Nitric Oxide Binding to Prokaryotic Homologs of the Soluble Guanylate Cyclase β1 H-NOX Domain*

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The heme cofactor in soluble guanylate cyclase (sGC) is a selective receptor for NO, an important signaling molecule in eukaryotes. The sGC heme domain has been localized to the N-terminal 194 amino acids of the β1 subunit of sGC and is a member of a family of conserved hemoproteins, called the H-NOX family (Heme-Nitric Oxide and/or OXygen-binding domain). Three new members of this family have now been cloned and characterized, two proteins from Legionella pneumophila (L1 H-NOX and L2 H-NOX) and one from Nostoc punctiforme (Np H-NOX). Like sGC, L1 H-NOX forms a 5-coordinate FeII-NO complex. However, both L2 H-NOX and Np H-NOX form temperature-dependent mixtures of 5- and 6-coordinate FeII-NO complexes; at low temperature, they are primarily 6-coordinate, and at high temperature, the equilibrium is shifted toward a 5-coordinate geometry. This equilibrium is fully reversible with temperature in the absence of free NO. This process is analyzed in terms of a thermally labile proximal FeII-His bond and suggests that in both the 5- and 6-coordinate FeII-NO complexes of L2 H-NOX and Np H-NOX, NO is bound in the distal heme pocket of the H-NOX fold. NO dissociation kinetics for L1 H-NOX and L2 H-NOX have been determined and support a model in which NO dissociates from the distal side of the heme in both 5- and 6-coordinate complexes.

The H-NOX (Heme-Nitric Oxide and/or OXygen-binding domain) family of heme proteins has been identified recently, first through sequence analysis (1) and then through biochemical characterization (2). This family was identified based on homology to the heme domain from soluble guanylate cyclase (sGC), the well characterized and conserved eukaryotic nitric oxide receptor (3, 4). H-NOX proteins have now been identified in many prokaryotes in addition to the well known eukaryotic sGCs. Several of these prokaryotic H-NOX proteins have now been cloned, expressed, and spectroscopically characterized (2, 5–7). In addition, the crystal structure of the H-NOX domain from Thermoanaerobacter tengcongensis (Tt H-NOX) has been solved to 1.77 Å resolution (8), providing the first structural data for the H-NOX family and, by homology, for the heme domain in the β-subunit of sGC. Taken together, these structural and biochemical results have suggested that some members of this family are able to use a homologous protein fold and an identical heme cofactor to discriminate between NO and O2 binding.

Although a great deal of information pertaining to the entire H-NOX family has been determined from studying the structure of Tt H-NOX (5, 8), specific structural data for an NO complex to any of the H-NOX proteins are still lacking. This is particularly relevant as NO is an important signaling molecule in eukaryotes, and sGC is the only firmly established NO receptor (3). Cytochrome c′ is a protein that shares ligand binding properties but not sequence or structural homology with sGC. A crystal structure of the FeII-NO cytochrome c′ complex shows NO bound to the proximal side of the heme (9), which has led to speculation that the same is true for sGC. Physical studies of NO binding to sGC have been hindered by poor expression systems for this large protein. Thus considerable effort has been spent on shorter constructs of sGC containing its heme-binding domain, such as β1-(1–385) (10) and β1-(1–194) (6). Most of the prokaryotic members of the H-NOX family, however, are stand-alone ~200 amino acid proteins that overexpress well in Escherichia coli. These close homologs of the sGC heme domain have proven generally useful in understanding ligand binding in the family.

In this study, we examine three additional prokaryotic H-NOX proteins from Legionella pneumophila (ORF1 and ORF2) and Nostoc punctiforme (Np H-NOX). Unlike all other prokaryotes with predicted H-NOX proteins identified to date, L. pneumophila has two predicted ORFs for H-NOX proteins (L1 H-NOX and L2 H-NOX). Np H-NOX is 39% identical to the H-NOX domain from sGC, and the H-NOX domains L1...
H-NOX and L2 H-NOX share 19 and 16% identity with sGC, respectively. The NO binding characteristics of these proteins will be important in determining function in these bacteria. In addition, given the high homology these proteins share with sGC, these results should have implications for the NO-heme complex of sGC.

EXPERIMENTAL PROCEDURES

Material and General Methods—Unless otherwise noted, all reagents were purchased in their highest available purity and used as received.

Protein Expression and Purification—PCR was used to amplify ZIP 421786 from N. punctiforme genomic DNA (ATCC, Manassas, VA) using Expand polymerase (Roche Applied Science). Upstream and downstream primers contained NdeI and NotI restriction sites, respectively. PCR was used to amplify YP 095089 (L1 H-NOX) and AAU28519 (L2 H-NOX) from L. pneumophila genomic DNA (ATCC) using Expand polymerase (Roche Applied Science). Upstream and downstream primers contained NdeI and XhoI restriction sites, respectively. All amplified PCR products were cloned into pET-20b (Novagen) and sequenced (sequencing core; University of California, Berkeley). Mutagenesis was carried out using the QuikChange protocol from Stratagene. Cell culture procedures and purification of Np H-NOX were carried out as described previously for the H-NOX protein from Vibrio cholerae. Cell culture procedures of both Legionella H-NOX proteins were carried out as described previously (2). Purification of the Legionella H-NOX proteins took advantage of the C-terminal His6 tag; proteins were purified by metal affinity (nickel-nitrilotriacetic acid) followed by gel filtration (Superdex 200 HiLoad 26/60).

