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Bacterial Heme-Based Sensors of Nitric Oxide

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Abstract

Significance: The molecule nitric oxide (NO) has been shown to regulate behaviors in bacteria, including biofilm formation. NO detection and signaling in bacteria is typically mediated by hemoproteins such as the bis-(3',5')-cyclic dimeric adenosine monophosphate-specific phosphodiesterase YybT, the transcriptional regulator dissimilative nitrate respiration regulator, or heme-NO/oxygen binding (H-NOX) domains. H-NOX domains are well-characterized primary NO sensors that are capable of detecting nanomolar NO and influencing downstream signal transduction in many bacterial species. However, many bacteria, including the human pathogen *Pseudomonas aeruginosa*, respond to nanomolar concentrations of NO but do not contain an annotated H-NOX domain, indicating the existence of an additional nanomolar NO-sensing protein (NosP).

Recent Advances: A newly discovered bacterial hemoprotein called NosP may also act as a primary NO sensor in bacteria, in addition to, or in place of, H-NOX. NosP was first described as a regulator of a histidine kinase signal transduction pathway that is involved in biofilm formation in *P. aeruginosa*.

Critical Issues: The molecular details of NO signaling in bacteria are still poorly understood. There are still many bacteria that are NO responsive but do encode either H-NOX or NosP domains in their genomes. Even among bacteria that encode H-NOX or NosP, many questions remain.

Future Directions: The molecular mechanisms of NO regulation in many bacteria remain to be established. Future studies are required to gain knowledge about the mechanism of NosP signaling. Advancements on structural and molecular understanding of heme-based sensors in bacteria could lead to strategies to alleviate or control bacterial biofilm formation or persistent biofilm-related infections. *Antioxid. Redox Signal.* 29, 1872–1887.

Keywords: nitric oxide, H-NOX, NosP, hemoprotein, NO sensor, signal transduction

The Physiological and Signaling Roles of Nitric Oxide

NITRIC OXIDE (NO) IS A FREE RADICAL, diatomic gas molecule. Initially recognized as a toxic gas, NO has since emerged as a ubiquitous signaling molecule in mammals. In the 1980s, it was first discovered to act as an endothelium-derived relaxing factor (68). NO is now considered to play a vast role in both cellular defense and signaling. For example, NO controls a range of physiological processes, including neurotransmission, vasodilation, platelet aggregation, immune response, and myocardial function (29, 53, 56, 86, 91). An important characteristic for a signaling molecule, NO can readily diffuse across cell membranes to trigger rapid intercellular signaling (5). In mammals, NO is endogenously produced by NO synthase from the amino acid precursor L-arginine (24).

NO is not exclusive to eukaryotes; bacteria can also produce and detect low levels of NO. Bacteria do not encode homologs of the eukaryotic NO synthases, but endogenous NO may be produced as an intermediate in respiratory denitrification (9). At elevated concentrations, NO causes nitrosative stress that can be toxic. Consequently, bacteria have evolved mechanisms to detect and detoxify elevated NO concentrations, such as the detection of NO by FNR-like transcription factors (fumarate and nitrate regulatory proteins) (20) and detoxification of NO by using NO-binding flavohemoglobins (74). Although NO detoxification has generally been documented to occur at micromolar concentrations of NO (30, 60, 74, 92, 93), in some bacteria NO detoxification pathways are known to be stimulated by nanomolar amounts of NO (37). Intriguingly, recent studies

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have indicated that bacteria, such as eukaryotes, can detect NO at low concentrations as a specific signaling molecule to control various bacterial physiological processes, such as biofilm formation, symbiosis, and quorum sensing (3, 63).

NO signaling is widely mediated by hemoproteins in both eukaryotic and prokaryotic organisms. Hemoprotein sensors are composed of a functional domain and an Fe(II)-protoporphyrin type IX cofactor-containing sensing domain. These two domains are either stand-alone proteins that interact in *trans* or fused on the same polypeptide. In bacteria, these sensors typically mediate signaling through functional domains such as cyclic (di)nucleotide-metabolizing enzymes, chemotaxis receptor proteins, DNA-binding transcription factors, or histidine kinases (36, 63). Typically, NO signaling involves reversible NO binding to a 5-coordinate, deoxy Fe(II) hemoprotein complex. An essential aspect of NO-specific signaling events is that heme-based sensors have evolved to preferentially recognize NO and to discriminate against other more prevalent diatomic signaling molecules such as molecular oxygen and carbon monoxide (CO) (102, 104).

