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Gaseous Ligand Selectivity of the H-NOX Sensor Protein from *Shewanella oneidensis* and Comparison to Those of Other Bacterial H-NOXs and Soluble Guanylyl Cyclase

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Abstract

To delineate the commonalities and differences in gaseous ligand discrimination among the heme-based sensors with Heme Nitric oxide/Oxygen binding protein (H-NOX) scaffold, the binding kinetic parameters for gaseous ligands NO, CO, and O₂, including K_D, k_{on}, and k_{off}, of *Shewanella oneidensis* H-NOX (*So* H-NOX) were characterized in detail in this study and compared to those of previously characterized H-NOXs from *Clostridium botulinum* (*Cb* H-NOX), *Nostoc* sp. (*Ns* H-NOX), *Thermoanaerobacter tengcongensis* (*Tt* H-NOX), *Vibrio cholera* (*Vc* H-NOX), and human soluble guanylyl cyclase (sGC), an H-NOX analogue. The K_D(NO) and K_D(CO) of each bacterial H-NOX or sGC follow the “sliding scale rule”; the affinities of the bacterial H-NOXs for NO and CO vary in a small range but stronger than those of sGC by at least two orders of magnitude. On the other hand, each bacterial H-NOX exhibits different characters in the stability of its 6c NO complex, reactivity with secondary NO, stability of oxyferrous heme and autoxidation to ferric heme. A facile access channel for gaseous ligands is also identified, implying that ligand access has only minimal effect on gaseous ligand selectivity of H-NOXs or sGC. This comparative study of the binding parameters of the bacterial H-NOXs and sGC provides a basis to guide future new structural and functional studies of each specific heme sensor with the H-NOX protein fold.

Graphical Abstract (synopsis)

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Supplementary Information

Supporting information includes 1) sequence alignment of the bacterial H-NOXs and sGC (Fig. S1); 2) analysis of the MCD spectrum of the resting *So* H-NOX (Fig. S2); 3) rapid-scan of ferrous *So* H-NOX reaction with stoichiometric NO in extended reaction time (Fig. S3); 4) EPR spectrum of 5c heme-NO complex in *So* H-NOX (Fig. S4); 5) visible ligand access channel in *So* and *Tt* H-NOXs (Fig. S5).

Heme Nitric oxide/Oxygen binding protein from *Shewanella oneidensis* (So H-NOX) binds CO and NO with high affinities but shows no O₂ binding under atmospheric pressure, although it does autoxidize. It exhibits multiple-step NO binding and its 6-coordinate NO complex converts to more stable 5-coordinate NO complex.

Keywords

H-NOX; gaseous ligand selectivity; soluble guanylyl cyclase; sliding scale rule

1. Introduction

Diatomic gases nitric oxide (NO), carbon monoxide (CO), and O₂ play messenger roles under many physiological and pathological conditions [1,2]. Living organisms have evolved different systems to respond to the environmental changes of these gaseous ligands [1,3]. Heme sensor proteins are the most important components of these gaseous messenger-sensing systems [1,4]. Heme Nitric oxide/Oxygen binding proteins (H-NOXs) are one of the six major groups of heme sensor proteins whose bindings with the gaseous messengers cause changes in the downstream effector proteins, evoking various responses [1,5].

All the bacterial H-NOXs show strong affinities for NO and CO [6], but only some H-NOXs exhibit strong affinities for O₂. Whether an H-NOX binds O₂ under atmospheric pressure correlates with its biological origin from either a facultative or obligate anaerobe [1,4]. H-NOXs from facultative anaerobes are encoded in the bacterial genomes as stand-alone proteins and in the same operons with either putative histidine kinases or diguanylate cyclases [4]. These H-NOXs usually do not show any affinity for O₂ under atmospheric pressure, exemplified by the ones found in proteobacteria *Vibrio cholera* (Vc H-NOX) and cyanobacteria *Nostoc* sp. PCC7120 (Ns H-NOX) [7,8,9,10]. On the other hand, H-NOXs found in obligate anaerobes are domains of methyl-accepting chemotaxis proteins (MCP) and often bind O₂ to form an oxyferrous complex, such as the ones from firmicutes *Clostridium botulinum* (Cb H-NOX) and thermophilic *Thermoanaerobacter tengcongensis* (Tt H-NOX, renamed *Caldanaerobacter subterraneus* H-NOX recently) [7,11,12,13].

In animals, the heme domain in the β subunit [β 1(1–194)] of soluble guanylyl cyclase (sGC), a heterodimer that consists of α and β subunits, shows significant sequence homology with the bacterial H-NOXs (Fig. S1). The fact that the heme proximal ligand

His105, heme interacting triad Tyr135/Ser137/Arg139, Pro118 that buttresses the heme, and four glycines, Gly3/Gly18/Gly71/Gly148 (all numbers are based on sGC sequence) are 100% conserved in the six heme sensors indicates that they are structural homologs. The bacterial H-NOXs have therefore been used as the model systems to understand the gaseous ligand selectivity of sGC [4,14,15,16]. On the other hand, the overall sequence identity among these six heme sensors is less than 35%, which may reflect their different biological functions. Whether the primary sequence divergences contribute to the gaseous ligand selectivity of H-NOXs and sGC has not been systematically evaluated.

sGC is the only known NO receptor in animals and its enzymatic activity that converts GTP to cGMP increases several hundred-fold above basal level upon binding to NO, a critical mediator for many physiological processes [15,16,17]. Although an analogue of the bacterial H-NOXs, sGC exhibits significantly weaker affinities for CO and NO than the formers, nonetheless, it is capable of selectively binding NO with complete exclusion of O₂ under atmospheric pressure [6,15,16,17].

In previous studies, we characterized the binding kinetics for the gaseous ligands of sGC, *Cb*, *Ns*, *Tt*, and *Vc* H-NOXs [6,9,10,11,18]. These studies bring attention to the similarities but more importantly the differences between the gaseous ligand selectivity of the bacterial H-NOXs and sGC. To better reveal the similarities and differences of the heme sensor proteins with H-NOX fold in bacteria and animals, in this study, we first characterized the binding kinetics of another bacterial H-NOX isolated from facultative anaerobe *Shewanella oneidensis* (*So* H-NOX), with NO, CO, and O₂, and then compared in details the binding parameters of sGC, *Cb*, *Ns*, *So*, *Tt*, and *Vc* H-NOXs. This comprehensive comparative study reveals that the gaseous ligand bindings of each of these heme sensor proteins obeys the “sliding scale rule”; on the other hand, each heme sensor protein exhibits its own characteristic gaseous ligand selectivity, including the extent of oxygen binding/autoxidation and the efficiency of multiple-step NO-binding.

2. Material and methods

2.1. Materials

CO and NO gases were from Matheson-TriGas Inc. (Houston, TX) and NO was pre-purified by passing through a NaOH trap [9]. Sodium hydrosulfite (Na₂S₂O₄), ferricyanide, imidazole, heme, δ -aminolevulinic acid, isopropyl-1-thio- β -D-galactopyranoside (IPTG), ampicillin, chloramphenicol, and egg lysozyme were from Sigma (St. Louis, MO). Restriction enzymes were from New England BioLabs (Beverly, MA). Vector pET43.1a and *E. coli* strain Rosetta 2(DE3)pLysS were from Novagen (Madison, WI). TALON Co²⁺ affinity resin was purchased from BD Biosciences Clontech (Palo Alto, CA). The 10DG desalting column and DC protein assay kit were from Bio-Rad Laboratories (Hercules, CA).