Sample Preparation—Preparation of the various protein complexes was carried out as published previously (2) with one minor exception, as follows. Rather than generate the FeII-NO complex with NO gas generated from the head space of a concentrated diethylylamine NONOate (Cayman) solution, diethylylamine NONOate was added directly to the protein solution, and then excess NO, diethylylamine, and diethylylamine NONOate were removed from the sample by using a PD10 desalting column. This was to ensure no free NO was in solution, and thus the only NO present in the sample was that bound to the heme in the protein.

Spectroscopy—All electronic spectra were recorded on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 constant temperature bath. For temperatures at 0 °C or lower, ethylene glycol (50%) was added to the constant temperature bath and 5% glycerol to the protein sample. Resonance Raman spectra were collected using 406.7 nm excitation from a Kr laser (Spectra-Physics model 2025). Raman scattering was detected with a cooled, back-illuminated CCD (LN/CCD-1100/KB; Roper Scientific) controlled by an ST-133 controller coupled to a subtractive dispersive double spectograph. The laser power at the sample was ~2 milliwatts. A microspinning sample cell was used to minimize photo-induced degradation. For the temperature dependence studies, the sample temperature was controlled by flowing either cooled (~10 °C) or heated (~40 °C) N2 gas over the Raman cell. Samples were equilibrated at the respective temperatures for 30–60 min prior to data acquisition. Typical data acquisition times ranged from 30 to 60 min, except for the L1 H-NOX NO-complex, which was signal-averaged for 3 h because of a high fluorescence background. Electronic absorption spectra were obtained both before and after the Raman experiments to ensure that sample integrity was maintained. Raman spectra were corrected for wavelength dependence of the spectrometer efficiency, and cyclohexane was used for instrument calibration. The reported frequencies are accurate to ±2 cm−1, and the resolution of the spectra is 8 cm−1. For each Raman spectrum, the raw data were base-line corrected, and the buffer background signal was subtracted.

Extinction Coefficient Determination—The extinction coefficients were determined similarly to the methods described previously for Tt H-NOX and Vc H-NOX (2). Specifically, for the 6-coordinate FeII-NO complex of L2 H-NOX, the electronic spectra of various dilutions of a sample of the aerobic FeII-NO complex (prepared as described above) at 0 °C were recorded. The electronic spectra of dilutions of a sample of horse heart metmyoglobin (ε409 nm = 181 cm−1 mmol−1) were also recorded and used as a standard for heme concentration. The heme content of each sample was determined by HPLC (Hewlett Packard Series II 1090 HPLC with a diode array detector). Each sample (75 μl) was applied to a C4 column (250 × 4.6 mm, 5 μm; Vydac) that had been equilibrated with 0.1% trifluoroacetic acid. The column was developed with a linear gradient of 0–75% acetonitrile over 25 min followed by a linear gradient of 75–100% acetonitrile over 5 min. The column was washed and re-equilibrated between runs with a gradient of 100–0% acetonitrile over 3 min followed by 5 min of 100% aqueous phase (0.1% trifluoroacetic acid). The flow rate was 1 ml/min.

NO Dissociation Rate—NO dissociation rates were measured as described previously (5). Briefly, FeII-NO complexes of protein (5 μM heme final concentration) diluted in anaerobic 50 mM triethanolamine, 50 mM NaCl, pH 7.5, buffer were rapidly mixed with a saturated carbon monoxide and 30 mM (final concentration) dithionite trap (Na2S2O4) in the same buffer (anaerobic). Each sample (75 μl) was applied to a C4 column (250 × 4.6 mm, 5 μm; Vydac) that had been equilibrated with 0.1% trifluoroacetic acid. The column was developed with a linear gradient of 0–70% CO2 over 1 h followed by a linear gradient of 70–100% CO2 over 5 min. The column was washed and re-equilibrated between runs with a gradient of 100–0% acetonitrile over 3 min followed by 5 min of 100% aqueous phase (0.1% trifluoroacetic acid). The flow rate was 1 ml/min.

RESULTS

Electronic Spectroscopy—UV-visible spectra of L2 H-NOX, L1 H-NOX, and Np H-NOX proteins as the FeII-unligated, FeII-
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![Electronic absorption spectra of H-NOX domains.](image)

**TABLE 1**

UV-visible peak positions (nm (at 20 °C)) for various FeII protein complexes

| Protein | Soret | β | α | Ref. |
|---------|-------|---|---|-----|
| FeII unligated complex | | | | |
| sGC | 431 | 555 | 36 |
| Ty H-NOX | 431 | 565 | 2 |
| Ve H-NOX | 429 | 568 | 2 |
| Np H-NOX | 430 | 555 | This work |
| L1 H-NOX | 428 | 557 | This work |
| L2 H-NOX | 428 | 557 | This work |
| L2 F142Y | 428 | 557 | This work |
| Hb | 430 | 555 | 37 |
| FeII-CO complex | | | | |
| sGC | 423 | 541 | 567 | 36 |
| Ty H-NOX | 424 | 544 | 565 | 2 |
| Ve H-NOX | 423 | 541 | 566 | 2 |
| Np H-NOX | 423 | 539 | 566 | This work |
| L1 H-NOX | 420 | 540 | 566 | This work |
| L2 H-NOX | 423 | 540 | 571 | This work |
| L2 F142Y | 422 | 539 | 569 | This work |
| Hb | 419 | 540 | 569 | 37 |
| FeII-NO complex | | | | |
| sGC | 398 | 537 | 572 | 36 |
| Ty H-NOX | 420 | 547 | 575 | 2 |
| Ve H-NOX | 398 | 540 | 573 | 2 |
| Np H-NOX | 416/400 | 543 | 576 | This work |
| L1 H-NOX | 398 | 540 | 571 | This work |
| L2 H-NOX | 399/416 | 544 | 575 | This work |
| L2 F142Y | 417 | 544 | 578 | This work |
| Hb | 418 | 545 | 575 | 37 |

