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# Heme Flattening Is Sufficient for Signal Transduction in the H-NOX Family

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Supporting Information

**ABSTRACT:** The H-NOX family of nitric oxide (NO) sensing proteins has received considerable attention because its members include the mammalian NO sensor, soluble guanylate cyclase. Despite this attention, the mechanism of signal transduction has not been elucidated. Structural studies of bacterial members of the family have revealed that the H-NOX heme cofactor is extremely distorted from planarity. Furthermore, it has been determined that heme distortion is maintained primarily by a conserved proline residue located in the proximal heme pocket. It has been suggested that changes in heme planarity may contribute to signal transduction. Here we demonstrate that heme flattening is, indeed, sufficient for signal transduction in the H-NOX family. Using our previously described H-NOX/diguanylate cyclase functional partners from *Shewanella woodyi*, we demonstrate that mutation of the conserved proline (P117 in *Sw*H-NOX) to alanine, which results in heme flattening, has the same affect on phosphodiesterase activity as NO binding to wildtype *Sw*H-NOX. This study demonstrates, for the first time, that heme flattening mimics the activated, NO-bound state of H-NOX and suggests that NO binding induces heme flattening as part of the signal transduction mechanism in the H-NOX family.

Diatomic gas (CO, NO, $O_2$) sensing and signaling is fundamental to all organisms, mediating a wide variety of transcriptional and regulatory events.[1−4] Heme proteins have evolved to be the specific receptors of these important gas signals (reviewed in ref 2). As high-resolution crystal structures of heme sensor proteins have become available, it has become evident that nearly all protein-bound hemes are nonplanar, an observation underscored by the fact that heme in solution is flat, and suggesting function.[5,6] The possible biological significance of nonplanar porphyrin structures, although largely overlooked, has been suggested.[7−9] For example, nearly identically distorted heme structures are observed in the crystal structures of cytochrome *c* from many species, which suggests a functional role for that distortion.[10] Nitrophorin, a nitric oxide (NO)-binding protein, has an extremely distorted heme structure that is thought to tune the redox potential of this protein.[11,12] Furthermore, CooA,[13] Dos,[14] and FixL[15,16] are examples of heme sensor proteins for which changes in heme structure have been suggested to play a role in signal transduction.[4,17,18]

H-NOX (heme-nitric oxide/oxygen binding) domains are a family of heme-based sensor proteins that include the heme domain of soluble guanylate cyclase (sGC), the well-studied mammalian NO sensor.[19−24] H-NOX domains are all about 190 amino acids with 15−40% identity to the heme domain of sGC and particularly high identity to sGC in the heme-binding region.[25] Like sGC, bacterial H-NOX proteins bind NO sensitively and selectively.[19] Upon NO binding, the cyclase domain of sGC is activated, converting GTP to cyclic GMP, which mediates downstream events via $Ca^{2+}$ channels, phosphodiesterases and kinases. Likewise, bacterial H-NOX domains regulate enzymatic activities to mediate downstream signal transduction. These enzymatic activities include histidine autophosphorylation[26] and cyclic di-GMP synthesis and hydrolysis.[27−29]

The structure of the H-NOX domain from *Thermoanaerobacter tengcongensis* (*Tt*H-NOX), reported to 1.7 Å resolution, revealed that the H-NOX heme is severely distorted from planarity.[24] Subsequent H-NOX structures (2.1 Å resolution) from *Nostoc sp.* (*Ns*) in the ligand-free, carbon monoxide (CO)-bound, and NO-bound forms revealed that ligand binding causes heme pivoting and bending to occur concomitantly with a shift in the N-terminal helices of the protein.[24,30] NMR solution structures of H-NOX from *Shewanella oneidensis* as well as additional high-resolution crystal structures of *Tt*H-NOX also suggest that ligand binding results in heme flattening that leads to N-terminal displacements.[31−33] These structures have led us and others to hypothesize that changes in heme conformation may contribute to signal transduction in the H-NOX family. It has never been conclusively demonstrated, however, that altering the H-NOX heme structure has an effect on the downstream enzymatic activity regulated by H-NOX. Here we reveal, for the first time, that heme flattening is sufficient to trigger signal transduction in the H-NOX family.

In this work, we study the role of H-NOX heme conformation in regulation of phosphodiesterase activity in the *Shewanella woodyi* H-NOX/DGC functional pair.[28] We have previously demonstrated that *Sw*DGC is a bifunctional diguanylate cyclase/phosphodiesterase enzyme[29] and that NO/H-NOX sensitively regulates its phosphodiesterase activity.[28] Diguanylate cyclase activity refers to the synthesis of cyclic di-GMP [c-di-GMP = bis-(3′-5′)-cyclic dimeric guanosine monophosphate] from two molecules of GTP and phosphodiesterase activity refers to the hydrolysis of cyclic di-GMP to the linear 5′-phosphoguanylyl-(3′-5′)-guanosine product.[34]