Currently, there are two known primary heme-based NO sensors that have been implicated in regulating bacterial communal behaviors at nanomolar concentrations of NO: the well-studied H-NOX (heme-NO/oxygen binding domain) family and the newly discovered NosP (NO-sensing protein) family. Bacterial H-NOX domains are members of the same superfamily of heme sensors that includes soluble guanylyl cyclase (sGC), the eukaryotic sensor of NO (47). In fact, the large majority of H-NOX proteins are encoded in eukaryotic genomes. NosP, however, is almost exclusively coded for in bacterial genomes. This article focuses on the structure and biochemistry of H-NOX and NosP proteins in bacteria. The bacterial heme-based NO sensors YybT, a bacterial bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP)-specific phosphodiesterase, and dissimilative nitrate respiration regulator (DNR), a transcriptional regulator in *Pseudomonas aeruginosa*, are included for completeness.

The H-NOX Family of NO Sensors

Bacterial H-NOX proteins were discovered based on their homology to eukaryotic sGCs. sGC is a heterodimeric heme-based sensor composed of an $\alpha 1$ and a $\beta 1$ subunit that binds one protoporphyrin IX group per dimer (43). The heme-binding domain of sGC has been localized to the first 194 amino acids of the $\beta 1$ subunit (50) with histidine 105 as the proximal ligand (107). In its resting state, sGC heme is in the reduced unligated ferrous state [Fe(II)-unligated]. sGC binds NO at the heme iron to form a ferrous-NO [Fe(II)-NO] complex. NO ligation to the heme iron causes cleavage of the Fe-His bond, which is believed to result in a conformational change that results in activation of the catalytic domain (16, 28, 85), wherein guanosine triphosphate (GTP) is converted to cyclic guanosine monophosphate (cGMP) (96). cGMP then mediates downstream events such as vasodilation, platelet aggregation, neurotransmission, and myocardial function via calcium channels, phosphodiesterases, and kinases (31, 56).

Bacterial heme proteins with ~15% to 40% identity to the heme domain from rat sGC were identified about 15 years ago (47). This family of hemoproteins was named H-NOX, and it includes eukaryotic sGCs as well as several hundred predicted open reading frames from bacterial genomes. H-

NOX domains are ~200 amino acids long. The bacterial members have a particularly high identity to sGC in the heme-binding region (87).

The first bacterial H-NOX domains characterized were from *Vibrio cholerae* (Vc_0270) and *Caldanaerobacter subterraneus* (at the time known as *Thermoanaerobacter tengcongensis*; TTE0680 basepairs 1-564) (51). As expected based on sequence similarity to sGC, it was reported that these H-NOX domains are histidine-ligated protoporphyrin IX hemoproteins that bind gaseous ligands at the ferrous iron atom of the heme cofactor. It was also discovered that the ferrous protein from *C. subterraneus*, in addition to binding NO, could stabilize a complex with molecular oxygen [Fe(II)-O₂]. Indeed, it was based on this observation that this family of heme sensors was named H-NOX.

Based on strong sequence homology to sGC, it was hypothesized that bacterial H-NOX proteins would also serve as sensors of NO (or possibly oxygen in the case of obligate aerobic organisms). Most H-NOX domains are encoded in a histidine kinase-containing operon, some H-NOX domains are encoded in a cyclic diguanylate monophosphate (c-di-GMP) synthase (diguanylate cyclase or DGC) and/or phosphodiesterase-containing operon (47), and H-NOX domains from some obligate anaerobes are encoded as the N- or C-terminal domain of methyl-accepting chemotaxis proteins (MCPs) (15, 47). Thus, it was hypothesized that NO binding to H-NOX might result in regulation of these associated signaling proteins.

Characterization of H-NOX domain heme complexes

Many bacterial H-NOX domains have now been cloned, expressed, purified, and spectroscopically characterized (2, 11, 23, 39, 51, 54, 57, 61, 76, 102, 103, 105, 109). A combination of ultraviolet-visible and resonance Raman spectroscopy has led to a general understanding about the properties of H-NOX proteins in complex with various gaseous ligands (Fig. 1). In general, ferrous H-NOX domains from facultative aerobes form high-spin 5-coordinate Fe(II)-unligated complexes (Fig. 1A) (11, 51). On addition of CO, these proteins form low-spin 6-coordinate Fe(II)-CO complexes; whereas on ligation to NO, the proteins form high-spin 5-coordinate Fe(II)-NO complexes (Fig. 1A) (51). Thus, H-NOX domains from facultative aerobes, such as sGC, form 5-coordinate NO complexes and rigorously exclude O₂ as a ligand (51, 76). The ferrous H-NOX domains from obligate anaerobes, however, are similar to the globins, forming stable low-spin 6-coordinate complexes with O₂, NO, and CO (Fig. 1B) (15, 51, 100).