CO and FeII-NO complexes at room temperature are shown in Fig. 1 and compared with sGC, Ty H-NOX, and other histidyl-ligated heme proteins in Table 1. Interestingly, although the FeII-unligated and CO complexes of each of these proteins are similar to sGC and all other H-NOX proteins characterized to date (Table 1), there are some significant differences in the FeII-NO complexes. Specifically, L1 H-NOX forms a 5-coordinate NO complex with a characteristic Soret absorbance maximum at 398 nm (Fig. 1B) like sGC, but L2 H-NOX (Fig. 1C) and Np H-NOX (Fig. 1A) appear to be composed of a mixture of 5- and 6-coordinate FeII-NO complexes at 20 °C (399 and 416 nm, respectively). L2 H-NOX is a relatively evenly distributed mixture, whereas Np H-NOX appears to be primarily a 6-coordinate complex with a small shoulder corresponding to the 5-coordinate complex. Given the degree of homology of these proteins to sGC (Np H-NOX is 39% identical), which exclusively forms a 5-coordinate FeII-NO complex, an additional investigation into the NO-binding characteristics of these proteins was carried out.

**Resonance Raman Spectroscopy**

The resonance Raman spectra of the FeII-NO complexes of L2 H-NOX, L1 H-NOX, and Np H-NOX are shown in Fig. 2. Table 2 details the assignment of the major heme skeletal modes and compares them to sGC, Ty H-NOX, and other histidyl-ligated heme proteins. The Raman spectra confirm observations made in the electronic absorption spectra that L1 H-NOX forms a 5-coordinate NO complex, whereas L2 H-NOX and Np H-NOX have mixed coordination states, the specifics of which are discussed below. The π-electron density marker, ν₃, is used to determine the oxidation state of the heme. The spin and coordination state markers, ν₁₀, ν₂ₗ, and ν₄, are sensitive to the core size of the heme macrocycle (13). Typical frequency shifts of 3–15 cm⁻¹ are often observed upon switching from 5- to 6-coordinate complexes for some of these skeletal markers.

In the low frequency spectrum of L2 H-NOX (Fig. 2A), two bands at 521 and 550 cm⁻¹ are assigned to the Fe-N stretching modes (νFe-N) for 5- and 6-coordinate NO complexes, respectively, based on their similarity to values reported previously for this vibrational mode (2, 14, 15). In the high frequency region of L2 H-NOX (Fig. 2D), ν₃, ν₂ₗ, and ν₁₀ₗ are primarily positioned at 1502, 1583, and 1633 cm⁻¹, respectively, and are characteristic of histidyl-ligated, 6-coordinate, low spin, FeII-NO heme complexes such as myoglobin and Ty H-NOX (Table 2). However, there are also shoulders that appear at 1508 and 1646 cm⁻¹, which correspond well to the marker values typically observed for the 5-coordinate nitrosyl heme complexes, such as sGC (Table 2). Together, these spectra strongly suggest that L2 H-NOX is a mixture of 5- and 6-coordinate complexes under room temperature conditions (~20 °C).
complexes, temperature-dependent studies of the Fe\textsuperscript{II}-NO complex of Np H-NOX and L2 H-NOX were carried out. Electronic absorption spectra indicate that both Np H-NOX and L2 H-NOX are in equilibrium between 5- and 6-coordinate complexes at physiologically relevant temperatures, as indicated by the Soret $\lambda_{\text{max}}$ shift with temperature (Fig. 3). The temperature was varied between 1 and 45 °C in the same sample several times showing that this 5- to 6-coordination behavior was fully reversible. Furthermore, the temperature-dependent coordination states are independent of NO concentration for both H-NOX proteins; no excess NO was added to any of the samples; the only NO in solution was that bound to the heme at the beginning of the experiment. The same results are obtained when the experiment is carried out anaerobically, indicating that O$_2$ has no effect on this process.

Quantification of the amount of 5- and 6-coordinate complexes present at each temperature requires measurement of the extinction coefficient; however, the only complex that can be isolated to accurately measure the extinction coefficient was L2 H-NOX at 0 °C. Thus the extinction coefficient of the 6-coordinate NO complex of L2 H-NOX was determined to be 139 mm\textsuperscript{-1} cm\textsuperscript{-1}, which was then used to estimate the mixture of each complex at a given temperature. At 40 °C, there is approximately a 50% mixture of each coordination state in the Fe\textsuperscript{II}-NO complex of L2 H-NOX. Even at $-12$ °C, L1 H-NOX shows no evidence of a 6-coordinate NO complex, although it is expected that at a sufficiently low temperature, the NO complex of L1 H-NOX would also convert to the 6-coordinate species.