2.2. Expression and purification of *So* H-NOX

The gene encoding *So* H-NOX was synthesized with codon optimization for *E. coli* expression and codons for a six-histidine tag were inserted upstream of the stop codon. The cDNA was first cloned into pBSK vector (Epoch LifeScience, Houston, TX) followed by

NdeI and *XhoI* digestion and subcloning into pET43.1a vector. The integrity of the resulting plasmid was then confirmed at Lone Star Labs (Houston, TX).

So H-NOX was expressed and purified following the procedure described previously [9,11]. Briefly, Rosetta2(DE3)pLysS strain of *E. coli* was transformed with the expression plasmid and grown overnight at 37 °C in TB medium with chloramphenicol (45 µg/mL) and ampicillin (150 µg/mL). TB medium with ampicillin was inoculated with the overnight culture and shaken at 37 °C until A₆₁₀ reached ~ 0.8. The temperature was then lowered to 20 °C, and the expression was induced with 1 mM IPTG in the presence of 2 µM heme and 0.2 mM δ-aminolevulinic acid. The cells were harvested 48 hours after induction.

The cells were lysed in 100 mM potassium phosphate (pH 7.5) containing 100 mM NaCl, 10% glycerol, and 1.2 mg/mL egg lysozyme. The supernatant after centrifugation was loaded onto Co²⁺ affinity resin, and *So* H-NOX was eluted with 250 mM imidazole, which was subsequently removed using a 10DG column. The amount of protein was determined with a DC protein assay kit using bovine serum albumin as the standard. The Soret extinction coefficient of *So* H-NOX was determined by the pyridine hemochrome assay [19]. Ferric *So* H-NOX was prepared by oxidizing the resting protein with ferricyanide followed by a cleanup with a 10DG column.

2.3. UV-Vis and magnetic circular dichroism (MCD) spectroscopy

The UV-Vis and MCD spectra of *So* H-NOX were recorded with a Hewlett-Packard 8452A diode-array spectrophotometer (Agilent Technologies, Santa Clara, CA) and a Jasco J-815 CD spectropolarimeter (Tokyo, Japan) with an Olis permanent magnet (Bogart, GA), respectively. The field strength of the magnet was calibrated as described before [9]. MCD expressed in molar delta absorption coefficient, $\Delta\epsilon$, in units of M⁻¹cm⁻¹tesla⁻¹, was obtained with a bandwidth of 5 nm, averaged from 4 repetitive scans, and calculated using the spectral analysis software coming with the instruments [9]. The CO complex of *So* H-NOX was prepared by flushing ferrous *So* H-NOX with CO gas in an anaerobic cuvette sealed with an air-tight septum. The Soret wavelength of the CO complex observed with spectrophotometer and resolved from rapid-scan data exhibited a difference of 2 nm, which was due to the different spectral resolutions of these two instruments and the spectral data deconvolution process.

2.4. Stopped-flow measurements

The binding kinetics of NO, CO, and O₂ to ferrous *So* H-NOX were studied at room temperature under anaerobic conditions with SX-18MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK) as described previously [9,11]. The dead time of the instrument is 1.5 ms. To make sure that *So* H-NOX started with a homogenous 5c ferrous heme in the kinetic characterizations of gaseous ligand binding, purified *So* H-NOX was first oxidized to its ferric state to release any bound ligand, cleaned up using a 10DG column, and subsequently reduced to its ferrous state by Na₂S₂O₄ titration. Only residual amount of unreacted Na₂S₂O₄ was present in the final samples [9]. NO binds to *So* H-NOX at a rate faster by several orders of magnitude than it reacts with Na₂S₂O₄ [20], any residual Na₂S₂O₄ left in the sample did not affect the kinetic measurement of NO binding to *So* H-

NOX. The rapid-scan spectral changes were monitored with a diode-array accessory, and the data were analyzed using Pro-Kineticist global analysis package (Applied Photophysics). The time courses were followed with a monochromator set at different wavelengths, and the observed rates, k_{obs} , were obtained by fitting to standard exponential function [11]. Second-order association rate constants were derived from the slopes in the secondary plots of k_{obs} versus [gaseous ligand] [9,11]. The formation rate constant of a 6c NO-heme-His complex, $k_{\text{on}}(\text{NO})$, in *So* H-NOX was measured under the true 2nd-order reaction conditions as described previously [9,11].

The dissociation rate constants were measured using competition methods by either reacting the 6c CO complex with 1 mM NO or the 5c NO complex with a mixture of 500 μM CO/12.5 mM $\text{Na}_2\text{S}_2\text{O}_4$ [9,11,21]. The dissociation rate constant of the unstable 6c NO complex was measured promptly after its formation using sequential stopped-flow method: *So* H-NOX was first reacted stoichiometric NO, and after a 100 ms delay, the 6c NO complex formed in the first mixing was reacted with the 500 μM CO/12.5 mM $\text{Na}_2\text{S}_2\text{O}_4$ mixture [9,11].

3. Results

3.1. Redox and spin states of resting *So* H-NOX

The electronic absorption spectrum of the resting *So* H-NOX exhibited a Soret peak at 422 nm with a shoulder at 400 nm and a split Q band with maxima at 554 nm and 570 nm (Fig. 1A). These spectral features indicated that the resting *So* H-NOX existed in mixed redox states. To characterize the electronic absorption of *So* H-NOX in different redox states, the resting *So* H-NOX was first oxidized with ferricyanide, in which the Soret peak showed a hypsochromic shift to 400 nm, typical of a ferric heme in an H-NOX protein (Fig. 1A). Ferric *So* H-NOX is featureless in the Q band region (Fig. 1A). Upon $\text{Na}_2\text{S}_2\text{O}_4$ reduction, ferrous *So* H-NOX (Scheme 1, A) exhibited a Soret peak at 430 nm and a broad Q band at 554 nm (Fig. 1A). The Soret peak wavelength of the resting *So* H-NOX was between those of ferric and ferrous *So* H-NOXs, and the split Q band of the resting *So* H-NOX further indicated that portion of it possessed a 6c heme, with a distal ligand. The reaction of ferrous *So* H-NOX with CO shifted its Soret band from 430 nm to 422 nm and split its Q band into two maxima at 550 nm and 568 nm (Fig. 2A); the spectral features of the produced CO complex were similar as the portion of the resting *So* H-NOX (Fig. 1A). On the other hand, EPR of the resting *So* H-NOX indicated that it did not contain any noticeable level of NO complex (Fig. 1C).