Thus far, all purified bacterial H-NOX domains, regardless of species of origin or coordination number of the Fe(II)-NO complex, display slow NO dissociation kinetics [e.g., $15.2 \times 10^{-4} \text{ s}^{-1}$ for *Shewanella woodyi* (Sw)-H-NOX, $21 \times 10^{-4} \text{ s}^{-1}$ for *Vibrio fischeri*-H-NOX, $3.6 \times 10^{-4} \text{ s}^{-1}$ for rat sGC, 0.05 s^{-1} for *Nostoc* sp.-H-NOX, 0.3 s^{-1} for Vc-H-NOX] (54, 95, 96, 103, 105, 110, 112) with an assumed diffusion-limited NO association rate constant of $\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Consequently, these domains are believed to possess nanomolar to picomolar affinity for NO, underscoring a role for them in NO sensing and signaling in bacteria.

The first H-NOX domain to be crystallized was from the thermophilic anaerobe *C. subterraneus*. The structure of the

FIG. 1. Ferrous-unligated-and-ligated forms of heme for H-NOX domains. (A) H-NOX domains from facultative aerobes form 5-coordinate ferrous-unligated complexes, 5-coordinate ferrous-NO complexes with NO present in either the distal or proximal heme pocket, and 6-coordinate ferrous-CO complexes with CO present in the distal pocket. (B) H-NOX domains from obligate anaerobes form 5-coordinate ferrous-unligated complexes and 6-coordinate ferrous-ligated complexes with NO, CO, and O₂. CO, carbon monoxide; H-NOX, heme-NO/oxygen binding; NO, nitric oxide.

Fe(II)-O₂ complex of the *Caldanaerobacter subterraneus* (Cs)-H-NOX domain [at the time known as *Thermoanaerobacter tengcongensis* (Tt)-H-NOX] was solved to 1.7 Å resolution (70), and it revealed that the H-NOX family has a novel protein fold that consists of seven α -helices (α A-F) and a four-stranded antiparallel β -sheet (Fig. 2A). The heme is buried within the protein fold, and it is surrounded on the distal side by the three helices α A, α D, and α G. The proximal side, however, consists of the β -sheet, the α F helix, and a one-turn helix, α E. Characteristics conserved across the H-NOX family are that the heme cofactor is significantly distorted from planarity and the propionic acid side chains of the heme are coordinated by a conserved YxS/TxR (x=any amino acid) amino acid motif (Fig. 2B). Also conserved among H-NOX proteins, the proximal histidine residue (H102 in Cs-H-NOX) serves as an axial ligand for the heme iron and is found on the N-terminal signaling helix, α F. A proline residue (P115 in Cs-

H-NOX) in close sequential proximity to the histidine is also strictly conserved across the H-NOX family (Fig. 2C).

To date, H-NOX domains from the obligate anaerobe *C. subterraneus*, and facultative anaerobes *Nostoc* sp., and *Shewanella oneidensis* have been characterized with molecular resolution. In the next few sections, we will discuss how key observations from these structural studies provide possible insight into ligand selectivity of H-NOX proteins and the molecular mechanisms by which H-NOX proteins initiate downstream signal transduction events, most notably heme flattening and Fe-His bond cleavage.

NO-selective signaling and activation mechanisms of H-NOX proteins

Ligand specificity is observed in H-NOX domains. sGC and the H-NOX domains from facultative anaerobes have

FIG. 2. Crystal structure of the Fe(II)-O₂ complex of Cs-H-NOX (PDB ID: 1U55). (A) Full protein structure showing six out of the seven α -helices (α A-D, α F), a four-stranded antiparallel β -sheet, and heme. (B) Zoomed-in view of the proximal heme pocket featuring the conserved residues Y131, S133, and R135 of the YxS/TxR motif, and (C) the heme-distorting residue P115 and the heme proximal ligand H102. (D) Zoomed-in view of the distal heme pocket featuring the W9/N74/Y140 hydrogen-bonding network with O₂. Cs, *Caldanaerobacter subterraneus*.

been demonstrated to exclusively bind NO and CO, but not molecular O₂ (Fig. 1A) (51, 54, 76, 95, 103, 105). The ability to exclude O₂ is crucial to these proteins functioning as sensitive NO sensors in an aerobic environment. Consequently, the ability to rigorously exclude O₂ by these H-NOX proteins has long been a topic of interest. The discovery of H-NOX domains that bind O₂ has offered researchers an opportunity to understand this molecular phenomenon: Understanding why *Cs*-H-NOX can stabilize O₂ as a ligand should translate into further understanding why sGC and facultative anaerobic H-NOX family members exclude O₂.