The temperature-dependent behavior was further investigated for L2 H-NOX between $-10$ and $-40$ °C by using resonance Raman spectroscopy. The high frequency spectra obtained at $-10$ °C for L2 H-NOX show $v_3$, $v_2$, and $v_{10}$ at 1500, 1586, and 1633 cm\textsuperscript{-1}, respectively. In corroboration with the electronic absorption spectra (Fig. 3B), these observed vibrational frequencies show a shift in the equilibrium population toward a 6-coordinate complex with decreasing temperature. The spin state marker, $v_3$, is clearly split with overlapped bands at 1502 and 1508 cm\textsuperscript{-1} upon increasing the temperature to $-20$ °C (Fig. 4, B and E). The vibrational frequency for $v_3$ increases to 1510 cm\textsuperscript{-1} when the temperature is raised to $-40$ °C, supporting an equilibrium shift toward the 5-coordinate complex (Fig. 4, C and F). A temperature-dependent

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**TABLE 2**

| Protein | CN | $v_{10}$ | $v_3$ | $v_2$ | $v_s$ | $v_{Fe-N}$ | Ref. |
|---------|----|---------|------|------|------|----------|-----|
| sGC     | 5  | 1646    | 1584 | 1509 | 1375 | 525      | 14, 31 |
| Tt H-NOX| 6  | 1625    | 1580 | 1496 | 1370 | 553      | 2   |
| Vc H-NOX| 5  | 1640    | 1580 | 1505 | 1372 | 523      | 2   |
| Np H-NOX| 5  | 1645    | 1580 | 1506 | 1372 | 528      | This work |
| L1 H-NOX| 5  | 1643    | 1581 | 1507 | 1373 | 522      | This work |
| L2 H-NOX| 5  | 1643    | 1581 | 1507 | 1373 | 522      | This work |
| Myoglobin| 6  | 1633    | 1583 | 1502 | 1375 | 550      | 500 |
| Ax cytc$^*$| 5  | 1638    | 1596 | 1504 | 1372 | 579      | 16  |
| Ax cytc$'$| 5  | 1638    | 1596 | 1504 | 1372 | 579      | 16  |
| Rm FixLN$^*$| 5  | 1646    | 1509 | 525  | 18   |
| Rm FixLN| 6  | 1632    | 1498 | 558  | 18   |

$^*$Ax cytc$'$ indicates cytochrome c' from Alcaligenes xylosoxidans; Rm FixLN indicates heme domain of FixL from Rhizogium meliloti.
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FIGURE 3. Temperature-dependent electronic absorption spectra of the Fe$^{II}$-NO complexes of H-NOX domains. Np H-NOX (12 μM) (A) and L2 H-NOX (7 μM) (B). Heme concentration is indicated in parentheses. Temperatures for Np H-NOX are 4 °C (--), 10 °C (---), 20 °C (-----), and 37 °C (----). The corresponding temperatures for L2 H-NOX are those shown with the same dash and color patterns using thick lines. In addition, 1, 15, 25, 30, 35, 40, and 45 °C are illustrated using thin lines. The 6-coordinate complex at 416 nm decreases simultaneously with increasing 5-coordinate complex at 399 nm as the temperature increases from 1 to 45 °C. This temperature dependence implicates Fe-His bond formation as an exothermic reaction.

A shoulder is also observed for ν$_{10}$ at 1646 cm$^{-1}$ with increasing temperature (Fig. 4, D–F). This shift in the vibrational frequency for ν$_{10}$ has similarly been observed for 5- and 6-coordinate heme-NO complexes in cytochrome c’ (16, 17) and FixLN (18).

**Dissociation Rates for NO from H-NOX Domains**—NO dissociation rates ($k_{off( NO)}$) were measured to further characterize the nature of the 5- and 6-coordinate NO complexes L1 H-NOX, L2 H-NOX, and Np H-NOX. A CO and dithionite trap (11, 12, 19) for the released NO, consisting of saturating CO and 30 mM dithionite, was employed to minimize recombination of the dissociated NO. The rate of NO dissociation was followed by the formation of the Fe$^{III}$-CO complex at 423 nm. The experiment was repeated at several different temperatures (0–40 °C) in order to vary the coordination state of the starting complex, and all measured rates were independent of CO and dithionite at all concentrations tested (3–300 mM).

Representative data for L2 H-NOX are shown in Fig. 5, and Table 3 summarizes the data for the H-NOX proteins in this study as well as other Fe$^{III}$-NO heme proteins. The following reaction can be used to describe the kinetic data at all temperatures. This is based on the published solutions for similar three-state systems (20, 21). NO dissociation from the H-NOX domain is assumed to proceed as shown in Reaction 1,

$$
A \xrightarrow{k_1} B \xrightarrow{k_3} C
\xrightarrow{k_2}
$$

**REACTION 1**

where $A$ is the 5-coordinate NO complex; $B$ represents the 6-coordinate NO complex, and $C$ represents the CO-trapped dissociated NO complex. $k_1$ represents the rate of histidine rebinding, $k_2$ the rate of histidine dissociation, and $k_3$ the rate of NO dissociation. The first-order observed rate constant, $k_{obs}$, can be readily derived from Reaction 1 provided that $A$ and $B$

are in equilibrium (21) as shown in Equation 1,

$$
k_m = \frac{k_1 k_3}{k_1 + k_2 + k_3} \quad \text{(Eq. 1)}
$$

If $B$ is the only or the dominant species, then Equation 2 is the result,

$$
k_m = k_3 \quad \text{(Eq. 2)}
$$

If $A$ is the only or the dominant species, then the rate constant is as shown in Equation 3,

$$
k_m = \frac{k_1 k_3}{k_2} \quad \text{if } k_3 \ll k_2 \quad \text{(Eq. 3)}
$$