So H-NOX was further characterized using MCD spectroscopy (Fig. 1B). Ferric *So* H-NOX exhibited a small Soret band, $21.6 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 400 nm to $-19.0 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 425 nm with a crossover at 410 nm, and a charge-transfer band at 627 nm ($-9.4 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$) (Fig. 1B), indicating a high-spin heme [22]. Upon reduction, heme in ferrous *So* H-NOX remained in high-spin state as indicated by its MCD spectrum (Fig. 1B) [22]. The major MCD features of ferrous *So* H-NOX included a strong Soret band, $-102.3 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 417 nm to $260.3 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 435 nm with a crossover at 425 nm, and a sizeable Q-band, $28.0 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 549 nm to $-41.3 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 594 nm with a crossover at 580 nm (Fig. 1B). The ferrous heme converted to the low-spin state upon

the binding to CO [22], exhibiting MCD features typical of a 6c CO heme complex (Fig. 1B). Compared to that of ferrous *So* H-NOX, MCD of its CO complex exhibited a significantly weaker Soret band, $95.1 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 415 nm to $-111.5 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 428 nm with a crossover at 422 nm, and Q-bands with significantly increased intensities: $31.5 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 522 nm, $50.5 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 561 nm to $-94.0 \text{ M}^{-1}\text{cm}^{-1}\text{tesla}^{-1}$ at 578 nm with a crossover at 568 nm (Fig. 1B). The resting *So* H-NOX showed mixed MCD features including those from ferrous *So* H-NOX and those that may be attributed to its complex with CO (Fig. 1B), and the MCD spectrum of the resting *So* H-NOX was approximately synthesized with the MCD spectra of ferrous *So* H-NOX and its CO complex (Fig. S2). MCD signals of ferric *So* H-NOX were not obvious in the resting *So* H-NOX due to its much smaller intensities.

The EPR at 10 K of the resting *So* H-NO clearly demonstrated the existence of ferric heme (Fig. 1C). The EPR signals at $g = 6.10$ and 1.99 in the resting *So* H-NOX marked the existence of a high-spin, 5c ferric heme with axial geometry since both ferrous *So* H-NOX and its CO complex are EPR silent (Fig. 1C). After oxidation with ferricyanide, the EPR signal at $g = 6.10$ increased dramatically (Fig. 1C), indicating that the majority of the resting *So* H-NOX was EPR-silent, either in the ferrous state or CO complex. The major EPR features of ferric *So* H-NOX indicated that large proportion of the heme was high-spin with axial geometry. On the other hand, EPR signals at $g = 2.26$ indicated the existence of a low-spin heme in ferric *So* H-NOX (Fig. 1C). Overall, the electronic absorption, MCD, and EPR data showed that *So* H-NOX was purified as a mixture of ferric and ferrous states, and CO complex.

3.2. Kinetics of CO binding to ferrous *So* H-NOX

Rapid-scan reaction of ferrous *So* H-NOX with CO exhibited a simple one-step spectral change with no spectral intermediate (Fig. 2A), Soret peak shifting from 430 nm of ferrous *So* H-NOX to 424 nm of a 6c CO-heme-His complex (Scheme 1, B). The observed rates, k_{obs} 's, of the A_{424} time courses (Fig. 2B) exhibited a linear dependence on $[\text{CO}]$, and the slope equaled to the second-order association rate constant, $k_{\text{on}}(\text{CO}) = (8.2 \pm 0.3) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 2B, inset and Table 1). The dissociation rate constant of the 6c CO-heme-His complex was measured with the NO displacement method, $k_{\text{off}}(\text{CO}) = 0.7 \pm 0.02 \text{ s}^{-1}$ ($n = 4$) (Fig. 2C and Table 1). The equilibrium dissociation constant $K_{\text{D}}(\text{CO})$ was calculated as $k_{\text{off}}(\text{CO})/k_{\text{on}}(\text{CO}) = (8.5 \pm 0.4) \times 10^{-7} \text{ M}$ (Table 1).

3.3. Reaction of *So* H-NOX with stoichiometric NO

In the rapid-scan reaction of ferrous *So* H-NOX with stoichiometric NO, the Soret peak quickly shifted to 420 nm (Fig. 3A). Deconvolution of the rapid-scan data resolved the spectrum of a new species, exhibiting a broad Soret peak at 420 nm with a decreased intensity than that of ferrous *So* H-NOX (Fig. 3A), consistent with the formation of a 6c NO-heme-His complex (Scheme 1, C) [9]. The accurate formation rate of the 6c NO complex, $k_{\text{on}}(\text{NO})$, was determined by following the time course of A_{428} in the reaction of $1 \mu\text{M}$ *So* H-NOX with stoichiometric NO (Fig. 3B). About 80% of the total A_{428} change was captured, enabling a good fit to the true 2nd-order reaction equation to obtain the $k_{\text{on}}(\text{NO})$ of $(1.5 \pm 0.4) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ ($n = 6$) (Fig. 3B and Table 1) [11]. The dissociation rate constant of

the 6c NO complex, $k_{\text{off}}(\text{NO})$, was measured using the sequential mixing stopped-flow method, following the A_{424} time course for the CO complex formation in the second mixing (Fig. 3C) [18]. The rate, $k_{\text{obs}} = 0.2 \pm 0.02 \text{ s}^{-1}$ ($n = 3$), was much slower than $k_{\text{on}}(\text{CO})$ of *So* H-NOX, confirming that the formation rate of the 6c CO-heme-His complex in this reaction was limited by $k_{\text{off}}(\text{NO})$ ($= k_{\text{obs}}$). The $K_{\text{D}}(\text{NO})$ of ferrous *So* H-NOX for NO was calculated as $k_{\text{off}}(\text{NO})/k_{\text{on}}(\text{NO}) = (1.5 \pm 0.4) \times 10^{-10} \text{ M}$ (Table 1).

The 6c NO-heme-His complex was not stable and converted incompletely to a 5c NO-heme complex (Scheme 1, *F*), as manifested by the shift of the Soret peak to 400 nm after a longer reaction time (Fig. S3). The conversion was due to the dissociation of the proximal histidine 103, and the rate, $1.8 \times 10^2 \text{ s}^{-1}$, was obtained by deconvoluting the rapid-scan data (Fig. S3).

3.4. Reaction of *So* H-NOX with excess NO

When ferrous *So* H-NOX was reacted with 10-fold NO, the 6c NO-heme-His complex formed within the dead time of our stopped-flow instrument. The only observed spectral change was the shift of the Soret peak from 420 nm to 400 nm (Fig. 4A), consistent with the conversion from the 6c NO complex to a 5c NO complex (Scheme 1, *E*). EPR spectroscopy of ferrous *So* H-NOX reacted with excess NO confirmed the formation of a 5c NO complex as shown by the EPR features typical of a 5c NO complex (Fig. S4). In the final 5c NO complex NO likely binds to the proximal side of heme as concluded by the sequential rapid-freeze quench EPR studies of the binding of ^{14}NO and ^{15}NO to sGC in the two successive mixing stages [23].

The kinetics of the ferrous *So* H-NOX reaction with excess NO was investigated by following the decay of the 6c NO complex, represented by the time course of A_{422} (Fig. 4B). The observed rates, k_{obs} 's, showed a linear dependence on $[\text{NO}]$ (Fig. 4B, inset). Formation of the 5c heme-NO complex at low $[\text{NO}]$ ($< 10 \mu\text{M}$) may proceed through two possible pathways; one through a quaternary intermediate, NO-heme--NO(--His) (Scheme 1, *D*), produced by the reaction between the 6c NO complex with secondary NO, and the other through a *bis*-NO complex intermediate (Scheme 1, *I*) from the reaction of secondary NO with the 5c NO-heme complex converted from the 6c NO complex (Fig. 3A). The latter pathway was feasible under relatively low $[\text{NO}]$, where k_{obs} was slower than the conversion rate ($1.8 \times 10^2 \text{ s}^{-1}$) from the 6c NO complex to 5c NO-heme. At higher $[\text{NO}]$, the pathway $C \rightarrow D$ (Scheme 1) dominated since k_{obs} became significantly faster than the rate of $C \rightarrow F$. Both the quaternary intermediate NO-heme--NO(--His) and the *bis*-NO intermediate in *So* H-NOX were formed transiently and unobservable due to their fast conversions to the 5c heme-NO complex (Scheme 1). In the $C \rightarrow D$ pathway, binding of secondary NO with the 6c NO-heme-His complex was overall the rate-limiting step due to the linear dependence of k_{obs} on $[\text{NO}]$ (Fig. 4B, inset). The 2nd-order formation rate of the quaternary intermediate, represented by the slope of the line, was $(1.9 \pm 1.6) \times 10 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4B, inset).

