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Is histidine dissociation a critical component of the NO/H-NOX signaling mechanism? Insights from X-ray absorption spectroscopy†

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Abstract

The H-NOX (Heme-Nitric oxide/Oxygen binding) family of diatomic gas sensing hemoproteins has attracted great interest. Soluble guanylate cyclase (sGC), the well-characterized eukaryotic nitric oxide (NO) sensor is an H-NOX family member. When NO binds sGC at the ferrous histidine-ligated protoporphyrin-IX, the proximal histidine ligand dissociates, resulting in a 5-coordinate (5c) complex; formation of this 5c complex is viewed as necessary for activation of sGC. Characterization of other H-NOX family members has revealed that while most also bind NO in a 5c complex, some bind NO in a 6-coordinate (6c) complex or as a 5c/6c mixture. To gain insight into the heme pocket structural differences between 5c and 6c Fe(II)–NO H-NOX complexes, we investigated the Extended X-ray Absorption Fine Structure (EXAFS) of the Fe(II)–unligated and Fe(II)–NO complexes of H-NOX domains from three species, *Thermotoga maritima*, *Shewanella woodyi*, and *Pseudomonas aeruginosa*. Although the Fe(II)–NO complex of *ThH*-NOX is formally 6c, we found the Fe–N_{His} bond is substantially lengthened. Furthermore, although NO binds to *SwH*-NOX and *PaH*-NOX as a 5c complex, consistent with histidine dissociation, the EXAFS data do not exclude a very weakly associated histidine. Regardless of coordination number, upon NO-binding, the Fe–N_{porphyrin} bond lengths in all three H-NOXs contract by ~0.07 Å. This study reveals that the overall heme structure of 5c and 6c Fe(II)–NO H-NOX complexes are substantially similar, suggesting that formal histidine dissociation may not be required to trigger NO/H-NOX signal transduction. The study has refined our understanding of the molecular mechanisms underlying NO/H-NOX signaling.

Introduction

Diatomic gas molecules such as nitric oxide (NO) regulate important biological signal transduction pathways through selective binding with cognate sensor proteins.^{1–9} The H-NOX (Heme-Nitric oxide/Oxygen binding) family of heme-based sensor proteins has been shown to play essential roles in gas molecule sensing in a variety of biological systems.^{3, 4, 10–13} The H-NOX domain can be defined as a ~200 amino acid protein sequence that binds a histidine-ligated ferrous protoporphyrin IX in a mixed α/β fold.^{14–16} This fold is exclusive to the H-NOX family.

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The best-studied H-NOX family member is soluble guanylate cyclase (sGC), the only confirmed NO sensor in mammals. Upon binding of NO to the H-NOX domain of sGC, the enzymatic activity of sGC is enhanced by over 200-fold, leading to a significant increase in the production of cyclic guanosine monophosphate (cGMP). Downstream, cGMP regulates important physiological processes including neurotransmission, smooth muscle relaxation, and inhibition of platelet aggregation.^{1, 2, 17, 18} In bacteria, H-NOX proteins have been demonstrated to regulate enzymatic activities including histidine autophosphorylation and cyclic-di-GMP synthesis and hydrolysis in response to NO binding.^{3, 4, 12, 13} While the downstream physiological consequences of these NO-mediated changes in enzymatic activity are still being elucidated, regulation of biofilm formation has been demonstrated.^{5, 12, 13}

In 2004, the crystal structure of the H-NOX domain from *Thermoanaerobacter tengcongensis* (*Tt* H-NOX) was solved to a resolution of 1.77 Å (Figure 1).¹⁶ This structure, as well as subsequent H-NOX structures,^{15, 19-22} has been widely used to model the sGC heme domain as well as other H-NOX family members, yielding many valuable insights into the H-NOX signaling mechanism.^{21, 23-31} Currently the prevalent hypothesis for NO/H-NOX signaling is that NO binding to H-NOX generates conformational changes to the heme group, most notably dissociation of the proximal histidine ligand.^{22, 30-34} Subsequently, these changes in heme structure result in a shift of the N-terminal helices on the surface of H-NOX.¹⁹⁻²² Presumably this change in the structure of the H-NOX domain triggers downstream signaling events through changes in domain-domain interactions, either within the protein itself (such as in sGC) or between separate proteins (such as interactions of H-NOX with effector enzymes like histidine kinases and di-guanylate cyclases). The molecular details of this mechanism remain to be fully elucidated, however. In particular, there is still significant disagreement over even the most fundamental components, such as whether NO binds to the proximal or distal face of the heme in its active signaling form.³⁵⁻³⁹

A problem that significantly impedes the resolution of the mechanism of H-NOX signaling is the lack of structural information for the Fe(II)-unligated and Fe(II)-NO complexes, which represent, respectively, the physiological resting state and active state of these NO sensors. To date, there has been only one instance of successful crystallization of both the Fe(II)-unligated and Fe(II)-NO H-NOX complexes, which was reported for *Nostoc sp* H-NOX (*Ns* H-NOX).²⁰ *Ns* H-NOX binds NO as a mixture of 5-coordinate (5c, with dissociation of the proximal His ligand) and 6-coordinate (6c, with retention of the Fe-N_{His} bond) complexes, with 6c-NO as the predominant species. As sGC and most other characterized H-NOX domains bind NO in a 5c complex,^{4, 14, 26, 33} and dissociation of His is thought to be important to trigger signal transduction in the H-NOX family, it is unclear how much relevant structural information can be extracted from this structure. Therefore, additional structural characterization of the heme pocket of the H-NOX Fe(II)-unligated and Fe(II)-NO complexes is highly desirable.

In this work, we use iron K-edge X-ray absorption spectroscopy (XAS) to obtain structural details of the heme pocket of H-NOX. XAS is a powerful technique for examining Fe-heme local structure and electrostatics in hemoproteins, providing information on Fe oxidation state, Fe spin state, the number and type of ligands bound to Fe, and Fe-ligand bond lengths.^{41, 42} In our previous work, we used iron K-edge XAS to examine the flow of electron density between the iron center and surrounding ligands and X-ray Absorption Near-Edge Structure (XANES) to predict iron-heme displacement upon ligand binding to *Tt* H-NOX.³⁰ Here we use Extended X-ray Absorption Fine Structure (EXAFS) analysis to obtain precise structures of the H-NOX heme iron for several different H-NOX domains.

Specifically, we investigate the Fe(II)–unligated and Fe(II)–NO complexes for H-NOX domains from three different species, *Thermoanaerobacter tengcongensis* (*Tt*), *Shewanella woodyi* (*Sw*), and *Pseudoalteromonas atlantica* (*Pa*). Our data challenge the requirement of histidine dissociation upon NO binding and support a model in which NO is bound to the distal side of the heme. Furthermore, the results suggest that contraction of the Fe–N_{porphyrin} bonds, as well as Fe–N_{His} bond lengthening (but not dissociation), are key structural changes that initiate signal transduction in the H-NOX family.

