposition of the *Ns* and *Tt* H-NOX structures revealing an N-terminal domain shift for helices α A– α C. Depicted are the *Ns* H-NOX structure (blue), partially shifted *Tt* H-NOX (green; monoclinic molecule B in PDBid 1U55), and maximally shifted *Tt* H-NOX (red, monoclinic molecule A in PDBid 1U55) (Pellicena *et al*, 2004). The arrow indicates the direction of the shift.

pivot Fe shift of 0.2–0.3 Å for NO and a larger 0.8–0.9 Å Fe shift upon CO binding observed in both independently refined molecules in the asymmetric unit (Figures 3C and 4A). The heme pivots while keeping the propionate group attached to pyrrole ring D (P_D) in place serve as the pivot point. Shifts over 1 Å are observed for heme atoms farthest away from the pivot point in the CO-bound structure. The heme Fe-center pivots away from W74, which appears to be necessary to provide the steric space for NO and CO to bind to the distal face of the heme (the distance between NO/CO and the side chain of W74 is 3.0–3.4 Å). CO's larger shift may be due to the larger atomic radius of its carbon atom and its preference to bind perpendicular to the plane of the heme (Kachalova *et al*, 1999) compared with NO binding at an angle. These larger changes to accommodate CO suggest that the perpendicularly oriented aromatic residue at position 74

close to the Fe serves as a molecular ruler; its presence could explain why sGC and the model heme compound bind NO with similar affinity, whereas CO binds to sGC ~ 5 orders of magnitude more weakly than the model heme compound (Martin *et al*, 2006). Steric hindrance, and the relieve thereof, is also used by myoglobin to decrease its affinity for CO (Kachalova *et al*, 1999). The heme shift is accompanied by a shift of the H105 side chain towards the heme (Figure 3C). The NO-bound H-NOX structure yields minimal additional changes, yet the larger CO-induced heme shift causes several structural changes in *Ns* H-NOX (Figures 3C–E). First, the α F residue H105 and its adjacent residues shift with the heme. Second, the α A region near Y2, whose main chain interacts with the heme's P_A propionate, shifts with the heme by about 0.5 Å. Third, the flanking α B– α C loop near residue E41 shifts about the same amount in concert with α A. Fourth, the

Figure 3 Structural consequences of NO and CO binding to Ns H-NOX. (A) NO binding to the distal face of the heme in the NO-bound Ns H-NOX structure. Omit $|F_o| - |F_c|$ electron density (contoured blue at 5σ) and $2|F_o| - |F_c|$ electron density (contoured gray at 1σ) for the heme and its two ligands, NO and H105, are shown. (B) As above, but for CO binding to H-NOX, omit density contoured at 4σ , $2|F_o| - |F_c|$ contoured at 1σ level. (C) Molecular shifts within the heme region of Ns H-NOX upon NO and CO binding. Superpositioning of free (blue), NO-bound (yellow), CO-bound Ns H-NOX (magenta) structures reveals a shift in the heme α A, including residues 1–3, and loop α B- α C containing residues E41. Hydrogen bonds between the heme propionate and main chain of Y2 and between the main chains of G3 and E41 are shown (dashed lines). (D) CO binding to Ns H-NOX leads to increased flexibility in the loop following helix α F and C-terminal part of this helix. Depicted is a close-up view of the heme region of the free Ns H-NOX structure (left) and CO-bound Ns H-NOX structure (right). The temperature factor of the main chain is color ramped from blue ($\sim 35 \text{ \AA}^2$), green ($\sim 45 \text{ \AA}^2$), to orange ($\sim 80 \text{ \AA}^2$). The heme and the H105 and F112 side chains are color coded by atom type. Figure generated using PYMOL (<http://www.pymol.org>).

interacting α F- β 1 loop region near F112 and C-terminal part of helix α F becomes substantially disordered, displaying poor electron density and increasing its refined temperature factors from $\sim 45 \text{ \AA}^2$ in the unbound structure, to $\sim 75 \text{ \AA}^2$ in the CO-bound structure (Figure 3D). This increased flexibility is caused by heme shifting away, leading to decreased van der Waals interactions between the heme and the side chains of F112 and V108. These shifts and increased flexibility are observed in both independently refined H-NOX molecules in the asymmetric unit. The CO-induced shifts involving helix

α A and loop α B- α C are in the same direction as the shifts in the oxygen-bound *Tt* H-NOX structure (Figure 2D), suggesting a conserved directional path of movement for this α A- α C region. These CO-induced structural changes likely provide the molecular basis for the four-fold activation of sGC by CO, yet the even smaller induced changes by NO potentially question the contentious, tonic signaling importance (Cary *et al*, 2006; Roy and Garthwaite, 2006) of the transient six-coordinated NO-bound intermediate before Fe-His bond breakage.