The high-resolution structure of the *Cs*-H-NOX Fe(II)-O₂ complex (70) revealed that the dioxygen ligand is bent with respect to the plane of the porphyrin, as expected from studies of the globin family. Further, the O₂ molecule was found to interact with a distal pocket hydrogen-bonding network (Fig. 2D). A primary H-bond is present between the Fe(II)-bound O₂ and the distal pocket tyrosine residue (Y140 in *Cs*-H-NOX) located in the α G helix. Tyr140 is part of a distal pocket hydrogen-bonding network with the tryptophan 9 (W9) and asparagine 74 (N74) residues located in the α A helix and α D helix, respectively. It appears that Tyr140 is held in the optimal position for H bonding with iron ligands through stabilizing H bonds from W9 and N74. Sequence alignments reveal that this hydrogen-bonding network is conserved only in H-NOX proteins from obligate anaerobes (70), leading to the hypothesis that this network is required for O₂ binding and its absence may result in ligand discrimination in H-NOX domains.

Support for this hypothesis was initially demonstrated by mutational analysis experiments in *Cs*-H-NOX. Mutation of the distal pocket Tyr140 to leucine in *Cs*-H-NOX (12) was found to increase the O₂ dissociation rate constant by 20-fold, whereas the NO dissociation rate constant remained unchanged. Further, introduction of a mutant tyrosine residue into the hydrophobic distal pockets of the rat sGC H-NOX domain (β 1 1-194) or the *Legionella pneumophila* L2-H-NOX domain enabled these domains to gain O₂-binding function (12). These observations suggest a compelling molecular basis for ligand selectivity in the H-NOX family: A distal pocket H-bonding residue is requisite for O₂ binding and is used to kinetically distinguish between NO and O₂. In the absence of this tyrosine, the O₂ dissociation rate is so fast that the O₂ complex is never formed, whereas the rate of NO dissociation remains unchanged, thus providing discrimination (12). A mutational study introducing a Tyr into the distal pocket of full-length sGC, however, seems to contradict the notion that the distal pocket tyrosine (and, by extension, the hydrogen-bonding network) is important for facilitating O₂ binding to H-NOX domains. When a tyrosine (I145Y) was introduced into the distal pocket of full-length sGC, as opposed to the heme binding domain of sGC only (β 1 1-194) (12), researchers did not find evidence for O₂ binding (58). Consequently, this finding suggests that oxygen binding to H-NOX domains is not solely mediated by the presence of the distal pocket tyrosine residue, but may also be dependent on other structural features.

Indeed, an alternate hypothesis for the role of the hydrogen-bonding network in H-NOX proteins has been proposed as part of the sliding-scale hypothesis (102). This hypothesis is based on the observation that it is consistently true that gas-binding heme proteins bind NO with about

~1000-fold greater affinity than CO, and CO with ~1000-fold greater affinity than O₂, even when the affinity for any one of these ligands varies by more than six orders of magnitude from one protein to another. Notable exceptions to this rule include proteins whose primary function is to bind O₂ (102). According to the sliding-scale hypothesis, to compensate for the much lower affinity that heme iron would usually have for O₂ than for the other gas ligands, selective structures in the protein are necessary to ensure that oxygen is the preferred binding partner. Therefore, hydrogen-bonding residues in the distal pocket are used to increase the affinity for O₂ to near the affinity for CO. This hypothesis is supported by data that show that when the distal pocket H-bonding residues in O₂-binding proteins, such as the globins, are mutated to non-polar residues, the sliding-scale ratios are restored (102). This hypothesis predicts, therefore, that the H-bonding network in H-NOX from anaerobic bacteria functions to favor O₂ binding with respect to NO- and CO binding, by lowering the binding rate constants and distorting the sliding scale, whereas H-NOX from aerobic bacteria conform to the sliding scale (110).

Heme distortion in H-NOX signaling

A particularly striking feature of the H-NOX fold is the severely distorted nature of the heme cofactor. Normal-coordinate structural decomposition analysis (89) revealed that the *Cs*-H-NOX heme has displacements of -1.0 Å saddling and -1.2 Å ruffling. It is not uncommon for heme proteins to cause distortion of the heme cofactor, but the degree of distortion in H-NOX proteins is significantly higher than that of other known heme proteins such as cytochrome c3 and nitrophorin (70).

This large degree of non-planarity can largely be attributed to two factors that are specific to H-NOX. First, the heme in H-NOX is buried within the protein, where the propionate groups are constrained by interactions with the highly conserved YxS/TxR motif at the proximal side of heme (Fig. 2B) (70). The arginine, serine, and tyrosine residues (Y131, S133, and R135 in *Cs*-H-NOX) have been shown to coordinate the propionate groups on pyrrole A and D of heme. Second, Van der Waals interactions between the absolutely conserved H-NOX proline residue (P115 in *Cs*-H-NOX) and one of the pyrroles of the heme causes the pyrrole to shift out of plane (64, 70). Indeed, the crystal structure of a *Cs*-H-NOX mutant with an alanine residue instead of the conserved proline residue results in significant heme flattening (42), strongly implicating this residue in maintaining heme structure in H-NOX domains.