Results and Discussion

Electronic spectroscopy suggests that H-NOX domains can bind NO in either a 5c or 6c complex

UV/visible spectra of hemoproteins are indicative of the Fe oxidation state as well as the bound ligand. The Fe(II)–unligated and Fe(II)–NO bound complexes for *Tt*H-NOX, *Sw*H-NOX, and *Pa*H-NOX are shown in Figure 2 and the peak absorbance maxima are compared with sGC in Table 1. The spectra for *Tt*H-NOX¹⁴ and *Sw*H-NOX¹³ have previously been published; they are included here for comparison. The Fe(II)–unligated state of all three H-NOX proteins has a Soret band maximum at ~430 nm and a merged α/β band at ~560 nm, indicative of a 5-coordinate, high-spin heme complex, similar to sGC and other characterized H-NOX domains (Table 1).^{14, 26, 33} The Soret band maximum at 419 nm and split α/β bands that results from addition of NO to the *Tt*H-NOX Fe(II)–unligated complex is consistent with a 6c Fe(II)–NO complex with a bound histidine ligand, as previously reported.¹⁴ However, the Fe(II)–NO complexes for *Sw*H-NOX and *Pa*H-NOX display Soret bands at ~399 nm and split α/β bands at ~570 nm and 543 nm respectively, which are similar to sGC (Table 1). Thus, the electronic spectra of *Sw*H-NOX and *Pa*H-NOX are consistent with formation of 5c complexes with the Fe–N_{His} bonds broken. sGC (bovine) shares 40% homology (24% identity) and 39% homology (22% identity), respectively, with *Sw*H-NOX and *Pa*H-NOX. Thus, these two H-NOX proteins are good models of the sGC heme domain; characterization of their interactions with NO should provide insight into the NO/H-NOX activation mechanism, including the H-NOX family member sGC.

XANES analysis of H-NOX ferrous complexes suggests electronic structure differences in the Fe(II)–NO complexes

X-ray absorption near-edge spectroscopy (XANES) provides qualitative insights into the oxidation state and electronic symmetry of the absorbing atom, as well as the identity of ligating atoms.^{43, 44} For example, the energy of the iron K-edge in iron-containing compounds indicates the energy required to excite the core shell (1s) electron of the Fe atom. Thus higher edge energies correspond to a greater energy requirement for electron excitation and a more positive effective nuclear charge at the iron center. We have previously determined the Fe K-edge positions for *Tt*H-NOX, with values for the Fe(II)–unligated and Fe(II)–NO complexes of 7124.1 eV and 7125.5 eV, respectively (Table 1).³⁰ The 1.4 eV increase in edge energy upon NO binding suggests extensive electron density flow from Fe to its surrounding ligands (including NO), generating a more positive heme iron center. Surprisingly, the K-edge energies for *Sw* and *Pa*H-NOX Fe(II)–NO complexes are more comparable to those of their Fe(II)–unligated counterparts, increasing by only 0.3 – 0.5 eV (Table 1). The small difference in iron K-edge energies caused by NO association does not exclude the possibility of a change in the electronic environment of Fe upon NO binding, however. The edge energy indicates the net electron density on heme iron; it does not reveal the detailed iron ligand field. The small change in K-edge energy upon NO ligation in *Sw* and *Pa*H-NOX can be attributed to the presence of only 5 iron ligands in the Fe(II)–NO complexes of *Sw* and *Pa*H-NOX, in contrast to a coordination number of 6 in *Tt*H-NOX. In *Sw* and *Pa*H-NOX, NO binding to the heme iron promotes elongation or

dissociation of the proximal histidine ligand, formally maintaining a 5c environment; i.e., the effect of NO association on iron electron density is roughly cancelled out by histidine dissociation, resulting in similar K-edge values. Therefore, the similar edge values for the Fe(II)–unligated and Fe(II)–NO complexes of *Sw* and *Pa* H-NOX are consistent with two distinct 5c ligand fields at the heme center.

The shape of the rising edge also provides insights into the electronic environment of the heme iron center. The XANES spectra of both the Fe(II)–unligated and NO-bound states of *Tt* H-NOX, shown in Figure 3a, exhibit rising white-line absorptions of similar intensity and steepness, whereas NO binding to *Sw* and *Pa* H-NOX elicits a broadening and decrease in the edge intensity relative to their respective unligated states (Figure 3b,c). In general, the shape and intensity of the K-edge white line is related to both coordination number and structural disorder at the absorbing atom, with higher coordination numbers and more ordered first coordination spheres yielding more intense white-line absorptions.^{45, 46} The observed edge shape change upon NO binding to Fe(II)–unligated *Sw* or *Pa* H-NOX likely reflects increased disorder and/or an increase in the distribution of bond lengths for the heme iron site. We favor this explanation over one involving a decrease in coordination number, presumably from 6c in the Fe(II)–unligated state to 5c in the NO-bound form, because our UV/visible observations suggest both Fe(II)–unligated and NO-bound *Sw* and *Pa* H-NOX are 5c.

For *Tt* H-NOX, the similar white-line absorption for both the Fe(II)–unligated and NO-bound forms probably results from the increased coordination number of the Fe(II)–NO form (from 5c in the unligated state to 6c in the NO-bound state, consistent with the UV/visible spectra) being balanced by an increase in the distribution of bond lengths for the iron center. Indeed, comparison of all three of the Fe(II)–unligated H-NOX complexes reveals edge absorptions of nearly identical sharpness and intensity, while the NO-bound form of *Tt* H-NOX exhibits a clearly steeper edge than those of NO-bound *Sw* and *Pa* H-NOX (Figure S1, Electronic Supplementary Information). This distinction likely reflects a somewhat different iron electronic environment in the NO complex of *Tt* H-NOX.

The pre-edge features, which are associated with a formally forbidden 1s-to-3d transition that gains intensity by mixing with the 4p orbitals of the iron center,⁴⁴ support our iron K-edge observations. The pre-edge peak for *Tt* H-NOX upshifts by ~1.5 eV (from 7112.3 eV to 7113.8 eV) and increases significantly in intensity upon NO binding. This is consistent with a substantial loss of centrosymmetry at the iron center as well as an increase in effective nuclear charge. This observation is in agreement with previous XANES observations on the NO-bound forms of both heme and non-heme iron proteins.^{47–49}

Both *Sw* and *Pa* H-NOX exhibit similar changes in their pre-edge peak properties. The Fe(II)–NO complex of *Pa* H-NOX exhibits an unusually high pre-edge energy of 7115.4 eV; we do not have a good explanation at present for this surprising result. The intensities of the pre-edge peaks for the NO complexes of both *Sw* and *Pa* H-NOX are greater than that of *Tt* H-NOX (Figure S1, Electronic Supplementary Information; note that any pre-edge intensity differences for the unligated states are quite subtle). We can infer that the iron site in the *Tt* H-NOX Fe(II)–NO complex has less electronic distortion than in either *Sw* or *Pa* H-NOX, which is again consistent with a 6c site in the former and 5c sites in the latter two species. In general, our XANES data are consistent with UV/visible data indicating that *Sw* H-NOX and *Pa* H-NOX form 5-coordinate Fe(II)–NO complexes, while the NO-bound state of *Tt* H-NOX is better described as 6-coordinate.