Figure 4 Evidence for heme pivoting and heme bending in the activation mechanism of H-NOX. **(A)** Top (distal) view of the heme groups of the superimposed H-NOX structures. Ns H-NOX residues M144 and W74 are shown, as well as the residues interacting with the propionate groups of the heme (M1, Y2, Y134, S136, and R138) and residues belonging to α A– α C (red). The five heme moieties are color coded as in Figures 2D and 3C. The pivot directions of Fe and heme are indicated by arrows. **(B)** Side-view stereo figure of the heme groups of the superimposed HNOX structures. The five heme moieties are color coded as in (A). The van der Waals interactions between the A8 side chain and CZ3 atom of W74 as well as between CE atom of M144 and the propionate P_D group in the unliganded Ns H-NOX are depicted as transparent dashed lines. The position of the α A helix in Tt H-NOX molecule A and its M1 side chain are shown in transparent blue to indicate their potential clash with the W74 side chain and the propionate P_A group, respectively. **(C)** Western blot analysis of wt and mutant sGC proteins. Cytosols of the wt and H-NOX β 1 heme domain mutants (F74W and I145M) for both the α 1 and β 1 subunits were probed with anti-sGC α 1 and anti-sGC β 1 antibodies.

Heme pivoting and heme-bending activation mechanism

Our Ns H-NOX structures, together with the Tt H-NOX structure (Nioche *et al*, 2004; Pellicena *et al*, 2004), likely represent the entire spectrum of activation: The oxygen-bound Tt H-NOX structure is thought to represent the activated state, whereas our new free and NO/CO-bound Ns H-NOX structures represent the basal and low-activity H-NOX

state, respectively. During activation, the N-terminal α A helix may progressively push against the heme, causing a drastic heme bend in the final step; α A shifts range from about 0.5 Å for the CO-activated state to over 4 Å for oxygen-bound Tt H-NOX (Figures 2D, 3C, 4As and B; see Supplementary Video 1a for the large-scale subdomain movement and Supplementary Video 1b for a close-up view of heme pivoting and bending). The superposition reveals that the heme and heme iron follow a trajectory in the order of presumed increasing cyclase activity (Figure 4A and Supplementary Video 1b). In addition to this heme pivot, we speculate that two heme bending motions are key for the final activation step: the heme bending at the side of the pyrrole A ring with attached propionate P_A provides the space to accommodate the large shift of the N-terminal α A– α C helices, whereas the pyrrole D ring attached to the propionate P_D group tilts in the opposite (distal) direction towards M144 (Figure 4B and Supplementary Video 1b). Although structurally conceivable as a signaling mechanism for H-NOX domains in general, Ns H-NOX stays predominantly in the six-coordinated NO-bound state (Figures 2B and 3A) and might perhaps be more sensitized for this transition once it interacts with its target output domain. Our activation mechanism offers a structural explanation for Ns H-NOX's limited ability to reach the acute state: the helical shift of α A would require, in addition to the heme bending, space in the direction of residue W74 as the α A residue A8 is postulated to shift about 3–4 Å towards W74 (Figure 4B). Owing to the size of W74 (which is the smaller F74 in rat sGC β 1) and the close 3.7 Å van der Waals interaction between the side chains of A8 and W74, the α A helix has limited space for this large shift in Ns H-NOX. In addition, the tilting of the pyrrole D ring with attached propionate P_D of the heme involves a 0.7 Å shift of this propionate group when comparing the unliganded Ns H-NOX to the activated oxygen-bound Tt H-NOX structure (Figure 4B). However, the CE atom of M144 forms a snug 3.4 Å van der Waals interaction with the C_{AD} atom of this propionate P_D group in the unliganded Ns H-NOX structure (Figure 4B), thereby causing steric hindrance for such a heme bending at this side as well.