or Equation 4,

$$
k_m = k_1 \quad \text{if } k_3 \ll k_2 \quad \text{(Eq. 4)}
$$

This same analysis was used to treat all of the data regardless of the coordination state of the starting complex. In the L2 H-NOX system, at low temperature when the starting complex is dominated by the 6-coordinate complex $B$, only one time constant for NO dissociation is measured, which is $k_3$ in Equation 2 and is assigned unambiguously as $k_{off}$. At higher temperatures, there is an ~50:50 mixture of 5- and 6-coordinate complexes, and two parallel time constants are measured (called $k_{m1}$ and $k_{m2}$). $k_{m1}$ is the same rate seen at lower temperatures (assuming Arrhenius temperature dependence) and is thus assigned as $k_3$ ($k_{off}$); this rate represents the portion of 6-coordinate molecules that are present in the starting mixture as complex $B$, analogous to the experiments at lower temperatures. The other rate, $k_{m2}$, is either $k_1$ or $(k_1/k_3) \times k_3$ from Equations 3 and 4 and represents the portion of the molecules that are present in the starting mixture as the 5-coordinate complex $A$. Most importantly, the amplitude of each rate constant represents 50% of the total absorbance change, which correlates well with the known abundances of 5- and 6-coordinate complexes present in solution at 40 °C, as estimated using the extinction coefficient of the 6-coordinate complex. Also, by taking into account a doubling of the rate for each 10 °C, $k_3$ ($k_{off}$) is exactly the same at all temperatures, strongly supporting the proposed mechanism in Reaction 1. The average rates and amplitudes of $k_{m1}$ (equivalent to $k_3$ and reported as $k_{off}$ in Table 3) from six independent experiments are $5.6 \times 10^{-4}$ s$^{-1}$ (100%) at 0 °C, $10.6 \times 10^{-4}$ s$^{-1}$ (100%) at 10 °C, and $87.6 \times 10^{-4}$ s$^{-1}$ (46%) at 40 °C. The average rate and amplitude of $k_{m2}$ (equivalent to either $k_1$ or $(k_1/k_3) \times k_3$) at 40 °C are $14.3 \times 10^{-4}$ s$^{-1}$ (54%).

For L1 H-NOX, at all temperatures, two parallel exponents (called $k_{m1}$ and $k_{m2}$) are needed to fit the data despite the fact that there is only one starting coordination state as assessed by UV-visible and resonance Raman spectroscopy. An F test was used to compare the data fit with one and two exponentials. This test confirms that the probability of the two exponential fit being correct is 100% ($p$ value <0.0001). If the NO dissociates according to the mechanism in Reaction 1, however, the kinet-
ics should fit to a single exponential with a $k_m$, defined by either Equation 3 or 4. The simplest way to explain the requirement for two time constants is to invoke a second species of $A$ that is kinetically distinct, leading to Reaction 2,

$$
\begin{align*}
A^* & \xrightarrow{k_a} A \xrightarrow{k_1} B \xrightarrow{k_2} C
\end{align*}
$$

where $A$ and $A^*$ are kinetically distinguishable isomers but that presumably both go through intermediate $B$ before being trapped as $C$. Reaction 1 is just a subset of Equation 4; fundamentally they are the same kinetic mechanism.

This mechanism is based on a published analysis of hydrogen exchange kinetics in proteins in which two kinetically distinct native conformations of a protein are in conformational equilibrium and can both undergo hydrogen exchange through some intermediate species (21). This analysis results in two observed time constants ($k_{m1}$ and $k_{m2}$), one with a rate constant of $k_a$ and the other with a rate constant equal to that of $k_m$ as defined in Equation 1.

NO dissociates from $L1$ H-NOX with two time constants, defined $k_{m1}$ and $k_{m2}$. One of these, $k_{m1}$, is the time constant representing the portion of the starting 5-coordinate complex present at the beginning of the experiment as $A$ that proceeds with a time constant of $k_m$ from Equation 1, which should simplify to either Equation 3 or 4. The other, $k_{m2}$, represents the 5-coordinate complex present as $A^*$ that proceeds with a time constant of $k_a$ from Reaction 2. Assuming a doubling of the rate for every 10 °C, the same two processes $k_{m1}$ and $k_{m2}$ were measured at each temperature. The slower of the two measured rates is assigned as the dissociation rate in Table 3, not because it can be assigned to $k_3$ in Reaction 2, but because it is the slowest rate in the dissociation process. Temperature dependences of the amplitudes of each process measured are consistent with $A$ and $A^*$ being in equilibrium as described in Reaction 2. The average rates and amplitudes of $k_{m1}$ (reported as $k_{off}$ in Table 3) from four independent experiments are $(2.7 \pm 0.5) \times 10^{-4}$ s$^{-1}$ (88%) at 0 °C, $(4.7 \pm 1.0) \times 10^{-4}$ s$^{-1}$ (85%) at 10 °C, and $(38.5 \pm 3.4) \times 10^{-4}$ s$^{-1}$ (49%) at 40 °C. The average rates and amplitudes of $k_{m2}$ are $(25 \pm 11) \times 10^{-4}$ s$^{-1}$ (12%) at 0 °C, $(44 \pm 17) \times 10^{-4}$ s$^{-1}$ (15%) at 10 °C, and $(370 \pm 150) \times 10^{-4}$ s$^{-1}$ (51%) at 40 °C.