The dissociation rate of NO from the quaternary intermediate was very small, indicated by the extremely small y-intercept of the secondary plot (Fig. 4B, inset). Moreover, formation of the quaternary intermediate never reached near equilibrium due to its fast conversion to the 5c heme-NO complex. Since the dissociation of proximal histidine 103 in the quaternary intermediate never became rate-limiting even at the highest $[\text{NO}]$ tested, the formation rate

of the 5c heme-NO complex (Scheme 1, $D \rightarrow E$) in the *So* H-NOX was not experimentally measured, but it had to be significantly faster than the k_{obs} at 30 μM NO, $\sim 600 \text{ s}^{-1}$ (Fig. 4B, inset). On the other hand, the dissociation rate of NO from the 5c heme-NO complex (Scheme 1, $E \rightarrow A$) was readily measured by CO displacement method following the time course of A_{424} , $k_{\text{off},5\text{c}}(\text{NO}) = (1.3 \pm 0.01) \times 10^{-3} \text{ s}^{-1}$ ($n = 2$) (Fig. 4C and Table 1).

3.5. Reaction of O_2 with ferrous *So* H-NOX

When ferrous *So* H-NOX was mixed with O_2 -saturated buffer (final $[\text{O}_2] = 600 \mu\text{M}$), the Soret peak shifted slowly from 430 nm to 400 nm without any observable oxyferrous intermediate (Fig. 5). The rapid-scan data was easily deconvoluted based on a simple one-step $a \rightarrow b$ model, in which “a” corresponded to ferrous *So* H-NOX and “b” to ferric *So* H-NOX (Fig. 5, inset). This indicated that ferrous *So* H-NOX does not bind O_2 even at the highest $[\text{O}_2]$ under our experimental conditions. On the other hand, the autoxidation of *So* H-NOX after long time exposure to O_2 indicated that O_2 does interact with ferrous *So* H-NOX, and the absence of an oxyferrous heme in *So* H-NOX was likely due to its extremely large $k_{\text{off}}(\text{O}_2)$. The autoxidation was due to the breaking down of the transiently formed oxyferrous complex (Scheme 1, G) to ferric heme and superoxide (Scheme 1, H) [24,25], and its rate at 600 μM O_2 , was 0.02 s^{-1} , obtained from the deconvolution of the rapid-scan data (Fig. 5, inset).

4. Discussion

The kinetics of the gaseous ligand bindings of *So* H-NOX are summarized in Scheme 1 and compared with those of *Cb*, *Ns*, *Tt*, and *Vc* H-NOXs, as well as sGC in detail below.

4.1. Affinities of all H-NOXs and sGC for NO and CO obey the “sliding scale rule”

Recently, the logarithms of $K_{\text{D}}/k_{\text{on}}/k_{\text{off}}$ of NO, CO, and O_2 versus the gaseous ligand type are found to exhibit a very similar pattern in more than hundred hemeproteins and model compounds. Such a general pattern has been summarized as “sliding scale rule” hypothesis that identifies five crucial factors governing the gaseous ligand selectivity of a hemeprotein [6,18]. The first factor is the identity of the proximal ligand, which determines whether a hemeprotein is capable of binding NO/CO/ O_2 selectively. The large $K_{\text{D}}(\text{CO})/K_{\text{D}}(\text{NO})$ and $K_{\text{D}}(\text{O}_2)/K_{\text{D}}(\text{CO})$ ratios, $10^3 - 10^4$ is observed for 5c hemeproteins that have a neutral proximal histidine ligand, whereas this ligand discrimination is lost in hemeproteins with imidazolate, cysteine thiolate, or tyrosine phenolate anion proximal ligands due to their strong ligand field strength. The next two crucial factors are distal steric hindrance and the proximal strain of heme. Steric hindrance determines whether the access and dissociate of a gaseous ligand to heme is impeded on the distal side. On the other hand, proximal strain adjusts the strength of the Fe-His bond in a hemeprotein, modulating the bond formation between Fe and a gaseous ligand on the distal side. In a hemeprotein, distal steric hindrance and proximal strain together can lead to dramatic $10^8 - 10^9$ -fold changes in K_{D} for all three gaseous ligands, but remarkably cause little change in the $K_{\text{D}}(\text{CO})/K_{\text{D}}(\text{NO})$ and $K_{\text{D}}(\text{O}_2)/K_{\text{D}}(\text{CO})$ ratios. The nearly constant $K_{\text{D}}(\text{CO})/K_{\text{D}}(\text{NO})$ and $K_{\text{D}}(\text{O}_2)/K_{\text{D}}(\text{CO})$ ratios determine that $\log K_{\text{D}}(\text{NO})$ - $\log K_{\text{D}}(\text{CO})$ - $\log K_{\text{D}}(\text{O}_2)$ lines of different hemeproteins remain approximately linear and parallel to each other in the “sliding scale rule” plot. The “sliding

scale rule” refers to the parallel $\log K_D(\text{NO})$ - $\log K_D(\text{CO})$ - $\log K_D(\text{O}_2)$ lines which “slide” along the y-axis. As a result, for a hemeprotein, determination of the K_D for one ligand (i.e. CO) allows estimation of the K_D values for the other two ligands, which is particularly useful for hemeproteins with very high ($> 10^{-3}$ M) K_D values for O_2 binding. Deviation from the “sliding scale” pattern is observed in a hemeprotein that either has an H-bond donor in the distal pocket or exhibits multiple-step NO binding, the 4th and 5th controlling factors in ligand selectivity. The presence of an H-bond donor(s) on the distal side of heme preferentially enhances O_2 affinity but has little or no effect on CO or NO binding, leading to deviation from the “sliding scale rule” at the $\log K_D(\text{O}_2)$ end. On the other hand, the ultra-high apparent affinities of some hemeproteins for NO, such as sGC and certain bacterial H-NOXs, are due to multiple-step NO binding in which the unstable 6c NO-heme-His complex either promptly converts to a 5c NO complex or react rapidly with secondary NO to generate a 5c NO complex. The K_D s of the initial 6c NO complexes in these hemeproteins follow the “sliding scale rule”, however, the subsequently generated 5c NO complexes have significantly smaller k_{off} constants, leading to much lower apparent K_D s.