Mutagenesis studies to test H-NOX activation mechanism

To test this hypothesis about the importance of heme bending and N-subdomain movement during activation, we substituted Ns H-NOX residues W74 and M144 with the smaller amino acids found in rat sGC β 1 (F and I, respectively). The smaller residues at these positions are speculated to more easily facilitate the N-terminal subdomain shift and heme bending. The W74F and M144I mutations in Ns H-NOX both resulted in considerable increases in the solet peak near 400 nm, representing increased levels of the five-coordinated NO-bound state (Figure 2B). These results are in agreement with our activation model, indicating that reaching the activated five-coordinated NO-bound state is directly correlated with heme bending and the N-terminal subdomain shift. To further validate this model, we carried out the reverse mutagenesis experiment by introducing the larger Ns H-NOX residues into rat sGC β 1 (F74W and I145M mutations) with the intent of restricting the heme bending and N-terminal subdomain shift. These mutations caused a significant loss of NO-stimulated guanylyl cyclase activity: from 21-fold stimulation in wild-type (wt) sGC to only two- and

five-fold stimulation for the F74W and I145M sGC mutants, respectively (Table II and Figure 4C). This observed drop is likely owing to the increased size of these side chains, thereby restricting the N-terminal subdomain shift and heme bending. In addition to this experimental validation, our activation model also provides a new explanation for the decreased activity of the previously characterized sGC mutants, where larger residues were introduced at the 74 and 145 positions. First, the sGC mutation I145Y exhibits decreased activity and diminished levels of the five-coordinated NO-bound state (Martin *et al*, 2006). Second, the F74Y mutation was also observed to cause a decrease in NO-stimulated activity (Rothkegel *et al*, 2006). Taken together,

Table II GC activity measurements of wt and mutants of rat sGC under basal and NO-stimulated conditions

	Basal (pmol/min mg)	+ NO donor SNAP (pmol/min mg)
<i>H-NOX mutants</i>		
wt $\alpha 1/\beta 1$	245 $\pm$ 15	5240 $\pm$ 35
$\alpha 1/\beta 1$ F74W	115 $\pm$ 7	263 $\pm$ 22
$\alpha 1/\beta 1$ I145M	155 $\pm$ 16	770 $\pm$ 40

these results suggest that the two sGC activation events, reaching the five-coordinated activated NO-bound state and the N-terminal helical subdomain movement concomitant with heme bending, are strongly correlated. These latter events are preceded by the ligand-induced heme pivoting event described earlier and together comprise a new activation model for sGC (Figure 5). A bent heme as a key feature of the activated state in sGC also provides an explanation for the ability of the elongated and flexible heme mimetic BAY58-2667 (Schmidt *et al*, 2004) to activate heme-free sGC. The strong heme bending motion in H-NOX is unprecedented and is to our knowledge not observed in other heme proteins (Jain and Chan, 2003). The presence of an energetically strained heme in the activated state might, in part, be key for the ability of sGC to be rapidly deactivated (Margulis and Sitaramayya, 2000) upon relaxing the heme. This could serve a beneficial physiological purpose to rapidly control blood pressure, neuronal signaling, and other processes.

Communication of the H-NOX domain to transmit the NO/CO signal to the catalytic guanylyl cyclase domain likely involves a direct interaction (Winger and Marletta, 2005). Such domain-domain communication likely involves H-NOX residues that shift upon ligand binding (as discussed in

Figure 5 Schematic diagram of NO- and CO-dependent activation of sGC. NO and CO bind to the distal face of the heme and cause the heme to pivot, with varying degrees, away from F74 (W74 in *Ns H-NOX*). The larger pivot for CO is likely a result of the need to accommodate its carbon atom, having a larger radius compared with nitrogen in NO, adjacent to F74 and CO's preference to bind perpendicular to the heme. The second step in the NO activation mechanism involves heme bending and N-subdomain movement (only helix αA is shown for illustrative purposes). Whether a similar heme bending event occurs in CO/YC-1-activated sGC is unknown and speculative. Also it is unknown whether a second NO molecule binds to the proximal side of the heme, thereby displacing the histidine ligand as well as the distal NO, as was postulated previously (Lawson *et al*, 2003; Russwurm and Koesling, 2004b). NO could therefore be bound to the distal or proximal face of the heme in the NO-bound five-coordinated activated state (both possibilities are depicted).

Figures 2D, 3C, and 4B), as the catalytic domain would need to sense NO-induced conformational differences. Our results therefore point to a potential role for residues in/near α F (containing H105) and α A- α C, which includes the region near residue D45 previously suggested to be a switch region (Pellicena *et al*, 2004; Rothkegel *et al*, 2006). Although speculative, a subset of these could perhaps interact on a similar site on the guanylyl cyclase domain, where G α modulates the adenylyl cyclase activity (Tesmer *et al*, 1997), and our insights provide a framework for future structure-based mechanistic studies.