The $Np$ H-NOX $k_{stoff}$ could not be obtained with the CO/dithionite trap, presumably because dithionite reacts with the bound NO complex (based on a dithionite concentration dependence; data not shown). This is the first example of an H-NOX nitrosyl complex that displays this reactivity with dithionite, suggesting this protein may have an altered distal pocket or electronic structure with NO. Therefore, an oxyglobin trap for released NO was employed to determine the NO dissociation rate (11, 22). This was also not an effective trap for the NO kinetics of $Np$ H-NOX, instead it seemed the NO dissociation rate was much slower than ambient globin oxidation under all experimental conditions attempted. This again suggests that in comparison with other characterized H-NOX family members, $Np$ H-NOX displays some unique NO ligand binding characteristics.
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FIGURE 5. Example of an experiment to measure the temperature-dependent dissociation rate for the FeII-NO complex of L2 H-NOX. A, 0 °C ($k_\text{m} = k_3 = k_\text{off} = 4.9 \times 10^{-4}$ s$^{-1}$, 100%); B, 10 °C ($k_\text{m} = k_3 = k_\text{off} = 10.6 \times 10^{-4}$ s$^{-1}$, 100%); and C, 40 °C ($k_\text{m} = k_3 = k_\text{off} = 83.0 \times 10^{-4}$ s$^{-1}$, 46%); $k_\text{off}$ is either $k_3$ or ($k_3 / k_2$) × $k_3$) = 12.3 x $10^{-4}$ s$^{-1}$, 54%) measured by electronic absorption spectroscopy using saturating CO and 30 mM Na$_2$S$_2$O$_4$ as a trap for the released NO. Measured rates and amplitudes are indicated in parentheses and were independent of CO and dithionite at all concentrations tested (3–300 mM). Heme concentration was 3 μM. For each temperature, the absorbance difference spectrum (the spectrum at time = 0 min is subtracted from the spectrum at each subsequent time point) of the FeII-CO complex growing over time is shown as well as a plot of the change in absorbance at 423–405 nm (the maximum and the minimum in the difference spectrum) versus time along with the exponential fit of that data.

TABLE 3
Properties of some temperature-dependent FeII-NO complexes in H-NOX proteins

| Protein | CN$^a$ | Soret | Temperature$^b$ | $k_{m,1}$ ($k_{\text{off}}$)$^c$ | $k_{m,2}$ $^d$ | Ref. |
|---------|-------|------|-----------------|------------------------|----------------|-----|
| sGC     | 5     | 399  | <-12            | (3.6 ± 0.8) x 10$^{-4}$ | (166 ± 44) x 10$^{-4}$ | Footnote 4 |
| Tt H-NOX| 6     | 420  | -60             | (5.6 ± 0.5) x 10$^{-4}$ | —              | 5   |
| Tt Y140L| 6     | 420  | -70             | (1.3 ± 0.3) x 10$^{-4}$ | —              |      |
| Np H-NOX| 6/5   | 416/400 | >45           | (10.3 ± 1.4) x 10$^{-4}$ | (87 ± 34) x 10$^{-4}$ | This work |
| L2 H-NOX| 5     | 398  | <-12            | (21.8 ± 0.5) x 10$^{-4}$ | (3.6 ± 1.0) x 10$^{-4}$ | This work |
| L2 H-NOX| 5/6   | 399/416 | ~40           | (5.1 ± 0.4) x 10$^{-4}$ | —              |      |
| L2 F142Y| 6     | 417  | >45             | (5.1 ± 0.4) x 10$^{-4}$ | —              |      |

$^a$ Coordination state at 20 °C.
$^b$ Temperature (°C) of CN transition (~50% mixture) is given.
$^c$ Rate of NO dissociation from the heme, $k_{\text{off(NO)}}$ s$^{-1}$ at 20 °C (estimated using a doubling of the rate for every 10 °C) using saturating CO and 30 mM Na$_2$S$_2$O$_4$ as a trap for the released NO. $k_{\text{m,1}}$ is assigned as $k_{\text{off(NO)}}$ either because it could be conclusively assigned as $k_{\text{off(NO)}}$ or because it is the slowest rate in the NO dissociation mechanism.
$^d$ Additional rate needed to fit the data, $k_{\text{m,2}}$ s$^{-1}$ at 20 °C (estimated using a doubling of the rate for every 10 °C). $k_{\text{m,2}}$ could be $k_4/|k_3| × k_3$ or $k_4$ according to Reactions 1 and 2.
$^e$ The NO complex at temperatures from 0 to 40 °C is 6-coordinate, so only one rate, $k_{\text{off(NO)}}$, is measured.
$^f$ Data could not be measured with this technique.

L2 H-NOX Mutagenesis—Mutation of a distal pocket phenylalanine in L2 H-NOX to tyrosine (L2 F142Y) shifts the equilibrium of the Fe$^{II}$-NO complex at 20 °C exclusively to the 6-coordinate complex (Fig. 1D). As reported previously (5), the F142Y mutation in L2 H-NOX is also responsible for a gain of O$_2$-binding function in the L2 H-NOX fold. The NO dissociation constant for L2 F142Y was determined at temperatures varying from 0 to 40 °C and fit to Reaction 1 as described above for wild type L2 H-NOX. These data are summarized in Table 3. An exclusively 6-coordinate Fe$^{II}$-NO complex is observed even at 40 °C, as evidenced by electronic absorption spectroscopy and the requirement of only one exponent to fit the $k_{\text{off(NO)}}$ data.