When $K_D(\text{NO})$ and $K_D(\text{CO})$ of *So* H-NOX are plugged into the “sliding scale” plot, its $\log K_D(\text{NO})$ - $\log K_D(\text{CO})$ line parallels with those of sGC, *Cb*, *Ns*, *Tt*, and *Vc* H-NOXs (Fig. 6A). Moreover, the $\log K_D(\text{NO})$ - $\log K_D(\text{CO})$ lines of the five bacterial H-NOXs cluster in a small region in the “sliding scale” plot but are well separated from that of sGC (Fig. 6A). The main factor that regulates the gaseous ligand discrimination in the bacterial H-NOXs and sGC is “proximal strain” rather than “distal steric hindrance” observed for cyt c’ [6] because the $k_{\text{on}}(\text{NO})$ s of the six heme sensors are all close to diffusion control. The ability of sGC, *Ns*, *Vc*, and *So* H-NOXs, all lacking a distal H-bond donor, to bind NO while excluding O_2 is achieved by simultaneous increase of the K_D values of all three gaseous ligands, to make the $K_D(\text{O}_2) \gg 260 \mu\text{M}$, above the solubility of O_2 in a regular buffer. Presence of the distal tyrosine as an H-bond donor in *Tt* and *Cb* H-NOXs causes their $\log K_D(\text{NO})$ - $\log K_D(\text{CO})$ - $\log K_D(\text{O}_2)$ lines to bend down on the oxygen end, in contrast to the lines of *Ns* H-NOX, heme model compound, and H61L leghemoglobin (Lb), indicating the selective effect of a distal H-bonding donor on $K_D(\text{O}_2)$, but not $K_D(\text{NO})$ or $K_D(\text{CO})$, as reported previously [6,10].

Similar to the other bacterial H-NOXs and sGC, *So* H-NOX has a $k_{\text{on}}(\text{NO})$ significantly faster than its $k_{\text{on}}(\text{CO})$ (Fig. 6B), due to the spin barrier to overcome in the association of CO with heme [26]. Again, the $\log k_{\text{on}}(\text{NO})$ - $\log k_{\text{on}}(\text{CO})$ lines of the five bacterial H-NOXs fall in a narrow region (Fig. 6B). *Tt* H-NOX has a $k_{\text{on}}(\text{O}_2)$ close to its $k_{\text{on}}(\text{NO})$, $4.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $1.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively (Table 1), exhibiting a V-shape pattern in the plot of its $\log k_{\text{on}}$ versus the ligand type. Similar V-shape patterns of $\log k_{\text{on}}$ versus the ligand type are also observed in heme model compound and H61L Lb (Fig. 6B) [18].

For the graphical analysis of k_{off} (Fig. 6C), we focused on those of CO complex and 6c NO complex because $k_{\text{off}}(\text{O}_2)$ was only measurable for *Tt* H-NOX due to the autoxidation or lack of binding in other H-NOXs and sGC. The significantly smaller $\log k_{\text{off}}(\text{O}_2)$ in *Tt* H-NOX than the extrapolated value from its $\log k_{\text{off}}(\text{NO})$ - $\log k_{\text{off}}(\text{CO})$ illustrates again the effect of distal H-bonding on the stabilization of an oxyferrous complex [6,10]. While all the bacterial H-NOXs, heme model compound, and H61L Lb have a $k_{\text{off}}(\text{NO})$ significantly

slower than its $k_{\text{off}}(\text{CO})$ (Fig. 6C) [18], sGC is unique by having a $k_{\text{off}}(\text{NO})$ faster than its $k_{\text{off}}(\text{CO})$, highlighting its distinctive kinetics in gaseous ligand binding. Overall, K_D , k_{on} , and k_{off} of the five bacterial H-NOXs and sGC are all consistent with the “sliding scale rule” hypothesis, but the parameters of sGC are significantly separated from those of the bacterial H-NOXs.

4.2. CO binding

As sGC, *Cb*, *Ns*, *Tt*, and *Vc* H-NOXs, *So* H-NOX binds CO in a one-step reversible process (Scheme 1, $A \rightarrow B$). The $k_{\text{on}}(\text{CO})$ s of the bacterial H-NOXs vary in a narrow range of ~4-fold, from 8.2×10^5 to $3.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, but are at least ~20-fold faster than that of sGC, $4.0 \times 10 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). The $k_{\text{off}}(\text{CO})$ of *So* H-NOX, 0.7 s^{-1} , is very similar to those of *Cb*, *Tt*, and *Vc* H-NOXs, varying from 0.5 to 1.0 s^{-1} , but all noticeably slower than that of *Ns* H-NOX, 3.6 s^{-1} , which itself is 3 times slower than that of sGC at 10.7 s^{-1} (Table 1). Overall, the bacterial H-NOXs bind CO with similar $K_D(\text{CO})$ s, all within a ~9-fold range from 1.6×10^{-7} to $1.4 \times 10^{-6} \text{ M}$, but at least ~190-fold tighter than sGC, $2.6 \times 10^{-4} \text{ M}$ (Table 1). Interestingly, it has been recently demonstrated that while the full length sGC from *Manduca sexta* (*Ms* sGC) and *Ms* sGC NT2, lacking 48 residues from the α -subunit N-terminus and the catalytic domains, have $K_D(\text{CO})$ s between $50 - 100 \mu\text{M}$, β H-NOX/PAS subunit of *Ms* sGC alone has a $K_D(\text{CO})$ of $0.2 \mu\text{M}$ [27], comparable to those of the bacterial H-NOXs. Thus, the α subunit of *Ms* sGC may negatively modulate its β subunit's affinity for CO. This negative modulation has important functional significance and is further discussed below.

4.3. NO binding

So H-NOX binds NO with a $K_D(\text{NO})$ of $1.5 \times 10^{-10} \text{ M}$ (Scheme 1, $A \rightarrow C$), which is similar to those of the other bacterial H-NOXs, varying in a narrow range from 2.3×10^{-1} to $2.7 \times 10^{-1} \text{ M}$. The bacterial H-NOXs bind NO at least 200-fold tighter than sGC with a $K_D(\text{NO}) = 5.4 \times 10^{-8} \text{ M}$ (Table 1). *So* H-NOX binds NO promptly at $1.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, faster than any other bacterial H-NOXs showing $k_{\text{on}}(\text{NO})$ from 1.5×10^8 to $1.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, and 10 times faster than that of sGC, $1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). On the other hand, the $k_{\text{off}}(\text{NO})$ of *So* H-NOX, 0.2 s^{-1} , while slightly slower than that of *Vc* H-NOX, 0.3 s^{-1} , is noticeably faster than those of the other bacterial H-NOXs varying from 3.4×10^{-3} to $5.0 \times 10^{-2} \text{ s}^{-1}$ (Table 1). However, the $k_{\text{off}}(\text{NO})$ s of all the bacterial H-NOXs are significantly slower than that of sGC, 27 s^{-1} (Table 1).

The 6c NO-heme-His complex in *So* H-NOX quickly converts to a 5c NO complex, presumably due to the dissociation of its proximal histidine ligand (Scheme 1, $C \rightarrow F$). Similar multiple-step in NO binding is also observed in sGC, *Cb*, and *Vc* H-NOXs, but not in *Ns* and *Tt* H-NOXs [9,11]. The conversion from the 6c NO complex to the 5c NO-heme complex is much faster in *So* H-NOX and sGC, $1.8 \times 10^2 \text{ s}^{-1}$ and 8.5 s^{-1} , respectively, than in *Vc* and *Cb* H-NOXs [9,11,23]. On the other hand, the significantly faster conversion rate in *So* H-NOX than that in sGC may have important implications for the mechanisms of their reactions with excess NO (see below). Such a conversion is not complete in *So*, *Cb*, and *Vc* H-NOXs (Fig. S3) [9,11], but complete in sGC [23].