In conclusion, we provide evidence for a molecular ruler mechanism in sGC favoring NO over CO binding, using steric hindrance involving an aromatic residue at position 74. Both ligands induce differential heme pivoting and heme bending, which appear to correlate with differences in sGC activation levels. CO induces a larger initial heme pivot shift compared with NO, whereas NO only proceeds to the five-coordinated fully activated state concomitant with a strongly bent heme and large N-terminal subdomain shift. These H-NOX heme pivoting/bending signaling insights for sGC activation offer new opportunities for sGC to be therapeutically exploited.

Materials and methods

Cloning and mutagenesis of *Ns* H-NOX

The genomic DNA of *Nostoc* sp PCC 7120 (*Anabaena* sp PCC 7120) was used for PCR of the 189-amino-acid H-NOX gene using the forward primer 5'-ggaattccatgatgttgtagtgaacaaagcc-3' and the backward primer 5'-ccggaattctcagtcgcatataaattcgagtc-3'. The amplified fragment was inserted into the pET22b (Novagen) expression vector. The W74F and M144I mutants were introduced using the QuickChange kit (Stratagene) and were confirmed by DNA sequencing.

Expression and purification of *Ns* H-NOX

The *Ns* H-NOX protein was expressed in *Escherichia coli* BL21 (DE3) Star cells (Invitrogen). After overnight 0.1 mM IPTG induction at 18°C, cells were pelleted and lysed in buffer A containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM β -mercaptoethanol. The cleared cell lysate was loaded onto a Q-Sepharose FF 5 ml column and the H-NOX protein was eluted using an NaCl gradient. The 420 nm peak fractions containing the H-NOX protein were concentrated and loaded onto a Sephadex 200 column with buffer A as the running buffer. The fractions with OD₄₂₀ > OD₂₈₀ were loaded onto a high-resolution MonoQ anion exchange column and an NaCl gradient was used for elution. Finally, the H-NOX protein was cleaned up using a high-resolution prepacked Sephadex75 column.

UV/Vis absorption spectroscopic analysis

A SHIMADZU UV-1700 UV-visible spectrophotometer was used for all spectral analysis performed at room temperature. The concentration of the wt and the mutant H-NOX proteins was 1 μ M in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM β -mercaptoethanol. The NO complex was prepared by adding the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) to a final concentration of 200 μ M. The CO complex was obtained by using a saturated CO solution that was prepared by bubbling CO gas (Praxair) through the above Tris buffer.

Ns H-NOX crystallization

Ns H-NOX crystals were grown at 20°C by the sitting-drop vapor diffusion method. The protein was concentrated to 15 mg/ml in 5 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM β -mercaptoethanol, and was mixed with an equal volume of the reservoir solution containing 1.4 M tri-sodium citrate dihydrate (pH 5.6–6.6). Native *Ns* H-NOX crystals were prepared for data collection by fast transfer

into cryo-protectant solution containing 1.36 M tri-sodium citrate dihydrate with 15% ethylene glycol. Selenomethionine-substituted protein crystals (produced similar to Van Duyne *et al*, 1993) were used for phasing. The F74W and M145I *Ns* H-NOX mutants unfortunately resisted crystallization.

Ns H-NOX structure determination and refinement

The native 2.1 Å resolution data set was collected at the Brookhaven NSLS synchrotron (beamline X29) and processed by HKL2000 (Otwinowski and Minor, 1997). For phasing, a SAD data set was collected from a selenomethionine-substituted crystal at the Advanced Light Source ALS (beamline 4.2.2.) and processed by D*trek (Pflugrath, 1999). SOLVE/RESOLVE (Terwilliger and Berendzen, 1999) was used to calculate initial phases and density modification, resulting in locating 10 selenium atoms yielding a figure of merit of 0.7 (Table I). A partial model of two molecules in the asymmetric unit generated by RESOLVE was used as a starting point for ARP/wARP (Perrakis *et al*, 2001) for additional automatic model building, using the 2.1 Å native data set. Iterative cycles of model building in Coot (Emsley and Cowtan, 2004) and refinement using REFMAC (Murshudov *et al*, 1997) yielded a model comprising H-NOX residues 1–182 for both molecules A and B, two heme moieties, and 230 water molecules (if above 3 σ observed in omit $F_o - F_c$ map and kept if peak remained above 1 σ in 2 $|F_o| - |F_c|$ map) with a final *R*-factor of 18.1% and *R*_{free} of 22.2% (see Table I for additional refinement statistics). One high-temperature factor water molecule was observed in the general vicinity of Fe in only molecule B, but was located at >4.5 Å from this Fe, weakly bound, and therefore was not included in the analysis.