Ever since the distorted nature of the H-NOX heme was first observed, it has been hypothesized that changes in heme structure may contribute to signal transduction in the H-NOX family. Crystal structures of H-NOX domains bound to various gaseous ligands support this hypothesis. Structures of the H-NOX domain from *Nostoc* sp. as the Fe(II)-NO, Fe(II)-CO, and Fe(II)-unligated complexes, in comparison with the O₂-bound *Cs*-H-NOX structure, reveal that ligand binding causes heme pivoting and bending to occur concomitantly with a shift in the N-terminal helices of the protein (57). These observations have led to the hypothesis that alterations in the degree of heme distortion are coupled to surface changes in H-NOX, which ultimately lead to changes in intermolecular

interactions with co-cistronic partner proteins and subsequent changes in signal transduction downstream.

The structure of *Cs*-H-NOX in the absence of O₂ was recently solved to 2.3 Å and provides evidence for this theory (42). In this structure, the researchers found that in the absence of O₂, the W9/N74/Y140 hydrogen-bonding network (shown in Fig. 2D) is displaced from the distal face of the heme, leading to an overall shift of the α A helix containing W9 and the α D helix containing N74. The α D helix also contains isoleucine 5, which is a major contributor to heme distortion and is conserved across the H-NOX family (65, 108). These rearrangements result in a heme and protein structure in the absence of O₂ that resembles the structure observed for the heme-flattened mutant *Cs*-H-NOX P115A (64). Thus, the study concluded that in the absence of a heme ligand, displacement of the N-terminal helices results in displacement of Pro115 and heme relaxation, leading to ligand-induced conformational shifts in the protein that are important for signal transduction.

Histidine dislocation in H-NOX signaling

Spectroscopic characterization of sGC and facultative anaerobic bacterial H-NOX family members indicates that the heme-iron bond to the proximal histidine ligand is broken on NO ligation to H-NOX (112). Thus, it has long been hypothesized that this event is an important molecular step in downstream signal transduction. The crystal structures of H-NOX proteins have solidified the theory that changes in the Fe-His bond are important in signaling, although as hereunder discussed, the details of these structural changes are not yet agreed upon.

Nuclear magnetic resonance (NMR) studies have contributed to our understanding of the role of Fe-His bond breakage, as well as heme structure, in H-NOX function. NMR structures of *Shewanella oneidensis* (So)-H-NOX in the CO-bound state for both wild-type and the H103G mutant (the latter protein being expressed and purified in the presence of imidazole) have been solved (27). The H103G mutant was used to mimic a post-NO bound protein with a cleaved His-Fe bond. CO complexes were used to avoid paramagnetic line broadening from the Fe(II)-NO complexes. These structures reveal that the distorted H-NOX heme cofactor relaxes upon Fe-His bond cleavage, which further supports the earlier findings regarding heme relaxation and signal transduction mechanism of H-NOX (27). Moreover, a crystal structure (solved to 2.0 Å) of the homologous proximal histidine mutant of *Cs*-H-NOX (H102G) has corroborated the NMR studies (65). Therefore, it appears that Fe-His bond cleavage may lead to heme relaxation upon NO binding, leading ultimately to structural changes on the surface of H-NOX and signal propagation.

On the other hand, however, X-ray absorption studies on several H-NOX proteins have indicated that Fe-His bond cleavage may not be a significant molecular event. Extended X-ray absorption fine structure (EXAFS) measurements of H-NOX from several species known to form either 5- or 6-coordinate complexes with NO were determined (23). *S. woodyi* and *Pseudalteromonas atlantica* H-NOX proteins are known to form 5-coordinate complexes with NO (2, 54), which is consistent with dissociation of the proximal histidine. However, the EXAFS structure was consistent with a residual weak interaction with the proximal histidine. *Cs*-H-

NOX, on the other hand, known to form a 6-coordinate complex with NO, also had appreciable Fe-His bond lengthening (23). In short, the Fe-His bond lengths of these three H-NOX structures were not significantly different, which is consistent with a hypothesis that Fe-His bond disruption is not the primary factor leading to signal transduction.