Finally, the edge energy for the *Tt* H-NOX Fe(II)–unligated state is ~0.6 eV greater than those of *Sw* and *Pa* H-NOX (Table 1). This suggests that there are large electrostatic

differences in the heme pocket in the respective H-NOX domains, with a more positive heme iron in *Tt*H-NOX. We reason that this difference is correlated with their distinct functions, as *Tt*H-NOX is predicted to be an O₂ sensor while *Sw* and *Pa* H-NOX are expected to be NO sensors. It has been suggested that the severe heme distortion in wildtype *Tt*H-NOX results in a lowered electron density at the heme iron,⁵⁰ so a higher edge energy could correlate with a larger heme distortion as well.

EXAFS analysis of 5c and 6c H-NOX Fe(II)–NO complexes reveals only subtle differences in a weak Fe–N_{HIS} bond

EXAFS is a powerful technique that has been extensively used to examine the coordination chemistry of metal centers in metalloproteins, including heme-based sensor proteins.^{43, 51, 52} Here we use it to identify the specific molecular details of heme iron conformational changes induced by NO binding to the three different H-NOX domains. The unfiltered $k^3\chi(k)$ EXAFS spectra and corresponding Fourier transforms (FT) for both the Fe(II)–unligated and NO-bound states of *Tt*H-NOX, *Sw* H-NOX and *Pa* H-NOX are shown in Figure 4, along with representative best fits.

The EXAFS features of the NO-bound state of all three H-NOX domains are similar to one another, aside from some modest differences in the amplitude of the EXAFS oscillations. The Fourier transforms contain an intense, sharp feature at a phase-uncorrected distance of $r' \sim 1.6$ Å, corresponding to first-shell ligands, and a set of peaks at $r' = 2.5 - 4.0$ Å that can be assigned to single- and multiple-scattering paths from the pyrrole moieties of the heme group.

While the $k^3\chi(k)$ EXAFS spectra of the Fe(II)–unligated complexes differ significantly from their Fe(II)–NO congeners, the Fourier transforms are more similar. The most notable difference in the FT is a loss of intensity and slight broadening in the feature at $r' \sim 1.6$ Å, which is most evident for the Fe(II)–unligated state of *Tt*H-NOX (both in relation to the NO-bound form and to the other Fe(II)–unligated spectra).

We carried out a detailed EXAFS analysis of the Fe(II)–NO complex of *Tt*H-NOX (summarized in Table 2). The EXAFS spectrum of NO-ligated *Tt*H-NOX can be simulated with a set of 4 Fe–N_p scatterers at 1.99 Å associated with the equatorial N donors of the heme, along with a set of single and multiple scattering paths associated with the C α , C β , and C γ atoms assignable to the pyrrole moieties and meso carbons of the heme. Inclusion of a single Fe–N/O scatterer at 1.75 Å attributable to bound NO⁵³ results in a significant improvement in fit quality (Table 2, fit 2). We note that the Debye-Waller factor (σ^2) for this shell is fairly large, which may reflect either sub-stoichiometric binding of NO or perhaps multiple modes of NO binding. It was not possible to identify unique multiple-scattering paths involving the Fe–NO unit that would enable determination of the Fe–NO bond angle, presumably due to the intensity of the long-range scattering paths from the heme group. The requirement for a short Fe–N/O scatterer that would significantly distort the centrosymmetry of the iron site agrees nicely with the observed increase in the intensity of the pre-edge feature upon NO binding.

Additional attempts were made to fit the inner shell to a six-coordinate Fe(II) site by inclusion of an axially bound Fe–N_{HIS} scatterer. A fit with a single shell of 5 (Fe–N_p + N_{HIS}) scatterers (Table 2, fit 3) resulted in a significant decrease in fit quality and a larger σ^2 value for the principal Fe–N shell, suggesting that the Fe–N_{HIS} bond, if present, has a bond length very different from those of the equatorial heme nitrogens. Indeed, a weak and fairly disordered Fe–N_{HIS} bond of 2.32 Å can be resolved (Table 2, fit 4), providing a modest improvement in fit quality.

The inclusion of a 6th bond, the Fe–N_{His} bond, is consistent with the electronic spectrum of the Fe(II)–NO complex of *Tt* H-NOX, thus we include it in our fit, but it is significant that the inclusion of a long Fe–N_{His} bond only modestly improves the fit relative to a 5c complex. We always obtain electronic spectra of each sample before and after XAS measurements to monitor any change in sample quality due to X-ray exposure, and we therefore know that this sample is NO-bound both before and after EXAFS data collection. This, as well as the fact that there are significant differences in EXAFS spectra of the Fe(II)–unligated and Fe(II)–NO complexes leads us to conclude that the apparent 5c nature of the Fe(II)–NO EXAFS is not due to NO dissociation during data collection. Instead, we conclude that the Fe–N_{His} bond is formally present, but severely weakened by the NO *trans* effect.

Similar analyses of the EXAFS were obtained for the NO complexes of *Sw* and *Pa* H-NOX (Table 3, Tables S1–S2 in Electronic Supplementary Information). EXAFS data for both species was adequately simulated by a short Fe–N scatterer at 1.72–1.75 Å from bound NO, 4 Fe–N_p scatterers at *ca.* 1.99 Å and a set of single and multiple scattering pathways associated with the heme macrocycle (the Fe••C_h component for the heme meso carbons was not required to fit *Pa* H-NOX Fe(II)–NO data). In addition, the magnitude of the Debye–Waller factors for the first-shell paths was, in general, identical to or smaller than those obtained for *Tt* H-NOX Fe(II)–NO, suggesting that the different shape of the XANES spectra for NO-bound *Sw* and *Pa* H-NOX reflects an altered iron site geometry compared to that of the *Tt* H-NOX Fe(II)–NO complex, rather than a greater level of disorder in the Fe site.

Interestingly, it was possible to include a long Fe–N (presumably representative of an Fe–N_{His} bond) scatterer at *ca.* 2.30 – 2.35 Å and slightly improve fit quality over the five-coordinate fits (Tables S1 & S2, Electronic Supplementary Information). However, we note that for both *Sw* and *Pa* H-NOX, the σ^2 values for this Fe–N_{His} shell were larger than that obtained for *Tt* H-NOX. We can therefore conclude from this analysis that, even if the axial His in NO-ligated *Sw* and *Pa* H-NOX is bound to the heme Fe, the binding of this residue is probably weaker than in *Tt* H-NOX. In view of our combined UV/visible and XANES spectra, as well as EXAFS analysis, we conclude that the Fe–N_{His} bond is formally broken in the Fe(II)–NO complexes of *Sw* and *Pa* H-NOX. However, we cannot ignore that there is still a very weak interaction between heme iron and the dissociated histidine.