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We have shown that *Sw*H-NOX and *Sw*DGC form a protein−protein interaction both in the absence and presence of NO.[28] When *Sw*H-NOX is present as the ferrous unligated complex, *Sw*DGC has relatively low phosphodiesterase activity ($k_{cat}/K_M$ = 1.65 ± 0.59 $s^{-1}$ $\mu M^{-1}$). However, when NO-bound ($Fe^{II}$-NO) *Sw*H-NOX is added to *Sw*DGC, the phosphodiesterase activity is increased by an order of magnitude ($k_{cat}/K_M$ = 15.5 ± 2.6 $s^{-1}$ $\mu M^{-1}$).[28] Furthermore, we have shown that this NO/H-NOX/DGC biochemical pathway regulates c-di-GMP metabolism and biofilm formation in *S. woodyi*.[28]

Inspection of the *Tt*H-NOX structure reveals that several amino acid residues such as I5, F78, H104, P115, Y131, S133, and R135 pack against the heme and contribute to heme distortion.[24] Previous studies have shown that among these residues, the proximal pocket proline residue, which is strictly conserved in all H-NOX proteins (P117 in *Sw*H-NOX; P115 in *Tt*H-NOX), makes tight van der Waals contact with the H-NOX heme and is primarily responsible for the nonplanar heme conformation in H-NOX.[24,32] As demonstrated first by computational analysis,[24] and then by mutation and structural analysis,[32] when this proline mutated to alanine, the heme pocket becomes less crowded and provides room for the heme to flatten.

To explore the relationship between heme structure and H-NOX signaling function, we expressed and purified the *Sw*H-NOX P117A mutant (*Sw*P117A). This mutant retained the ability to sensitively and selectively bind NO, with spectroscopic and ligand binding properties similar to *Sw*H-NOX wildtype and other characterized H-NOX proteins (Figure S1). We tested the ability of this heme-flattened mutant to regulate the phosphodiesterase activity of *Sw*DGC. The steady-state kinetics of *Sw*DGC phosphodiesterase activity were assessed in a continuous assay that we have previously described.[28]

Initially, we tested the effect of four H-NOX constructs, $Fe^{II}$-unligated and $Fe^{II}$-NO complexes of both *Sw*H-NOX wildtype and *Sw*P117A (each at 20 $\mu$M), on the initial velocity of c-di-GMP (50 $\mu$M) hydrolysis by *Sw*DGC (50 nM). As shown in Figure 1, the initial velocity of c-di-GMP turnover in the presence of *Sw*P117A as either the $Fe^{II}$-unligated or the $Fe^{II}$-NO complex is similar to the initial velocity observed in the presence of *Sw*H-NOX as the $Fe^{II}$-NO complex. All three of these initial velocities are approximately 2-fold faster than the initial velocity of c-di-GMP turnover by *Sw*DGC in the presence of *Sw*H-NOX as the $Fe^{II}$-unligated complex under the same conditions.

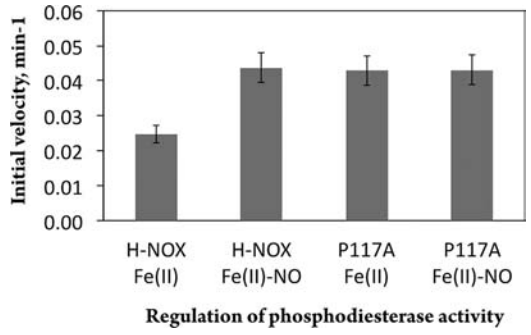


**Figure 1.** Heme flattening in *Sw*H-NOX mimics ligation by NO for regulating the phosphodiesterase activity of *Sw*DGC. Initial velocity of *Sw*DGC phosphodiesterase activity ([*Sw*DGC] = 50 nM, [c-di-GMP] = 50 $\mu$M) in the presence of *Sw*H-NOX or *Sw*P117A (20 $\mu$M) as the $Fe^{II}$-unligated or $Fe^{II}$-NO complex at 25 °C. Error analysis was determined from at least three independent trials.

To fully characterize the effect of heme flattening on H-NOX signal transduction, we performed a complete steady-state kinetic characterization of *Sw*DGC (50 nM) phosphodiesterase activity in the presence of *Sw*P117A (10 $\mu$M) in the $Fe^{II}$-unligated form (Table 1 and Figure S2). We found that in the presence of *Sw*P117A in the $Fe^{II}$-unligated complex, *Sw*DGC has a $k_{cat}$ of 3.36 ± 0.08 $s^{-1}$ and a $K_M$ of 0.17 ± 0.03 $\mu$M ($k_{cat}/K_M$ = 19.8 ± 2.9 $s^{-1}$ $\mu M^{-1}$). As summarized in Table 1, the catalytic efficiency ($k_{cat}/K_M$) of *Sw*DGC phosphodiesterase activity is the same, within experimental error, when regulated by *Sw*P117A as when regulated by NO-bound wildtype *Sw*H-NOX.