The most definitive evidence that the Fe-His bond is broken upon NO binding comes from the crystal structure of the Fe(II)-NO complex of *So*-H-NOX (41). This structure demonstrates that on NO binding, the proximal histidine dissociates, resulting in a 5-coordinate complex with NO bound to the proximal side of the heme. The authors acknowledge, however, that this might be an artifact of the crystallization conditions that required extremely high NO concentrations. Nonetheless, NO bound to the proximal side is consistent with proposals from previous studies of H-NOX domains from *Nostoc* sp. and *V. cholerae*. In this study, multiple rate constants were apparent in NO-binding reactions in the presence of a large excess of NO, but not at stoichiometric NO concentrations (102, 103, 110). The authors explain the results by comparing them with studies performed on cytochrome c (104). In cytochrome c', weak binding of NO to the distal side of heme weakens the Fe-His bond, allowing for a second NO molecule to displace the proximal histidine ligand as well as the distal-pocket NO, leading to NO bound in the proximal heme pocket (102, 104). It is important to note that, if NO is bound to the proximal pocket of H-NOX, it is likely a key contributing factor in NO/O₂ ligand discrimination, as O₂ would not be able to sufficiently weaken the Fe-His bond to allow for a second binding and displacement reaction to occur (102–104, 110).

However, there is also evidence against the proximal pocket NO binding model. Interestingly, the H-NOX domains from *Nostoc punctiforme* (*Np*-H-NOX) (11), *L. pneumophila* (*L2*-H-NOX) (11), and *Vibrio parahaemolyticus* (*Vp*-H-NOX) (Ueno *et al.*, unpublished data) form mixtures of 5- and 6-coordinate coordinate complexes with NO at room temperature. With each of these H-NOX domains, it has been observed that as temperatures were reduced close to 0°C, the 6-coordinate NO-bound forms were enriched, whereas as temperatures increased, the percentage of the 5-coordinate NO-bound form increased. This equilibrium is fully reversible with temperature in the absence of free NO. This observation is hypothesized to be due to a thermally labile proximal Fe(II)-His bond and suggests that in both the 5- and 6-coordinate Fe(II)-NO complexes, NO is bound in the distal heme pocket of the H-NOX fold.

Finally, the *So*-H-NOX structure also indicates the presence of a water-assisted hydrogen-bonding network involving several amino acid side chains and the protein backbone, including the proximal histidine ligand located on the α F helix and the conserved proline (P116 in *So*-H-NOX) located on the loop between the β 1 strand and α F helix (41). This water is lost on dissociation of the proximal histidine ligand, as the contacts between the histidine and other portions of the protein are disrupted. A molecular modeling study (7) of sGC has shown that various mutations of residues within this water network result in loss of heme binding and/or loss of sGC activation. Consequently, a general NO activation mechanism was proposed (Fig. 3) (41). In the Fe(II)-unligated state, this water-assisted hydrogen-bonding network holds the proline residue in Van der Waals contact with the heme, promoting heme distortion in H-NOX. NO binding and concomitant Fe-His

FIG. 3. General NO activation mechanism of H-NOX proteins. Before NO binding, the H-NOX heme cofactor is distorted (1). NO binds, leading to histidine dissociation and the histidine and proline shift away from the Fe(II) heme complex, resulting in a flattened H-NOX heme cofactor (2). This conformational rearrangement results in a signal output to the functional domain (3).

bond dissociation eliminates the water H-bonding network, thereby unrestricting proline and allowing heme to relax (41). Overall, the water molecule and its coordinating residues were, therefore, concluded to be needed for accomplishing the protein conformational changes necessary for signaling. This mechanism requires Fe-His dissociation, but it does not indicate whether NO is bound to the proximal or distal side.

Characterization of signaling downstream of NO-dependent H-NOX domains

Notably, as already described, much of the progress toward elucidating the NO activation mechanisms of H-NOX proteins has been through the characterization of the H-NOX domain from the obligate anaerobe *C. subterraneus* in which H-NOX signaling takes place through a fused MCPs. To date, there has been limited success in measuring the H-NOX-

mediated output of MCPs because of the difficulty of purifying active transmembrane proteins. However, one study, using an orthogonal signaling assay, showed that *Cs*-H-NOX was able to regulate an H-NOX-associated histidine kinase (HahK) from *Vibrio cholera* in an O_2 -dependent manner (42). The rest of this section will focus on NO-specific signaling pathways involving stand-alone H-NOX proteins from facultative aerobic organisms. For more extensive details on the molecular mechanisms by which H-NOX proteins explicitly regulate their associated signaling pathways, the following reviews are available (3, 63, 73).

C-di-GMP signaling. Although they are in the minority, some facultative aerobic bacteria code for an H-NOX domain in the same operon as c-di-GMP-metabolizing enzymes, namely DGCs and/or phosphodiesterases (Fig. 4). We have recently termed these enzymes, collectively, HaCEs for H-NOX-associated c-di-GMP processing enzymes. C-di-GMP is a secondary messenger molecule in bacteria that regulates biofilm formation (84).