EXAFS analysis of Fe(II)-unligated H-NOX provides support for 5c heme iron centers, with evidence for weakly-bound solvent in the hydrophilic *Tt* H-NOX active site

We next turned to the analysis of EXAFS data obtained for the Fe(II)–unligated forms of *Tt*, *Sw* and *Pa* H-NOX, the results of which are shown in Figure 4 and summarized in Tables 2 and 3, as well as Tables S3 and S4 in the Electronic Supplementary Information. Similar to our observations with the NO-ligated Fe(II) state, the $k^3\chi(k)$ EXAFS data obtained for Fe(II)–unligated *Tt* H-NOX is well simulated by a set of single- and multiple-scattering pathways associated with the 4 pyrrole moieties of the heme group, with a disordered set of 4 Fe–N_p scatterers at approximately 2.02 Å (Table 2). In contrast to our analysis of the NO-bound form, it is not necessary to include multiple-scattering from a three-body path involving the iron, C α (alpha carbons of each pyrrole), and N_{pyrrole} (Fe–C α /N_p), and indeed fit quality improves markedly when this path is removed (data not shown). The observed 0.06 Å increase in the average Fe–N_{pyrrole} bond length relative to the NO-ligated form is consistent with a high-spin Fe(II) center in unligated *Tt* H-NOX.

Addition of a fifth scatterer to the Fe(II)–unligated *Tt* H-NOX first shell, corresponding to the putative Fe–N_{His} interaction, did not produce a change in the average Fe–N bond length of 2.02 Å. However, it did lead to a noticeable decrease in fit quality as judged by the

magnitude of F , along with an increase in σ^2 that presumably reflects a greater distribution of bond lengths (Table 2, fit 6). Attempts to model the Fe–N_{His} scatterer with a different bond length than the principal Fe–N_p shell were unsuccessful, affording an unrealistically long Fe–N distance of 2.69 Å. Indeed, computational studies predicted an Fe–N_{His} bond length of 2.05 Å for unligated Fe(II) *Tt*H-NOX, which is very similar to the average 2.02 Å bond length obtained in our analysis.

Studies of structurally well-defined model complexes have shown that the best-fit coordination number can be underestimated in systems with significant disorder in the first coordination sphere.^{45, 55, 56} We therefore favor the 5-coordinate model of fit 6 over the 4-coordinate sites of fits 5 and 7 (Table 2). Finally, there is evidence for a very long Fe–O/N interaction at *ca.* 2.38 Å (Table 2, fit 8) that could conceivably be assigned to a weakly bound solvent ligand. Inclusion of this sixth ligand affords a modest improvement in the quality of fit relative to a 5-coordinate fit model.

The complete EXAFS analyses for the unligated Fe(II) forms of *Sw* and *Pa* H-NOX are broadly similar to that of *Tt*H-NOX, in that the EXAFS can be well simulated with a set of 4 – 5 Fe–N/O scatterers at ~2.06 Å, as well as single- and multiple-scattering paths associated with the heme pyrroles. One notable difference is that it is not possible to obtain chemically reasonable bond lengths for a putative solvent ligand that would complete a potential highly distorted 6-coordinate Fe(II) site. In both cases, the Fe–O distance refined to a value of 2.6 – 2.7 Å, clearly too long to be associated with any significant bonding interaction (see Tables S3-S4, Electronic Supplementary Information).

This difference relative to *Tt*H-NOX can be attributed to variations in the distal pocket environments of *Tt*, *Sw* and *Pa* H-NOX. As shown in Figure 5, *Tt*H-NOX has a relatively hydrophilic distal pocket with a hydrogen bonding (H-bonding) network involving Trp9, Asn74 and Tyr140 that is known to stabilize iron ligands.²³ This same network could conceivably stabilize a solvent water molecule as well. In contrast, *Sw* and *Pa* H-NOX are predicted by homology modeling to have very hydrophobic distal pockets and to lack the H-bonding network seen in *Tt*H-NOX (*Sw* H-NOX has Leu/Leu/Phe and *Pa* H-NOX has Leu/Met/Phe in the positions homologous to *Tt*H-NOX Trp9/Asn74/Tyr140). Therefore, it is not surprising that there is no significant Fe-solvent interaction at the distal pocket of *Sw* and *Pa* H-NOX, probably because the pocket is too hydrophobic to facilitate the presence of water.

In addition, the NO binding kinetics further confirms a more polar distal pocket in *Tt*H-NOX. It was determined that the NO dissociation rate constants (k_{off}) are $5.6 \times 10^{-4} \text{ s}^{-1}$, $15.4 \times 10^{-4} \text{ s}^{-1}$ and $8.6 \times 10^{-4} \text{ s}^{-1}$ for *Tt*²⁶, *Sw*¹³ and *Pa* (unpublished data), respectively. The NO association rate constants (k_{on}) are not known, but are thought to be diffusion limited ($\approx 10^8 \text{ M}^{-1} \text{ s}^{-1}$).^{32, 34} Therefore, we can infer that NO binds to *Tt*H-NOX most tightly as a result of having the smallest k_{off} value, which can be attributed to the stabilizing effect of the H-bonding network in the distal pocket of *Tt*H-NOX (Figure 5a).

Implications for the signal transduction mechanism in the H-NOX family

Our EXAFS analysis leads to the conclusion that the Fe(II)–NO complexes of all three H-NOX domains are similar. *Tt*, *Sw*, and *Pa* H-NOX Fe(II)–NO complexes can all be summarized as having a strong 5c site consisting of porphyrin-derived and NO ligands as well as some contribution towards a 6th ligand from a weak (to extremely weak) Fe–N_{His} bond. The fact that they can all be described as having a weakly associated His bond leads us to consider that dissociation of the His ligand may not represent the most important structural change upon NO ligation, and thus it may not be the most important factor triggering signal transduction in H-NOX domains.

This is in agreement with the report of the co-existing 5-coordinate and 6-coordinate NO complexes in the *Ns* H-NOX crystal structure,²⁰ as well as the mixture of 5c/6c structures that has been observed for Fe(II)–NO H-NOX complexes from *Nostoc punctiforme* and *Legionella pneumophila*.²⁶ Also consistent with the notion that dissociation of the Fe-His bond may not be imperative for activation of H-NOX signal transduction, weak activation of sGC by CO (forms a 6c complex) as well as full activation of sGC by CO plus an allosteric ligand (still 6c) has been reported.⁵⁴ Thus, the formal coordination number of the heme iron would not seem to be critical for triggering signal transduction in the H-NOX family.

Furthermore, if our model is correct, it seems unlikely that NO is bound in the proximal pocket in the active, signaling state of sGC and other H-NOX proteins, as has been proposed in other studies.^{35–39} To be certain, our EXAFS data does not rule out the possibility that the short Fe–N/O scatterer, assigned to NO, is bound in the proximal pocket. However, the presence of a weak 6th bond, assigned here to Fe–N_{His}, suggests that the proximal coordination site is open (or partially occupied with His). An alternate explanation is that there is a weak 6th bond in the distal pocket, with NO bound in the proximal pocket. A solvent molecule is perhaps the most obvious candidate, although weak association with distal residues such as Tyr140 in *Tt* H-NOX or Met74 in *Pa* H-NOX is also a possibility (Figure 5; there is no obvious potential ligand in *Sw* H-NOX). These distal pocket residues are all >3 Å from the heme iron based on crystal structures of *Tt* and *Ns* H-NOX, but it is conceivable that if NO were to bind to the proximal side of the heme, there would be a change in these distal pocket distances. Nonetheless, the most likely scenario is that the weak 6th ligand is, indeed, from the proximal pocket His in all three H-NOXs. Thus, the model most consistent with all the available data^{20, 26} is that in sGC and other H-NOX domains, NO binds in the distal pocket.