The 2.6 Å NO:H-NOX complex data set was collected at the Advanced Photon Source APS (beamline 19ID) and processed by HKL2000 (Otwinowski and Minor, 1997). The NO complex crystals were prepared by soaking wt H-NOX crystals in the similar cryo-protectant solution with 20 mM of the NO donor SNAP (Cayman) while varying the soaking time from 1, 2, 4, 6, 8, 10 min to several hours before crystal freezing by dunking it in liquid nitrogen for X-ray data collection. Tens of data sets were collected and analyzed. We found the 2 min soaking time point to be the most structurally informative regarding resolution of diffraction and NO electron density. The 2.1 Å native H-NOX structure was used as a starting point for crystallographic refinement with REFMAC. Strong NO density in the vicinity of Fe at the distal side of the heme was observed (7.7 σ peak for molecule A and 4.5 σ for molecule B) (Figure 3A). One NO molecule for each heme was positioned in the electron density and further refined using REFMAC. The final refined model contained residues 1–182 for both molecules A and B, two heme molecules, two NO molecules, and 64 waters. CO-bound crystals were obtained in a similar manner. The cryo-protection solution was bubbled with CO (Praxair) for an extended period of time to achieve a saturated CO soaking solution. The soaking time was varied and the most optimal time for H-NOX crystals was 45 min, and a 2.5 Å resolution data set was collected at APS. Initial refinement revealed strong $|F_o| - |F_c|$ difference density, representing CO at the distal side of the heme (6.3 σ and 7.0 σ for molecules A and B, respectively). Both CO molecules were refined at 100% occupancy. The final refined model contained residues 1–182 of both molecules, two heme molecules, two CO molecules, and 122 water molecules. Heme distortion calculations were calculated using normal-coordinate structural decomposition (Jentzen *et al*, 1998). Owing to the 2.6/2.5 Å resolution of the NO/CO H-NOX complex structures, the angles are likely only accurate to within about 10° of the refined values, yet these refined angles are within the range of the Fe–N/C–O angles observed for other NO and CO heme-protein complexes (Carlsson *et al*, 2005; Copeland *et al*, 2006). The program ESCET (Schneider, 2000) was used in the analysis of conformational changes among the different H-NOX structures.

Mutagenesis of rat sGC and transfection in COS-7 cells

Templates were cDNAs encoding the α 1 and β 1 subunits of rat sGC cloned into the mammalian expression vector pCMV5 (Yuen *et al*, 1994). The F74W and I145M mutations were introduced by PCR (Quickchange, Stratagene) and checked by DNA sequencing. COS-7 cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin

(100 U/ml). Cells were transfected for 48 h with Superfect reagent, according to the supplier's protocol (Qiagen).

Cytosol preparation and Western blot analysis

COS-7 cells were washed twice with ice-cold PBS and then scraped off the plate in cold lysis buffer: PBS buffer contained protease inhibitors, 50 mM HEPES (pH 8.0), 1 mM EDTA, and 150 mM NaCl. Cells were broken by sonication (three pulses of 3 s). The resulting lysate was centrifuged at 16 000g for 10 min at 4°C to collect the cytosol. To determine the expression levels for wt sGC or sGC mutants in COS-7 cells, 15 µg of cytosol was resolved on 10% SDS-PAGE and analyzed by immunoblotting with anti-sGC (anti- α_1 subunit and anti- β_1 subunit; Cayman Chemicals).

sGC activity assay

GC activity was determined by the formation of [α -³²P]cGMP from [α -³²P]GTP, as previously described (Lamothe *et al*, 2004). Reactions were performed for 5 min at 33°C in a final volume of 100 µl, in 50 mM HEPES (pH 8.0) reaction buffer containing 500 µM GTP, 1 mM DTT, and 5 mM MgCl₂. Typically, 40 µg of COS-7 cytosol transfected with either wt or mutants was used in each assay reaction. All assays were performed in duplicate and each experiment was repeated twice. Enzymatic activity was stimulated

with the NO donor SNAP (Calbiochem) at 100 µM. sGC activity is expressed in pmol/min mg and as mean $\pm$ s.e.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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