FIG. 4. Effect of H-NOX domains on diguanylate cyclase activity. (A) Ferrous-unligated H-NOX does not change the diguanylate cyclase activity of the *L. pneumophila* HaCE protein. Ferrous-unligated H-NOX in *S. woodyi* upregulates diguanylate cyclase activity, whereas the phosphodiesterase activity remains unchanged. (B) Ferrous-NO bound H-NOX downregulates diguanylate cyclase activity in *L. pneumophila*, *S. woodyi*, and *A. vitis*, and it upregulates phosphodiesterase activity in *S. woodyi* and *A. vitis*. Bold arrows signify upregulation of enzymatic activity. c-di-GMP, cyclic diguanylate monophosphate; EAL or HD-GYP, conserved amino acids in the catalytic site of some phosphodiesterases; GGDEF, conserved amino acids in the catalytic sites of diguanylate cyclases; GTP, guanosine triphosphate; HaCE, H-NOX-associated c-di-GMP processing enzymes; pGpG, 5'-phosphoguanylyl-(3'-5')-guanosine.

This genomic organization suggests that NO/H-NOX might regulate c-di-GMP signaling networks, which is an intriguing hypothesis because NO regulation of biofilm formation by nanomolar levels of NO has been documented in numerous bacteria (3, 5, 6, 59). Biofilms occur when bacteria assemble within a self-secreted exopolysaccharide matrix, frequently surface attached (10). Biofilms cause many public health problems because in the biofilm state bacteria are up to 1000-fold more tolerant to antibiotics, as well as host cell immune defenses (44, 94). Genetics studies support the hypothesis that H-NOX may regulate biofilm formation. Deletion of *hnoX1* results in a hyper-biofilm phenotype in *L. pneumophila* (14), whereas the deletion of *hnoX* in *S. woodyi* (54) or *Agrobacterium vitis* (Nesbitt *et al.*, unpublished data) results in reduced biofilm formation compared with the respective wild-type strain, implicating H-NOX in biofilm formation.

HaCE generally have one or both of two enzymatic domains that directly participate in c-di-GMP regulation: a DGC domain—which contains a GGDEF amino acid motif—that synthesizes c-di-GMP from two molecules of GTP, and/or a c-di-GMP phosphodiesterase domain—which contains either an EAL or a HD-GYP amino acid motif that hydrolyzes c-di-GMP into pGpG (5'-phosphoguanylyl-(3'-5')-guanosine) or GMP (guanosine-5'-monophosphate). H-NOX regulation of HaCE activity has been biochemically observed in the bacteria *L. pneumophila* (14), *S. woodyi* (54), and more recently, *A. vitis* (Nesbitt *et al.*, unpublished data) (Fig. 4).

In *L. pneumophila*, *Legionella pneumophila* (*Lpg*)-HaCE has only DGC activity (it contains a catalytically inactive phosphodiesterase domain) (14). In both *S. woodyi* and *A. vitis*, however, *Sw*-HaCE (54) and *Agrobacterium vitis* (*Av*)-HaCE (Nesbitt *et al.*, unpublished data) proteins were found to exhibit both DGC and phosphodiesterase activities *in vitro*. In *Lpg*-HaCE, DGC activity is unaffected in the presence of Fe(II)-unligated *L1*-H-NOX and NO-bound *L1*-H-NOX causes a decrease in *Lpg*-HaCE DGC activity (14). In *S. woodyi*, Fe(II)-unligated H-NOX causes *Sw*-HaCE DGC activity to be upregulated (~10-fold) whereas phosphodiesterase activity remains unchanged (54), and Fe(II)-NO *Sw*-H-NOX results in

Sw-HaCE DGC downregulation and phosphodiesterase activation (~15-fold), presumably leading to a dramatic decrease in intracellular c-di-GMP concentration in the presence of NO (54). In *A. vitis*, *Av*-HaCE DGC activity is unaffected by *Av*-H-NOX whereas phosphodiesterase activity is upregulated in the presence of Fe(II)-NO bound *Av*-H-NOX (Nesbitt *et al.*, unpublished data).

In light of the *So*-H-NOX and *Cs*-H-NOX structures revealing that NO binding may induce heme flattening and subsequent protein conformational changes (27, 64, 66) that translate into changes in downstream signaling events in H-NOX signaling pathways, our lab investigated the role of the H-NOX heme structure in the H-NOX/HaCE signaling pathway from *S. woodyi*. In this study, as expected, the heme-flattened mutant P117A *Sw*-H-NOX led to upregulation of the phosphodiesterase activity of *Sw*-HaCE (62), which is the very same effect that NO-bound H-NOX has on HaCE activity (54). This study, therefore, provided the first direct evidence for the role of heme relaxation in H-NOX signal transduction.