Finally, in our previous XANES analysis of *Tt* H-NOX, we observed that NO binding leads to only a subtle change in the displacement of the iron atom out of the mean heme plane.³⁰ This agrees with the contraction in the average Fe–N_p bond lengths upon NO binding (1.97 Å for –NO complex, versus 2.03 Å for –unligated complex). The combined effect of NO binding and Fe–N_{His} bond weakening would be expected to drive the iron atom towards the distal side. However, the contraction of the Fe–N_p bonds shrinks the heme cavity, and therefore partially prevents the heme iron atom from moving more completely into the distal pocket. This likely enables H-NOX proteins to provide mechanistic control over the extent of heme distortion³¹ and heme-induced protein conformational change,^{19–22} thus regulating signal transduction.

Conclusion

The heme domain of sGC and other H-NOX domains constitute an important family of hemoproteins that sense diatomic molecules such as NO, and in response, regulate biological signal transduction pathways. The mechanism of activation of these domains by diatomic gas binding is still unclear, however. To explore the detailed molecular basis of NO/H-NOX signaling pathways, we used EXAFS to investigate the Fe(II)–unligated and Fe(II)–NO complexes of three different H-NOX proteins, *Tt* H-NOX, *Sw* H-NOX, and *Pa* H-NOX. We found that NO forms a similar complex with all three H-NOXs. This complex is best described as a strong 5c ligand set, consisting of 4 Fe–N bonds with the heme and 1 Fe–N bond to NO, as well as a weak 6th ligand, most likely from an incompletely dissociated proximal Fe-His bond. According to our EXAFS analysis, this 6th Fe-His bond in *Tt* H-NOX is slightly stronger than the corresponding 6th ligand in *Sw* and *Pa* H-NOX. This, coupled with UV/visible and XANES data that support a 6th ligand to *Tt* H-NOX but not to *Sw* or *Pa* H-NOX lead us to conclude that *Tt* H-NOX forms a 6c complex with NO while *Sw* and *Pa* H-NOX bind NO in a 5-coordinate manner, with the proximal histidine dissociated, as has been reported for sGC.

Nonetheless, based on these data, we propose that there is no significant difference in the structures of the 5c and 6c NO complexes in H-NOX domains. Instead, they are only distinguished by slight separation along a continuum from a strong Fe–N_{His} bond to no Fe–N_{His} bond. This discovery suggests that histidine dissociation may not be required to trigger NO/H-NOX signal transduction, as has been widely hypothesized. Instead we propose that Fe–N_{His} lengthening together with contraction of the Fe-heme bonds are the relevant structural changes upon NO ligation that promote signal transduction. These data also suggest a mechanism for NO-ligation mediated control of heme flattening, which has been shown to be involved in NO/H-NOX signal transduction.³¹

Experimental

Protein cloning, expression, and purification

Tt H-NOX^{30, 57} and *Sw* H-NOX¹³ were expressed and purified as previously described. PCR was used to amplify *PatL_1532* from *Pseudoalteromonas atlantica* genomic DNA (ATCC) using Expand polymerase (Roche). Upstream and downstream primers contained NdeI and XhoI restriction sites, respectively. The amplified PCR product was cloned into pET-20b (Novagen) and sequenced. Cell culture procedures for *Pa* H-NOX were carried out as previously described for other H-NOX proteins.^{13, 26, 30, 57} Purification of the *Pa* H-NOX protein took advantage of the C-terminal His tag and was carried out as previously described for other H-NOX proteins.^{13, 26, 630, 57} All proteins were >95% pure as estimated by SDS-PAGE.

Sample preparation

Preparation of Fe(II)-unligated and Fe(II)-NO complexes was carried out as previously described.^{26, 57}

Electronic spectroscopy

All UV/visible spectra were recorded on a Cary 100 Bio spectrophotometer equipped with a constant temperature bath set to 20 °C.

X-ray absorption spectroscopy

H-NOX protein samples were exchanged into XAS sample buffer (50 mM HEPES, 50 mM NaCl, 20% glycerol, pH 7.5) and concentrated to 1–2 mM using spin columns from Millipore (Microcon Ultracel YM-3, 3000 molecular weight cut off (MWCO) and 0.5 mL maximum volume). The concentrated protein samples were transferred to copper sample holders (10 mm wide × 5 mm high × 0.5 mm thick) with Kapton windows, flash frozen in liquid nitrogen, and stored at –80 °C before XAS experiments. Flash freezing is not expected to significantly alter the structure of the H-NOX protein or Fe-heme complexes.

XAS data collection was carried out at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL) on beamline X3B. A sagittally focused Si (111) double crystal monochromator was used for energy selection, with a downstream nickel-coated mirror providing vertical focusing and rejection of higher harmonics. Frozen samples were cooled to 20–40 K during data collection with a closed-cycle liquid helium Displex cryostat. Iron K_α fluorescence was detected with Canberra 13 or 31 element solid-state germanium detectors. An iron foil was used as an internal reference to account for any energy shifts in the monochromator, with the reference energy set to 7112.0 eV. Data were collected in 10 eV steps in the pre-edge region (6912 – 7092 eV), 0.3 eV steps in the edge region (7092 – 7162 eV) and steps of 0.05 eV above the edge (7162 – 7859 eV). 6–9 scans for each complex were taken and averaged for data analysis. In order to minimize radiation damage to the samples, we made multiple samples for the same complex and collected at

most 2 scans per X-ray exposed spot; samples were routinely monitored for evidence of photo-reduction, as indicated by red-shifts in the edge energies and/or a loss of pre-edge peak intensity. XAS data were aligned, background-removed, normalized, and merged using Athena.⁵⁸ The Fe K-edge energies were determined from the first derivative of the average XAS spectra after smoothing (in some cases the zero-crossing of the smoothed second derivative was used for verification). The derivative spectra were smoothed using Origin 7.0.