**Table 1. Steady-State Kinetic Parameters of *Sw*DGC Phosphodiesterase Activity as a Function of *Sw*H-NOX Regulation**

| H-NOX[a] | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($s^{-1}$ $\mu M^{-1}$) | ref |
|---|---|---|---|
| *Sw*P117A $Fe^{II}$-unligated | 3.36 ± 0.08 | 19.8 ± 2.9 | this work |
| *Sw*H-NOX $Fe^{II}$-unligated | 1.04 ± 0.08 | 1.65 ± 0.59 | 28 |
| *Sw*H-NOX $Fe^{II}$-NO | 4.65 ± 0.14 | 15.5 ± 2.6 | 28 |

[a]H-NOX regulates *Sw*DGC phosphodiesterase activity.[28] In wildtype *Sw*H-NOX, NO ligation activates phosphodiesterase activity. The P117A mutation flattens the heme of *Sw*H-NOX. This heme flattening has the same effect on *Sw*DGC activity as NO ligation.

These observations suggest that heme flattening due to the P117A mutation has an effect on the protein−protein interaction between *Sw*H-NOX and *Sw*DGC. Further, these data suggest that heme flattening results in a protein conformation that closely mimics the structure of the *Sw*H-NOX NO-bound protein. Therefore, we conclude that heme flattening is sufficient for triggering signal transduction in the H-NOX family. We propose that NO binding to wildtype H-NOX results in heme flattening, which triggers a change in the global conformation of H-NOX (Figure 2). This change in the overall conformation of H-NOX then affects protein−protein interactions and results in signal transduction.

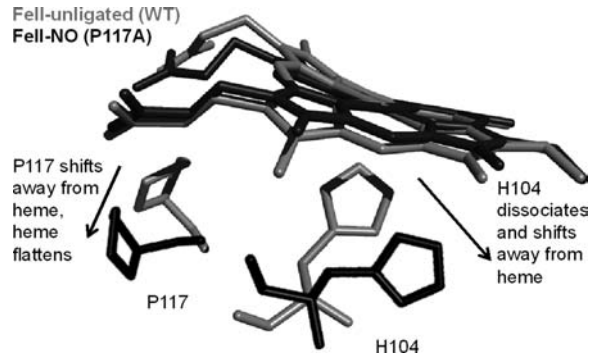


**Figure 2.** Our model for signal transduction in the H-NOX family. Upon NO binding, the proximal histidine ligand is broken leading to an opening of the proximal pocket and effective removal of the heme-distorting proline residue. This results in heme flattening, which triggers an overall change in protein conformation and downstream signal transduction.

There is a straightforward explanation for how NO binding could result in heme flattening in H-NOX. In *Sw*H-NOX, as in


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sGC and most other members of the H-NOX family,[19,30,31] upon NO binding, the proximal histidine (H104 in *Sw*H-NOX) bond to the heme iron is broken, resulting in a five-coordinate $Fe^{II}$-NO complex (Figure S1).[28] It is very likely that when the histidine bond is broken, this causes the helix containing H104 to move slightly away from the heme, which would also cause P117 to move slightly away from the heme. This small movement of P117 away from the heme would be sufficient to relieve the tight van der Waals interactions between the proline and heme and thus allow the heme to relax and flatten.

This heme-led protein conformational change mechanism is consistent with the structural data available for *Ns*H-NOX[30] and *Tt*H-NOX.[24,32] The differences observed between the crystal structures of *Ns*H-NOX[30] as the $Fe^{II}$-unligated and $Fe^{II}$-NO complexes are extremely similar to the differences observed between the structures of wildtype and the P115A mutant of *Tt*H-NOX.[24,32] These structural data all lead to the conclusion that heme flattening can be induced either by mutation of the conserved proximal pocket proline to alanine, or by NO binding to the ferrous heme. Either way, heme flattening results in conformational changes that are propagated to the surface of H-NOX by a concomitant shift in the N-terminal helices.[30−33] The model illustrated in Figure 2 is based on the data presented here as well as the changes in the conserved proline and histidine residues observed in the *Ns*H-NOX[30] and *Tt*H-NOX[24,32] structures.

Our proposal provides a general mechanism for NO regulation of H-NOX family proteins. We expect this same mechanism underlies NO activation of sGC as well as NO/H-NOX regulation of histidine kinases, diguanylate cyclases, and methyl-accepting chemotaxis proteins.

In summary, we demonstrate that heme flattening mimics the activated, NO-bound, state of *Sw*H-NOX in regulating the enzymatic activity of its functional partner, *Sw*DGC. These data suggest that NO binding induces heme flattening as part of the signal transduction mechanism in the H-NOX family. Here we provide the first direct evidence that heme structure plays a role in the NO signal transduction mechanism of H-NOX.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental procedures, electronic spectra of *Sw*P117A, and steady-state kinetic analysis of *Sw*DGC activity regulated by *Sw*P117A. This material is available free of charge via the Internet at http://pubs.acs.org.

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