The $k_{\text{off(NO)}}$ rates for Tt H-NOX and Tt Y140L are also included in Table 3 because they are relevant in the comparison of the effects of distal pocket mutagenesis on the geometry of Fe$^{II}$-NO complexes in the H-NOX family. The ligand binding properties of Tt H-NOX have been characterized previously (2); both of these proteins have been kinetically studied in the context of O$_2$ binding in the H-NOX family (5), and the NO dissociation rates at 20 °C (both are exclusively 6-coordinate complexes at this temperature) have been reported (5). Here
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The NO dissociation was followed at temperatures between 0 and 70 °C. Tt H-NOX very slowly transitions from a 6-coordinate to a 5-coordinate FeІІ-NO complex at 70 °C over the course of about 30 min. After more than 60 min at 70 °C, however, the Tt Y140L mutant undergoes a transition from a 6-coordinate complex to only about an ~50:50 mixture of 5- and 6-coordinate complexes. Furthermore, the k_{off(NO)} results for Tt H-NOX and Tt Y140L (Table 3) indicate that loss of the distal pocket tyrosine slows the dissociation rate, although not significantly.

DISCUSSION

The prokaryotic H-NOXs have greatly advanced our understanding of ligand binding in this family, including providing key insight into the ligand discrimination against O_{2} in sGC (5). In this study, we have presented spectroscopic and kinetic data to characterize the NO ligand binding properties for three additional H-NOX family members, Ll H-NOX, L2 H-NOX, and Np H-NOX. Ll H-NOX is very similar to sGC and the previously characterized H-NOX protein from V. cholerae (Vc H-NOX), forming a stable 5-coordinate NO complex. Np H-NOX and L2 H-NOX, however, display some atypical behavior especially with respect to FeІІ-NO complexes. Both of these proteins display a physiologically accessible thermal equilibrium between 5- and 6-coordinate FeІІ-NO complexes. A temperature dependence on the coordination state of an FeІІ-NO complex has been observed in other heme sensor proteins as well (18, 23, 24).

L. pneumophila is a facultative intracellular pathogen that is ubiquitous in warm fresh water, but this microbe can survive temperatures between 0 and 63 °C. At the preferred physiological growth temperature (~37 °C), L2 H-NOX is a mixture (~50:50) of the 5- and 6-coordinate FeІІ-NO complexes, but the coordination state of NO complex would be primarily 6-coordinate at the lower end of possible physiological growth temperatures and primarily 5-coordinate at the warmer extreme of possible growth temperatures. The preferred growth temperature of cyanobacteria like N. punctiforme may vary from ~15 to ~40 °C; in this range Np H-NOX remains mostly 6-coordinate, although the transition to 5-coordinate occurs at the higher temperatures in this range. Ll H-NOX, like sGC H-NOX and Vc H-NOX, is a 5-coordinate NO complex at all temperatures in the physiological range. Function of the prokaryotic H-NOXs remains to be established, but it is interesting the NO complexes of Np H-NOX and L2 H-NOX would vary from 5- to 6-coordinate over the temperature range of growth.

Distal versus proximal NO coordination in sGC has been the focus of some debate (9) because sGC shares some kinetic and spectroscopic properties with cytochrome c'. and the crystal structure of the 5-coordinate FeІІ-NO complex of cytochrome c' shows that NO binds to the proximal face of the heme in this protein. Based on work with cytochrome c', distal pocket steric crowding has been suggested as the driving force for conversion of a distal pocket-bound 6-coordinate complex into proximal pocket-bound 5-coordinate NO complex (25). Distal pocket steric crowding is not correlated with FeІІ-NO complex coordinate number in the H-NOX family, however. The structure of Tt H-NOX indicates that the distal heme pocket is quite packed (8), despite the fact that Tt H-NOX forms a 6-coordinate NO complex. On the other hand, homology modeling of the H-NOX domain of sGC (6) as well as studies using nitrosoalkanes as bulky distal pocket ligands for sGC indicate that the distal pocket of sGC, which forms a 5-coordinate NO complex, is quite accommodating (26).

The (i) temperature dependence of the NO complex coordination number, (ii) kinetics, and (iii) mutational data presented above and discussed below suggest that in both the 5- and 6-coordinate complexes, NO is bound in the distal pocket of the H-NOX fold. The fact that the L2 H-NOX and Np H-NOX proteins are able to reversibly convert between 5- and 6-coordinate NO complexes under aerobic conditions with no additional NO in solution is best explained by a model with NO bound on the distal side of the heme, and the Fe-His bond is broken and re-formed using thermal energy (Fig. 6). It seems unlikely that the NO would dissociate from the distal side of the heme, migrate to the proximal side, displace the axial histidine ligand, and then rebind to the other face of the porphyrin, because this would require the transient formation of a 4-coordinate heme. Photodissociation of CO from a proximal 6-coordinate myoglobin complex shows that CO can migrate to the proximal pocket, though not bound to the iron (27, 28).

This temperature-dependent change in coordination state suggests that formation of the Fe-His bond in the H-NOX fold is an exothermic reaction. The equilibrium between 5- and 6-coordinate NO complexes in various H-NOX family members should be observable, although for some the temperature might be quite low. In fact, in sGC a small amount of the 6-coordinate NO complex is observed in EPR experiments at ~196 °C (29).

A model in which NO is bound to the distal side of the heme is also most consistent with the NO dissociation kinetics observed in this study. Using temperature to select between a starting 5- or 6-coordinate NO complex in L2 H-NOX, k_{off(NO)} had the expected temperature dependence (doubling for every 10 °C) but no dependence on coordination state. Instead, at higher temperatures where the FeІІ-NO complex is present as an equilibrium mixture of 5- and 6-coordinate complexes, an additional (parallel), slower exponent is required to fit the data, which is consistent with a population of the sample that proceeds to the final product with a different slow kinetic step, presumably the additional process of Fe-His bond formation to form the 6-coordinate NO complex before NO dissociation. The simplest kinetic model Reaction 1 to explain these observations is consistent with NO bound in the distal pocket, regardless of whether or not the histidine is bound in the proximal pocket in L2 H-NOX.