EXAFS analysis were carried out using EXAFSPAK.⁵⁹ Theoretical phase and amplitude parameters were calculated using FEFF 8.40⁶⁰ for simple models of the active sites of H-NOX that were based on the coordinates of the O₂-bound state of *Tt*HNOX.¹⁶ These parameters were used by the opt program of the EXAFSPAK package. Structural models were refined iteratively during the course of the analysis to reflect the EXAFS-derived metrical parameters. For most refinements, the coordination number of a given shell was a fixed parameter, and was varied iteratively while bond lengths (*r*) and Debye-Waller factors (σ^2) were allowed to freely float. The amplitude reduction factor *S*₀ was fixed at 0.9, while the edge shift parameter ΔE_0 was allowed to float as a single value for all shells in fits of a given sample. Therefore, in any given fit, the number of floating parameters was typically equal to (2 × num shells) + 1. Two additional constraints were applied to fits where multiple-scattering from the heme group was simulated to reduce the number of free parameters and provide a geometrical restraint. Specifically, the values of *r* for the Fe••Ca (or Fe••Ca) single scattering paths and the Fe••Ca/*N*_p (or Fe••Ca/*N*_p) multiple scattering paths were constrained to a constant difference from one another, while σ^2 for the multiple scattering path was constrained to be twice that of the single scattering path. *E*₀, the point at which *k* = 0 Å⁻¹, was taken as the value used during normalization of the data in Athena, and was typically between 7123 – 7126 eV for datasets examined here. The goodness of fit *F* was defined simply as $[\sum k^6(\chi_{\text{exptl}} - \chi_{\text{calc}})^2 / \sum k^6 \chi_{\text{exptl}}^2]^{1/2}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Biobibliographic references

1. Ignarro LJ. Biochem. Pharmacol. 1991; 41:485–490. [PubMed: 1847633]
2. Waldman SA, Murad F. Pharmacol. Rev. 1987; 39:163–196. [PubMed: 2827195]
3. Wang Y, Dufour YS, Carlson HK, Donohue TJ, Marletta MA, Ruby EG. Proc. Natl. Acad. Sci. U.S.A. 2010; 107:8375–8380. [PubMed: 20404170]
4. Price MS, Chao LY, Marletta MA. Biochemistry. 2007; 46:13677–13683. [PubMed: 17988156]
5. Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, Kjelleberg S. J. Bacteriol. 2009; 191:7333–7342. [PubMed: 19801410]
6. Gong W, Hao B, Chan MK. Biochemistry. 2000; 39:3955–3962. [PubMed: 10747783]
7. Gong W, Hao B, Mansy SS, Gonzalez G, Gilles-Gonzalez MA, Chan MK. Proc. Natl. Acad. Sci. U.S.A. 1998; 95:15177–15182. [PubMed: 9860942]

8. Hao B, Isaza C, Arndt J, Soltis M, Chan MK. *Biochemistry*. 2002; 41:12952–12958. [PubMed: 12390021]
9. Lanzilotta WN, Schuller DJ, Thorsteinsson MV, Kerby RL, Roberts GP, Poulos TL. *Nat. Struct. Biol.* 2000; 7:876–880. [PubMed: 11017196]
10. Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, Bargmann CI. *Nature*. 2004; 430:317–322. [PubMed: 15220933]
11. Karow DS, Pan D, Davis JH, Behrends S, Mathies RA, Marletta MA. *Biochemistry*. 2005; 44:16266–16274. [PubMed: 16331987]
12. Carlson HK, Vance RE, Marletta MA. *Mol. Microbiol.* 2010; 77:930–942.
13. Liu, N.; Xu, Y.; Hossain, S.; Huang, N.; Coursolle, D.; Gralnick, J.; Boon, EM. Manuscript in revision. 2012.
14. Karow DS, Pan D, Tran R, Pellicena P, Presley A, Mathies RA, Marletta MA. *Biochemistry*. 2004; 43:10203–10211. [PubMed: 15287748]
15. Nioche P, Berka V, Vipond J, Minton N, Tsai A-L, Raman CS. *Science*. 2004; 306:1550–1553. [PubMed: 15472039]
16. Pellicena P, Karow DS, Boon EM, Marletta MA, Kuriyan J. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:12854–12859. [PubMed: 15326296]
17. Denninger JW, Marletta MA. *Biochim. Biophys. Acta*. 1999; 1411:334–350. [PubMed: 10320667]
18. Schmidt H, Walter U. *Cell*. 1994; 78:919–925. [PubMed: 7923361]
19. Erbil WK, Price MS, Wemmer DE, Marletta MA. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:19753–19760. [PubMed: 19918063]
20. Ma X, Sayed N, Beuve A, van den Akker F. *EMBO J.* 2007; 26:578–588. [PubMed: 17215864]
21. Olea C, Boon EM, Pellicena P, Kuriyan J, Marletta MA. *ACS Chem. Biol.* 2008; 3:703–710. [PubMed: 19032091]
22. Olea C, Herzik MA, Kuriyan J, Marletta MA. *Protein Sci.* 2010; 19:881–887. [PubMed: 20162612]
23. Boon EM, Huang SH, Marletta MA. *Nat. Chem. Biol.* 2005; 1:53–59. [PubMed: 16407994]
24. Boon EM, Marletta MA. *Curr. Opin. Chem. Biol.* 2005; 9:441–446. [PubMed: 16125437]
25. Boon EM, Marletta MA. *J. Inorg. Biochem.* 2005; 99:892–902. [PubMed: 15811506]
26. Boon EM, Davis JH, Tran R, Karow DS, Huang SH, Pan D, Miazgowiec MM, Mathies RA, Marletta MA. *J. Biol. Chem.* 2006; 281:21892–21902. [PubMed: 16728401]
27. Capece L, Estrin DA, Marti MA. *Biochemistry*. 2008; 47:9416–9427. [PubMed: 18702531]
28. Tran R, Boon EM, Marletta MA, Mathies RA. *Biochemistry*. 2009; 48:8568–8577. [PubMed: 19653642]
29. Weinert EE, Plate L, Whited CA, Olea C, Marletta MA. *Angew. Chem. Int. Ed.* 2010; 49:720–723.
30. Dai Z, Boon EM. *J. Inorg. Biochem.* 2011; 105:784–792. [PubMed: 21497576]
31. Muralidharan S, Boon EM. *J. Am. Chem. Soc.* 2012 doi 10.1021/ja211576b.
32. Zhao Y, Brandish PE, Ballou DP, Marletta MA. *Proc. Natl. Acad. Sci. U.S.A.* 1999; 96:14753–14758. [PubMed: 10611285]
33. Stone JR, Marletta MA. *Biochemistry*. 1994; 33:5636–5640. [PubMed: 7910035]
34. Stone JR, Marletta MA. *Biochemistry*. 1996; 35:1093–1099. [PubMed: 8573563]
35. Lawson DM, Stevenson CEM, Andrew CR, Eady RR. *EMBO J.* 2000; 19:5661–5671. [PubMed: 11060017]
36. Tsai A-L, Berka V, Sharina I, Martin E. *J. Biol. Chem.* 2011 in press.
37. Tsai A-L, Berka V, Martin E, Olson JS. *Biochemistry*. 2011; 51:172–186. [PubMed: 22111978]
38. Marti MA, Capece L, Crespo A, Doctorovich F, Estrin DA. *J. Am. Chem. Soc.* 2005; 127:7721–7728. [PubMed: 15913362]
39. Pixton DA, Petersen CA, Franke A, van Eldik R, Garton EM, Andrew CR. *J. Am. Chem. Soc.* 2009; 131:4846–4853. [PubMed: 19334778]
40. Söding J, Biegert A, Lupas AN. *Nucleic Acids Res.* 2005; 33:W244–W248. [PubMed: 15980461]