In the reaction of *So* H-NOX with excess NO, secondary NO either reacts with the 6c NO-heme-His complex to form a quaternary NO-heme--NO(--His) intermediate (Scheme 1, $C \rightarrow D$) or with the 5c NO-heme complex, at relatively low [NO], to form a *bis*-NO intermediate (Scheme 1, $F \rightarrow J$); both intermediates subsequently convert to a 5c NO complex promptly. Although the final 5c NO complex is indistinguishable by UV/Vis spectroscopy from that generated with stoichiometric NO, sequential rapid-freeze quench EPR using ^{14}NO and ^{15}NO to react with sGC demonstrates that NO resides on the proximal side (or the “dark side”) in the final complex in reaction with excess NO rather than on the distal side of the heme as in the reaction with stoichiometric NO [23]. Similar reactions of a 6c NO-heme-His complex with excess NO are also observed in sGC, *Cb*, and *Vc* H-NOXs [9,11,17], but not in *Ns* or *Tt* H-NOX [10]. In both *So* H-NOX and sGC [17], formation of the quaternary intermediate is rate-limiting, making the rates of the overall reaction [NO]-dependent. On the other hand, since the conversion of 6c NO complex to 5c NO-heme complex (Scheme 1, $C \rightarrow F$) is about 21 times faster in *So* H-NOX than in sGC, the binding of secondary NO with the 5c NO-heme complex is feasible in *So* H-NOX but not in sGC [23]. In *Cb* and *Vc* H-NOXs, conversion to the 5c heme-NO complex becomes rate-limiting at high [NO], leading to a saturable kinetics of k_{obs} on [NO] [9,11]. The intermediate NO-heme--NO(--His) forms with similar rates in *Cb*, *So*, and *Vc* H-NOXs, $1.8 \times 10^7 - 2.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, but much slower in sGC at $2.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1) [9,11,17]. On the other hand, *So* H-NOX exhibits a very small NO dissociation rate from the quaternary intermediate (Scheme 1, $E \rightarrow D$) compared to *Cb* and *Vc* H-NOXs or sGC (Table 1), highlighting the significantly different proteinaceous environments around heme in these proteins. In sGC, *Cb*, *So*, and *Vc* H-NOXs, multiple-step NO binding dramatically increases the apparent NO affinities due to the much slower $k_{\text{off},5\text{c}}(\text{NO})$.

4.4. Interaction with O₂

So H-NOX does not have any affinity for O₂ under atmospheric pressure, partly due to its lack of a distal H-bond donor to stabilize an oxyferrous heme, same as sGC, *Ns*, and *Vc* H-NOXs. However, the “sliding scale rule” provides a way to predict the $K_{\text{D}}(\text{O}_2)$ of *So* H-NOX based on the comparison with *Ns* H-NOX, whose $\log K_{\text{D}}(\text{O}_2)$, measured with a high pressure cell, falls on the linear extrapolation of its $\log K_{\text{D}}(\text{NO})$ - $\log K_{\text{D}}(\text{CO})$ line (Fig. 6A) [10]. Linearly extrapolating of *So* H-NOX’s $\log K_{\text{D}}(\text{NO})$ - $\log K_{\text{D}}(\text{CO})$ line yielded its predicted $K_{\text{D}}(\text{O}_2) \sim 4.5 \text{ mM}$ (Fig. 6A). The $K_{\text{D}}(\text{O}_2)$ of *Ns* H-NOX is measureable using a high pressure cell due to its extremely slow autoxidation rate, $1.4 \times 10^{-5} \text{ s}^{-1}$; *So* H-NOX, on the other hand, exhibits sizeable autoxidation rate, $\sim 2 \times 10^{-2} \text{ s}^{-1}$ when reacted with $600 \mu\text{M}$ O₂, preventing measuring its $K_{\text{D}}(\text{O}_2)$ with the same method. The $K_{\text{D}}(\text{O}_2)$ of *Vc* H-NOX, which also has a considerable autoxidation rate, $\sim 4 \times 10^{-3} \text{ s}^{-1}$ when reacted with $600 \mu\text{M}$ O₂ (data not shown), can be predicted similarly to be $\sim 1.4 \text{ mM}$ (Fig. 6A). The $K_{\text{D}}(\text{O}_2)$ of sGC has been predicted using the “sliding scale” plot to be $\sim 1 \text{ M}$ (Fig. 6A), which is unmeasurable even using high pressure cell although sGC has a very slow autoxidation rate [6]. Both *Cb* and *Tt* H-NOXs exhibit strong affinities for O₂, $K_{\text{D}}(\text{O}_2) = 5.3 \times 10^{-5}$ and $4.4 \times 10^{-8} \text{ M}$, respectively, due to their distal tyrosines, which are capable of stabilizing oxyferrous hemes. Interestingly, *Cb* H-NOX exhibits an autoxidation rate, $2 \times 10^{-2} \text{ s}^{-1}$ when reacted with $600 \mu\text{M}$ O₂, similar to that of *So* H-NOX, whereas no autoxidation is observed for the oxyferrous heme in *Tt* H-NOX. In contrast to CO and NO, each exhibiting highly

similar binding affinities among the five bacterial H-NOXs, the bindings of O₂ to the bacterial H-NOXs are widely diverse, likewise for their rates of autoxidation. Even for *Cb* and *Tt* H-NOXs that show significant O₂-binding, their K_D(O₂)s are quite different. These results indicate wide functional differences in terms of O₂-sensing among these heme sensors despite their structural homology, especially in the heme domain.

4.5. Possible access channel(s) for gaseous ligands

The affinity of a heme sensor protein for a gaseous ligand is determined by its k_{on} and k_{off} constants, and a k_{on} is significantly affected by the accessibility of the gaseous ligand to heme, especially so for $k_{\text{on}}(\text{NO})$ and $k_{\text{on}}(\text{O}_2)$ which are not hindered by spin barrier as $k_{\text{on}}(\text{CO})$ [26]. All the bacterial H-NOXs and sGC exhibit $k_{\text{on}}(\text{NO})$ close to the diffusion limit, indicating an essentially unhindered NO accessibility for heme. Triangulated protein molecular surface plots from the crystal structures of *So*, *Ns*, and *Tt* H-NOXs all reveal an opening leading towards the side of heme, without any large amino acid sidechain(s) in the way (Figs. 7A and S6) [8,28,29,30,31]. Such a short channel should provide a facile path to heme as the main access channel for the gaseous ligands. This short path for gaseous ligands also enables easy accessibility to both sides of the heme, providing good basis for the binding of secondary NO to the proximal side of the heme observed in sGC, *So*, and *Vc* H-NOXs [23]. The recently proposed “bifurcated Y-shaped channel” leading to the distal side of the heme is based on the crystallographic analysis for equilibrium xenon binding site(s) in *Ns* H-NOX and computation studies (Fig. 7B) [32,33]. However, locations of Xe in a protein crystal do not necessarily reveal the kinetic access path(s) of gaseous ligands to the heme as exemplified in the gaseous ligand bindings to globins [34]. Moreover, both $k_{\text{on}}(\text{CO})$ and $k_{\text{off}}(\text{CO})$ increase only marginally in several *Ns* H-NOX mutants with tryptophan replacing the residues lining the projected access channels by Xe-probe, hardly supporting these channels as the main access channels for gaseous ligands [32]. Using CO as the probing ligand may not provide correct information for gaseous ligand accessibility due to its intrinsic slow $k_{\text{on}}(\text{CO})$ [26], likely becoming the overall rate-limiting step (Fig. 6B). NO should be a better probing ligand for gaseous ligand accessibility due to its large intrinsic $k_{\text{on}}(\text{NO})$, which is not limited by any spin barrier. Locating this short passage to the heme edge (Fig. 7A and Fig. S6) in the 3D structures of the three H-NOXs and the near diffusion-controlled $k_{\text{on}}(\text{NO})$ s, $10^8 - 10^9 \text{ M}^{-1}\text{s}^{-1}$ for all the six heme sensors (Table 1), strongly imply that gaseous ligand accessibility plays an insignificant role in gaseous ligand selectivity.