In Ll H-NOX, Fe-His bond formation is sufficiently exothermic that lowering the temperature failed to generate any 6-coordinate NO complex. Therefore, according to Reaction 1, the kinetics should fit with a single exponential corresponding to the slow kinetic step in the mechanism of NO dissociation. In all cases, however, the kinetics had two time constants. Thus a second 5-coordinate species (A and A*) was required to describe the NO dissociation mechanism (see Reaction 2). In fact, A and A* can only be distinguished kinetically because they are spectroscopically identical; the electronic absorption (Fig.
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1) and resonance Raman (Fig. 2) spectroscopy both indicate only one complex, 5-coordinate, in solution at all temperatures tested. We propose Reaction 2 as the simplest general mechanism for NO dissociation from H-NOX proteins. Importantly, Reaction 1 is a subset of Reaction 2; fundamentally they are the same kinetic mechanism.

\[ \text{A}^* \text{ (399 nm)} \]

\[ \text{A (399 nm)} \]

\[ \text{B (416 nm)} \]

\[ \text{C (424 nm)} \]

\[ \text{L2 H-NOX, low temp.} \]

\[ \text{L2 H-NOX, high temp., L1 H-NOX low temp.} \]

\[ \text{L1 H-NOX, high temp.} \]

\[ \text{FIGURE 6. Schematic representation of the spectroscopy and kinetics of a temperature-dependent Fe}^{II}\text{-NO complex in an H-NOX domain. The initial ferrous-unligated complex binds NO leading to either a 5-coordinate (A) or a 6-coordinate (B) complex that can be distinguished by electronic spectroscopy; in addition, kinetic experiments have indicated a second species of the 5-coordinate complex (A*). The dashed arrows are not meant to indicate an NO binding mechanism, they are meant to represent that at the beginning of the NO dissociation experiments examined here, three different NO-bound H-NOX complexes are possible. Electronic absorption spectroscopy as well as kinetic data clearly indicate that species A and B are in thermal equilibrium, and kinetic data suggest that A and A* are also in thermal equilibrium. Using different H-NOX constructs at different temperatures (the specifics of which are indicated in the illustration), the various steps of this mechanism were identified. The dissociation mechanism depicted here and discussed in the text is the simplest mechanism that fits all of the data.} \]

\[ \text{L1 H-NOX is an NO sensor. To test the possibility that A* could be a 5-coordinate complex in which the dissociated histidine ligand was protonated, a pH dependence on the NO dissociation kinetics for L1 H-NOX and L2 H-NOX from pH 6.9 to 8.9 was carried out (data not shown), but the NO dissociation kinetics were found to be independent of pH over this range. It remains a formal possibility that A* is a 5-coordinate complex with NO bound in the proximal heme pocket, as has been proposed for cytochrome c' (16, 17, 25), but based on the discussion above, this seems unlikely here.} \]

\[ \text{Fig. 6 illustrates the simplest mechanism for NO dissociation from all members of the H-NOX family that takes into account all the data known at the present. Each step of this mechanism was separately probed by the experiments presented here. NO dissociation from L2 H-NOX at 0 and 10 °C proceeds simply as B to C, whereas at 40 °C, a population proceeds as B to C, and another population proceeds as A to B to C. NO dissociation from L1 H-NOX, at all temperatures measured, has two popu-} \]
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that like sGC, these H-NOX proteins are also NO sensors, perhaps loss of the Fe-His bond is not the structural switch. Consistent with this, CO and YC-1 are well known to fully activate sGC without breaking the Fe-His bond (33, 34). Furthermore, there is recent evidence that suggests there may be an additional binding site on sGC, either for NO or a nucleotide, that is proposed to serve as an allosteric site for activation of sGC (19, 35).

In summary, three additional members of the H-NOX family of heme proteins, related in sequence and structure to the heme domain from sGC, have been cloned and characterized. Percent sequence identity with sGC, however, is not the best predictor for ligand binding characteristics, as UV-visible and resonance Raman spectroscopy demonstrate that L1 H-NOX domain from L. pneumophila, which shares 19% sequence identity with sGC, is spectroscopically (and kinetically)4 nearly identical, forming a 5-coordinate, temperature-independent (from −12 to 40 °C) Fe(II)-NO complex. In contrast, L2 H-NOX from L. pneumophila, which shares 34% identity with L1 H-NOX and 16% identity with sGC, and the H-NOX protein from N. punctiforme, which is 39% identical to sGC, form a temperature-dependent mixture of 5- and 6-coordinate Fe(II)-NO complexes. At low temperature, they are primarily 6-coordinate, and at high temperature, a shift toward the 5-coordinate geometry is observed. This temperature-dependent process is rationalized in terms of enthalpy, such that lower temperatures favor the more exothermic reaction, in this case Fe(II)-His bond formation. A kinetic analysis of NO dissociation from both 5- and 6-coordinate complexes of H-NOX family members suggests that each of these proceed according to a mechanism (Fig. 6) in which NO is bound to the distal heme pocket and NO dissociation occurs via the distal pocket 6-coordinate complex. As the heme domain of sGC is also an H-NOX domain, these data suggest that NO is also bound to the distal side of the heme in sGC.

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