41. Chance B, Powers L, Ching Y, Poulos T, Schonbaum GR, Yamazaki I, Paul KG. Arch. Biochem. Biophys. 1984; 235:596–611. [PubMed: 6097192]
42. Chance M, Parkhurst L, Powers L, Chance B. J. Biol. Chem. 1986; 261:5689–5692. [PubMed: 3700368]
43. Scott, RA. Physical Methods in Bioinorganic Chemistry. Spectroscopy and Magnetism. Que, L., Jr., editor. University Science Books; Sausalito, CA: 2000. p. 465-527.
44. Westre TE, Kennepohl P, DeWitt JG, Hedman B, Hodgson KO, Solomon EI. J. Am. Chem. Soc. 1997; 119:6297–6314.
45. Stemmler TL, Sossong TM, Goldstein JI, Ash DE, Elgren TE, Kurtz DM, Penner-Hahn JE. Biochemistry. 1997; 36:9847–9858. [PubMed: 9245417]
46. Jacquamet L, Aberdam D, Adrait A, Hazemann J-L, Latour J-M, Michaud-Soret I. Biochemistry. 1998; 37:2564–2571. [PubMed: 9485406]
47. Aitken JB, Thomas SE, Stocker R, Thomas SR, Takikawa O, Armstrong RS, Lay PA. Biochemistry. 2004; 43:4892–4898. [PubMed: 15109246]
48. Rich AM, Armstrong RS, Ellis PJ, Lay PA. J. Am. Chem. Soc. 1998; 120:10827–10836.
49. Shu L, Chiou Y-M, Orville AM, Miller MA, Lipscomb JD, Que L. Biochemistry. 1995; 34:6649–6659. [PubMed: 7756296]
50. Olea C, Kuriyan J, Marletta MA. J. Am. Chem. Soc. 2010; 132:12794–12795. [PubMed: 20735135]
51. Miyatake H, Mukai M, Adachi S-i, Nakamura H, Tamura K, Iizuka T, Shiro Y, Strange RW, Hasnain SS. J. Biol. Chem. 1999; 274:23176–23184. [PubMed: 10438488]
52. Nakajima H, Honma Y, Tawara T, Kato T, Park S-Y, Miyatake H, Shiro Y, Aono S. J. Biol. Chem. 2001; 276:7055–7061. [PubMed: 11096066]
53. McCleverty JA. Chem. Rev. 2004; 104:403–418. [PubMed: 14871130]
54. Ibrahim M, Derbyshire ER, Marletta MA, Spiro TG. Biochemistry. 2010; 49:3815–3823. [PubMed: 20353168]
55. Riggs-Gelasco PJ, Stemmler TL, Penner-Hahn JE. Coord. Chem. Rev. 1995; 144:245–286.
56. Riggs-Gelasco, P. Ph.D. dissertation. University of Michigan; 1995.
57. Dai Z, Boon EM. J. Am. Chem. Soc. 2010; 132:11496–11503. [PubMed: 20684546]
58. Ravel B, Newville M. J. Synchrotr. Radiat. 2005; 12:537–541.
59. George, GN. Stanford Synchrotron Radiation Lightsource. SLAC National Accelerator Laboratory; Stanford, CA: 2000. EXAFSPAK.
60. Ankudinov AL, Ravel B, Rehr JJ, Conradson SD. Phys. Rev. B. 1998; 58:7565.

Figure 1.

Crystal structure of *Tt* H-NOX and homology models for *Sw* and *Pa* H-NOX (structure for *Tt* H-NOX was generated using PDB 1U55; homology models were generated using HHPRED⁴⁰). The structures for *Tt*, *Sw*, and *Pa* H-NOX are colored in cyan, green and purple, respectively. The heme cofactor is colored in red and shown in its position in *Tt* H-NOX.

Figure 2.

UV/visible spectra of *Tt* (A), *Sw* (B) and *Pa* (C) H-NOX in Fe(II)-unligated (solid) and Fe(II)-NO (dash) states.

Figure 3. XANES spectra of the Fe(II)-unligated (solid lines) and NO-bound (dashed lines) states of H-NOX from *Tt* (a), *Sw* (b), and *Pa* (c). The insets depict an expansion of the pre-edge transition for each sample.

Figure 4.

Representative best fits to the Fourier transforms of the Fe K-edge EXAFS data ($k^3\chi(k)$, left) and unfiltered EXAFS spectra ($k^3\chi(k)$, right) for the NO-bound (a) and Fe(II)–unligated (b) states of H-NOX from *Tt*, *Sw*, and *Pa*, as indicated by the labels. Experimental data is shown with dotted lines and fits are shown with solid lines. The spectra in each panel are shown with identical vertical scaling. Fit parameters are given in Table 2 (fit 4 for NO-bound, fit 8 for Fe(II)–unligated) for *Tt* H-NOX and Table 3 for both NO-bound and Fe(II)–unligated *Sw* H-NOX and *Pa* H-NOX.

Figure 5.

Model of the distal pocket of *Tt* (a), *Sw* (b) and *Pa* (c) H-NOX. These models are based on the crystal structure of *Tt* H-NOX (PDB: 1U55) and homology models for *Sw* and *Pa* H-NOX, as shown in Figure 1. *Tt* (a), *Sw* (b) and *Pa* (c) H-NOX are colored in cyan, green and purple, respectively. The heme macrocycle in *Tt* H-NOX is used for all three H-NOXs to demonstrate the relative position of the distal pocket residues. Only in *Tt* H-NOX is there a hydrophilic distal pocket forming a H-bonding network (shown with yellow dashed lines).

Table 1

UV/visible spectra peak positions and iron edge/pre-edge energies for *Tt*, *Sw* and *Pa* H-NOX in Fe(II)-unligated and Fe(II)-NO states.

	Soret ^a	β ^a	α ^a	E_{edge} ^b	$E_{\text{pre-edge}}$ ^b
Fe(II)-unligated					
<i>T. tengcongensis</i>	429	560	7124.1	7112.3	
<i>S. woodyi</i>	430	564	7123.4	7112.2	
<i>P. atlantica</i>	431	561	7123.5	7112.6	
sGC	431	555	ζ_{ND}	ζ_{ND}	
Fe(II)-NO					
<i>T. tengcongensis</i>	419	547	577	7125.5	7113.8
<i>S. woodyi</i>	399	543	572	7123.7	7113.4
<i>P. atlantica</i>	398	543	569	7124.0	7115.4
sGC	398	537	572	ζ_{ND}	ζ_{ND}

^a units: nm for UV/visible spectra.

^b units: eV for X-ray edge/pre-edge energies.

ζ_{ND} Not determined.