4.6. Summary

The comparison of the gaseous ligand bindings in *So* H-NOX with those of *Cb*, *Ns*, *Tt*, and *Vc* H-NOXs reveals the striking similarity in CO and the first-step NO bindings in the bacterial H-NOXs. On the other hand, each bacterial H-NOX exhibits different characters in the stabilities of its 6c NO complex, reactivity with secondary NO, stabilities of oxyferrous heme, and autoxidation to ferric heme. sGC, with a β heme domain analogous to the bacterial H-NOXs (Fig. S1), has significantly different gaseous ligand binding kinetics (Table 1), leading to much weaker affinities for all three gaseous ligands and the significant up-shift of its log K_D(NO)-log K_D(CO)-log K_D(O₂) line in the “sliding scale” plot (Fig. 6A). The weaker affinities for gaseous ligands in sGC are likely due to the negative modulating effects of its α subunit as implicated in the studies of *Ms* sGC and its deletion mutants [27].

It is worthwhile to verify whether the gaseous ligand affinities of sGC's β subunit alone follow the "sliding scale rule" and are comparable to those of the bacterial H-NOXs. On the other hand, characterizing the gaseous ligand bindings of the full length bacterial sensor proteins from *Cb* or *Tt*, each containing the H-NOX and MCP domains should provide insights into possible regulatory role(s) of other domain(s) on the gaseous ligand selectivity of these bacterial H-NOX domains. Any regulatory effect(s) due to the other domain(s) in these bacterial sensor proteins can be verified by the vertical shifts of their log $K_D(\text{NO})$ -log $K_D(\text{CO})$ -log $K_D(\text{O}_2)$ lines in the "sliding scale" plot. These ideas can be tested in the future. Such studies should provide insights into how gaseous ligand signaling pathway(s) in bacteria, especially in obligate anaerobes, through H-NOX binding to gaseous ligands is controlled. The structural components contributing to the five factors of gaseous ligand selectivity identified by the "sliding scale rule" can be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

H-NOX	heme nitric oxide and oxygen binding protein
<i>Cb</i> H-NOX	H-NOX from <i>Clostridium botulinum</i>
Lb	leghemoglobin
<i>Ns</i> H-NOX	H-NOX from <i>Nostoc</i> sp. PCC7120
<i>So</i> H-NOX	H-NOX from <i>Shewanella oneidensis</i>
<i>Tt</i> H-NOX	H-NOX from <i>Thermoanaerobacter tengcongensis</i>
<i>Vc</i> H-NOX	H-NOX from <i>Vibrio cholera</i>
sGC	human soluble guanylyl cyclase
5c NO-heme	five coordinate NO complex with NO ligand on the distal side of heme
5c heme-NO	five coordinate NO complex with NO ligand on the proximal side of heme

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Highlights

- The gaseous ligand bindings of Heme Nitric oxide/OXygen binding protein (H-NOX) from *Shewanella oneidensis* (*So* H-NOX) were characterized in detail in this study.
- The gaseous ligand selectivity of *So* H-NOX, four other bacterial H-NOXs previously characterized, and H-NOX analogue soluble guanylyl cyclase (sGC) obey the “sliding scale rule”.
- NO and CO binding parameters of all five bacterial H-NOXs cluster together, but are distant from those of sGC, indicating additional modulator of gas selectivity in sGC.
- This comparative study reveals distinct gaseous ligand binding behaviors of each bacterial H-NOX, including the stability of the 6-coordinate NO complex, reactivity with secondary NO, stability of oxyferrous heme and autoxidation to ferric heme.

Figure 1. Electronic absorption and MCD spectra of *So* H-NOX and its complex with CO
 The electronic absorption spectra (A), MCD spectra (B), and EPR (C) of *So* H-NOX and its 6c CO-heme-His complex are represented by different colors: resting *So* H-NOX, black; ferric *So* H-NOX, red; ferrous *So* H-NOX, green and 6c CO-heme-His complex, blue. Ferrous *So* H-NOX and its CO complex are EPR silent.

Figure 2.

Binding of CO to ferrous *SoH*-NOX. (A) Rapid-scan data of 3.8 μM ferrous *SoH*-NOX with 90 μM CO. (B) Time courses of A_{424} in the reactions of 3.8 μM ferrous *SoH*-NOX with 12.5, 25, 37.5, 50, 64.5 and 75 μM CO (bottom to top). Inset: the dependence of k_{obs} on $[\text{CO}]$ (circle) and the linear regression (line). The standard deviation of four measurements for each k_{obs} is represented by the vertical separation between the horizontal bars. The error range of the linear regression is reported for the derived secondary rate constant $k_{\text{on}}(\text{CO})$.

(C) Rapid-scan reaction of $\sim 3.8 \mu\text{M}$ ferrous *So* H-NOX/ $90 \mu\text{M}$ CO with 1 mM NO. Inset: time course of A_{424} (black) and the fit to the standard exponential function (red).

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Figure 3.

Kinetics of the 6c NO-heme-His complex in *So H-NOX*. (A) The absorption spectra of ferrous *So H-NOX* (solid line) and its 6c NO-heme-His complex (dashed line), resolved from the rapid-scan data of the reaction of 1.8 μM ferrous *So H-NOX* with stoichiometric NO. The y-axis represents the extinction coefficients calculated based on absorbance and [*So H-NOX*]. (B) Time course of A_{428} (circles) of 1.0 μM ferrous *So H-NOX* with stoichiometric NO and the fit to the second-order reaction mechanism (line) [9]. (C) The time course of A_{424} (circles) in the sequential stopped-flow reaction: 1.8 μM ferrous *So H-*

NOX first mixed with stoichiometric NO, aged for 100 ms, and then further reacted with a 500 μM CO/12.5 mM $\text{Na}_2\text{S}_2\text{O}_4$ mixture. The line represents the fit to the standard exponential function.

Figure 4.