Table 2

Selected EXAFS fits to the NO-bound and Fe(II)-unligated states of *Tt*H-NOX.^a

Tt H-NOX Fe(II)-NO											
fit 1			fit 2			fit 3			fit 4		
shell	r	σ^2	shell	r	σ^2	shell	r	σ^2	shell	r	σ^2
4 Fe-N _p	1.99	3.9	1 Fe-NO	1.75	5.1	1 Fe-NO	1.73	3.0	1 Fe-NO	1.74	5.4
			4 Fe-N _p	1.97	3.3	4 Fe-N _p + 1 Fe-N _{HIS}	1.97	5.1	4 Fe-N _p	1.97	3.4
									1 Fe-N _{HIS}	2.32	6.5
8 Fe••Ca	3.01	3.3	8 Fe••Ca	2.99	3.6	8 Fe••Ca	2.99	3.6	8 Fe••Ca	2.99	3.5
16 Fe••Ca/N _p	3.18	6.8	16 Fe••Ca/N _p	3.16	7.2	16 Fe••Ca/N _p	3.16	7.2	16 Fe••Ca/N _p	3.16	7.1
4 Fe••C _h	3.10	37.5	4 Fe••C _h	3.34	4.9	4 Fe••C _h	3.34	4.9	4 Fe••C _h	3.35	4.5
8 Fe••C β	4.36	6.3	8 Fe••C β	4.31	5.5	8 Fe••C β	4.31	5.4	8 Fe••C β	4.31	5.5
16 Fe••C β /N _p	4.39	12.6	16 Fe••C β /N _p	4.34	11.0	16 Fe••C β /N _p	4.33	10.8	16 Fe••C β /N _p	4.33	11.0
F		0.369	F		0.286	F		0.315	F		0.270
ΔE_0		8.40	ΔE_0		3.76	ΔE_0		3.43	ΔE_0		3.48
Tt H-NOX Fe(II)-unligated											
fit 5			fit 6			fit 7			fit 8		
shell	r	σ^2	shell	r	σ^2	shell	r	σ^2	shell	r	σ^2
4 Fe-N _p	2.02	7.2	4 Fe-N _p + 1 Fe-N _{HIS}	2.02	10.0	4 Fe-N _p	2.02	7.2	4 Fe-N _p + 1 Fe-N _{HIS}	2.03	10.3
									1 Fe-N _{HIS}	2.38	3.2
8 Fe••Ca	3.11	13.4	8 Fe••Ca	3.12	15.9	8 Fe••Ca	3.09	15.0	8 Fe••Ca	3.12	14.2
4 Fe••C _h	3.40	-0.3	4 Fe••C _h	3.40	-0.4	4 Fe••C _h	3.40	-0.5	4 Fe••C _h	3.40	-0.8
8 Fe••C β	4.39	3.7	8 Fe••C β	4.39	4.0	8 Fe••C β	4.39	3.8	8 Fe••C β	4.39	3.3
16 Fe••C β /N _p	4.42	7.5	16 Fe••C β /N _p	4.42	8.0	16 Fe••C β /N _p	4.42	7.7	16 Fe••C β /N _p	4.42	6.7
F		0.381	F		0.436	F		0.362	F		0.411

Tt H-NOX Fe(II)-NO										
fit 1			fit 2			fit 3			fit 4	
shell	r	σ^2	shell	r	σ^2	shell	r	σ^2	shell	r
ΔE_0		4.95	ΔE_0		4.94	ΔE_0		4.98	ΔE_0	
										4.98

^a *r* is in units of Å; σ^2 is in units of 10^{−3} Å²; ΔE_0 is in units of eV. All fits are to unfiltered data. F represents a goodness-of-fit parameter. Fourier transform ranges: *k* = 2.0 – 13.85 Å^{−1} (resolution = 0.134 Å) for Tt H-NOX Fe(II)–NO; *k* = 2.0 – 12.0 Å^{−1} (resolution = 0.157 Å) for Tt H-NOX Fe(II)–unligated. Values of *r* for the Fe••Ca/N_p multiple scattering paths were constrained to a constant difference from one another, while σ^2 for the multiple scattering path was constrained to be twice that of the single scattering path. A similar constraint was placed on the C β and C β /N_p combination. Inclusion of the Fe••Ca/N_p multiple scattering paths was not required for fits of Fe(II)–unligated data.

Table 3
Best EXAFS fits to the NO-bound and Fe(II)-unligated states of H-NOX from S_W and P_d .^a

S _W H-NOX and P _d H-NOX Fe(II)-unligated and Fe(II)-NO complexes											
S _W Fe(II)-NO			P _d Fe(II)-NO			S _W Fe(II)-unligated			P _d Fe(II)-unligated		
shell	r	σ ²	shell	r	σ ²	shell	r	σ ²	shell	r	σ ²
1 Fe-NO	1.75	1.8	1 Fe-NO	1.72	5.3						
4 Fe-N _p	1.99	2.0	4 Fe-N _p	1.98	4.0	4 Fe-N _p + 1 Fe-N _{HIS}	2.06	5.7	4 Fe-N _p + 1 Fe-N _{HIS}	2.06	5.2
8 Fe••Ca	3.02	3.0	8 Fe••Ca	3.03	2.8	8 Fe••Ca	3.11	6.6	8 Fe••Ca	3.10	3.1
16 Fe••Ca/N _p	3.19	6.1	16 Fe••Ca/N _p	3.20	5.6						
4 Fe••C _h	3.38	3.5				4 Fe••C _h	3.41	0.55	4 Fe••C _h	3.40	2.37
8 Fe••Cβ	4.36	4.8	8 Fe••Cβ	4.37	7.1	8 Fe••Cβ	4.42	3.8	8 Fe••Cβ	4.42	3.9
16 Fe••Cβ/N _p	4.39	9.6	16 Fe••Cβ/N _p	4.39	14.2	16 Fe••Cβ/N _p	4.45	7.6	16 Fe••Cβ/N _p	4.45	7.7
F	0.342		F		0.406	F		0.301	F		0.306
ΔE ₀	7.65		ΔE ₀		6.21	ΔE ₀		3.88	ΔE ₀		3.31

^a r is in units of Å; σ^2 is in units of 10^{-3} Å²; ΔE_0 is in units of eV. All fits are to unfiltered data. F represents a goodness-of-fit parameter. Fourier transform ranges: $k = 2.0 - 12.0$ Å⁻¹ (resolution = 0.157 Å) for S_W H-NOX Fe(II)-unligated; $k = 2.0 - 13.0$ Å⁻¹ (resolution = 0.142 Å) for P_d H-NOX Fe(II)-unligated; $k = 2.0 - 13.85$ Å⁻¹ (resolution = 0.134 Å) for S_W H-NOX Fe(II)-NO; $k = 2.0 - 12.5$ Å⁻¹ (resolution = 0.150 Å) for P_d H-NOX Fe(II)-NO. Values of r for the Fe••Ca single scattering and Fe••Ca/N_p multiple scattering paths were constrained to a constant difference from one another, while σ^2 for the multiple scattering path was constrained to be twice that of the single scattering path. A similar constraint was placed on the C β and C β /N_p combination. Inclusion of the Fe••Ca/N_p multiple scattering paths was not required for fits of Fe(II)-unligated data.