Kinetics of the 5c heme-NO complex in the *So* H-NOX reaction with excess NO. (A) The spectrum of the 5c heme-NO complex in *So* H-NOX (dashed line) formed in the reaction of 1.8 μM ferrous *So* H-NOX (solid line) with 18 μM NO. The y-axis represents the calculated extinction coefficients based on absorbance and [*So* H-NOX]. (B) Time courses of A_{422} during the reactions of 1.8 μM ferrous *So* H-NOX with 5, 10, 15, 20, 25, and 30 μM NO (from top to bottom). Inset: the dependence of k_{obs} on [NO] (circle) and the linear regression (line). The standard deviation of four measurements for each k_{obs} is represented by the

vertical separation between the horizontal bars. The error range of the linear regression is reported for the derived secondary rate constant. (C) The dissociation kinetics of the 5c heme-NO complex in *So* H-NOX by reacting 2.6 μM 5c heme-NO complex with a 500 μM CO/12.5 mM $\text{Na}_2\text{S}_2\text{O}_4$ mixture and following the time course of A_{424} .

Figure 5.

Reaction of ferrous *SoH*-NOX with O_2 . The spectral change during the reaction of 2.6 μM ferrous *SoH*-NOX with 600 μM O_2 . Spectra captured at 0.1, 10, 15, 20, 30, 50, 70, 100 and 200 s are shown. The arrows indicate the directions of the spectral changes. Inset: the optical species deconvoluted based on a (black) $\rightarrow$ b (red) model.

Figure 6.

Relationship of the $\log K_D$, $\log k_{on}$, and $\log k_{off}$ of NO, CO, and O₂ for the bacterial H-NOXs and sGC versus the ligand type. The logarithms of the measured K_D (A), k_{on} (B), and k_{off} (C) of NO/CO/O₂ to the ferrous bacterial H-NOXs are plotted versus the ligand type. Parameters measured for H61L Lb and heme model Fe(II) PP(1-MeIm) are also plotted for comparisons and demonstrating the large dynamic ranges of K_D , k_{on} , and k_{off} which are modulated by the heme binding environments. sGC, black triangle up; *Cb* H-NOX, blue diamond; *Ns* H-NOX, red triangle down; *So* H-NOX, yellow triangle up; *Tt* H-NOX, green square; *Vc* H-NOX, pink square; Fe(II) PP(1-MeIm), purple circle, and H61L Lb, black circle. The kinetic parameters for the heme sensors other than *So* H-NOX can be found in references [9,11,18] and the literatures cited therein. The dashed lines represent the predicted $K_D(O_2)$ s for sGC, *So*, and *Vc* H-NOXs based on the “sliding scale rule”. The clusters of H-NOXs’ $K_d(NO)/K_d(CO)$, $k_{on}(NO)/k_{on}(CO)$, and $k_{off}(NO)/k_{off}(CO)$ are circumscribed by red dashed lines.

Figure 7.

Gaseous ligand access channel(s) of *Ns* H-NOX. (A) The protein backbone of *Ns* H-NOX (PDB 2O09) is shown together with its protein surface (light orange) to reveal a large opening to the side of its heme (green) facing the carboxylate of the propionate side chain. The probe size is 2 Å. Red: oxygen, blue: nitrogen, iron: purple. (B) The surface of *Ns* H-NOX (gray) superimposed with its peptide backbone from a ~90° rotation along the vertical axis from (A), showing the distal pockets (DP) where Xe atoms (light brown balls) reside [32,33]. The contours of the proposed channels extending from the DPs to heme are colored in wheat and labeled “Tunnel 1” and “Tunnel 2”. The heme is colored red and the short direct excess channel in (A) is indicated with a red arrow. Panel (B) is adapted from Fig. 4a of reference [33] with permission from John Wiley and Sons (license number 3902590265564). The structures are plotted using the graphics tools from Protein Data Bank, based on fast Euclidean Distance Transform [28].

Scheme 1.

Binding of gaseous ligands to ferrous So H-NOX. Each heme species is labeled in red. Intermediates *D*, *G* and *I*, bracketed in parentheses, are transient and unobservable in experiments. Intermediate *D* may be a quaternary complex as proposed in [23]. The pathway from *F* through *I* to *E* may be feasible at low [NO]. Values in parentheses are estimated. Since the conversion from *D* to *E* is never rate limiting, its rate is significantly faster than the k_{obs} observed at the highest [NO] (Fig. 4B, inset).

Table 1

Kinetic parameters of gaseous ligand binding for the bacterial H-NOXs and sGC.

H-NOXs	Gaseous Ligand	k_{on} , M ⁻¹ s ⁻¹	k_{off} , s ⁻¹	K_D , M	comments
<i>Cb</i> H-NOX ^a	NO	1.5×10^8	1.2×10^{-2}	8.0×10^{-11}	6c NO-heme-His complex
		1.8×10^7	2.0×10^4		Intermediate NO-heme--NO(–His)
			1.1×10^{-3}	$(7.3 \times 10^{-12})^\dagger$	5c heme-NO complex
	CO	1.5×10^6	1.0	6.7×10^{-7}	
	O ₂	N/A	2.5×10^3	5.3×10^{-5}	
<i>Ns</i> H-NOX ^b	NO	3×10^8	5×10^{-2}	1.7×10^{-10}	6c NO-heme-His complex
		2.4×10^6	1.9	8×10^{-7}	2 nd 6c NO-heme-His complex
	CO	3×10^6	3.6	1.4×10^{-6}	
	O ₂	N/A	N/A	1.3×10^{-2}	
<i>Tl</i> H-NOX ^c	NO	1.5×10^8	3.4×10^{-3}	2.3×10^{-11}	6c NO-heme-His complex
	CO	3.3×10^6	0.5	1.6×10^{-7}	
	O ₂	4.3×10^7	1.9	4.4×10^{-8}	
<i>So</i> H-NOX ^d	NO	1.5×10^9	0.2	1.5×10^{-10}	6c NO-heme-His complex
		1.9×10^7	small		Intermediate NO-heme--NO(–His)
	CO	8.2×10^5	1.3×10^{-3}	$(8.6 \times 10^{-13})^\dagger$	5c heme-NO complex
	O ₂	N/A	N/A	8.5×10^{-7}	
				$\sim 4.5 \times 10^{-3\#}$	
<i>Vc</i> H-NOX ^e	NO	1.1×10^9	0.3	2.7×10^{-10}	6c NO-heme-His complex
		2.5×10^7	4.5×10^3		Intermediate NO-heme--NO(–His)
			$10^{-3} - 10^{-2}$	$(9.1 \times 10^{-13} - 10^{-12})^\dagger$	5c heme-NO complex
	CO	1.1×10^6	0.85	7.7×10^{-7}	
	O ₂	N/A	N/A	$\sim 1.3 \times 10^{-3\#}$	
<i>sGC</i> ^f	NO	1.4×10^8	27	5.4×10	6c NO-heme-His complex
		0.6×10^6	26		Intermediate NO-heme--NO(–His)
			$10^{-4} - 10^{-3}$	$(7.1 \times 10^{-13} - 7.1 \times 10^{-12})^\dagger$	5c heme-NO complex

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H-NOXs	Gaseous Ligand	k_{onr} M ⁻¹ s ⁻¹	k_{off} s ⁻¹	K_D M	comments
	CO	4×10^4	10.7	2.6×10^{-4}	
	O ₂	N/A	N/A	$\sim 1.0^{\#}$	
^a					ref. [11];
^b					ref. [10,18];
^c					ref. [11];
^d					this study;
^e					ref. [9];
^f					ref. [18].
[†]					$K_D(\text{NO})(\text{apparent})$.
[*]					estimated based on computer simulations.
[#]					estimated based on the "sliding scale rule".