Two-component signaling. Most H-NOX genes are co-cistronic with genes encoding two-component signaling histidine kinases (HahK). Two-component signaling networks are signaling systems that bacteria use to sense and respond to various environmental stimuli, including nutrient availability, pH, osmolarity, and host factors (8). Interestingly, thus far, most H-NOX two-component signaling systems have been implicated in c-di-GMP metabolism and biofilm formation in many bacteria, which is functionally consistent with H-NOX/HaCE signaling systems (40, 72, 80).

Simple two-component signaling networks comprised a sensor histidine kinase and a response regulator protein (Fig. 5). The sensor histidine kinase detects the environmental stimulus *via* its sensory domain, and it responds by utilizing adenosine triphosphate (ATP) as a phosphodonator to catalyze the autophosphorylation of a conserved histidine residue in the protein's kinase domain. Phosphotransfer from the histidine residue to a conserved aspartic acid residue in the receiver domain of a cognate response regulator protein

FIG. 5. H-NOX domains and two-component signaling. (A) Ferrous-NO bound H-NOX inhibits autokinase activity of HahK which, in turn, decreases phosphate flux to the EAL, HtH, and HD-GYP response regulator proteins in *S. oneidensis* and *V. cholerae* (*V. cholerae* only encodes the EAL and HD-GYP response regulator homologs). (B) Ferrous-NO-bound H-NOX inhibits HahK and, therefore, phosphate flux to the HD-GYP response regulator. Both the EAL and the HtH response regulator homologs are present in *P. atlantica*, but they have not yet been characterized. ADP, adenosine diphosphate; ATP, adenosine triphosphate; HahK, H-NOX-associated histidine kinase.

FIG. 6. H-NOX domains involved in quorum sensing and symbiosis. (A) In *V. harveyi* and *V. parahaemolyticus*, ferrous-NO-bound H-NOX inhibits phosphate flux through a quorum-sensing circuit. (B) In *Silicibacter sp. TrichCH4B* and *V. fischeri*, ferrous-NO-bound H-NOX inhibits phosphate flux through a HqsK-signaling pathway, which ultimately regulates the symbiosis of *Silicibacter sp. TrichCH4B* and *V. fischeri* with *T. erythraeum* and *E. scolopes*, respectively. HqsK, H-NOX-associated quorum-sensing histidine kinase; Hpt, histidine-containing phosphotransfer protein.

then results in activation of the appropriate cellular response (52). A common variation in these signaling systems, sometimes called three-component signaling (26, 67, 98), is when the histidine kinase sensory domain is replaced by an accessory sensory protein that acts to directly detect the environmental stimuli and, *via* protein-protein interaction, conveys this information to a histidine kinase (26, 98).

HahKs do not possess sensory domains, and, thus, H-NOX functions as the sensory domain to regulate these three-component systems in an NO-dependent fashion. To date, these simple H-NOX/HahK signaling systems have been biochemically characterized in *S. oneidensis* (72), *V. cholerae* (61, 72), and *P. atlantica* (2) (Fig. 5). In all three bacteria, in the absence of H-NOX, the HahK proteins exhibit ATP-dependent autophosphorylation activity (2, 72, 76) that is strongly inhibited in the presence of NO-bound H-NOX (Fig. 5). More recently, however, there is evidence that in addition to NO sensing, *Vc*-H-NOX may act as a redox sensor. This was first suggested based on findings that *Vc*-H-NOX is able to regulate *Vc*-HahK in both a heme-dependent (upon NO binding) and a heme-independent (upon cysteine oxidation) manner (61). Both the Fe(II)-NO and Fe(III) *Vc*-H-NOX complexes are able to inhibit *Vc*-HahK autophosphorylation activity. Future studies need to be conducted, however, to determine whether this dual functionality of *Vc*-H-NOX is physiologically relevant in regulating biofilm formation and pathogenicity in *V. cholerae*.

Another common deviation from simple two-component histidine kinase-response regulator architecture is found in hybrid histidine kinase signaling (Fig. 6). In this study, the histidine kinase has a receiver domain within the same polypeptide (52). These hybrid kinase proteins (in response to a stimulus) autophosphorylate their conserved histidine residues and then transfer the phosphate intra-molecularly to a conserved aspartic acid residue contained within their receiver domains. The phosphate is then subsequently transferred to a conserved histidine residue in a histidine-containing phosphotransfer protein (Hpt), which then further engages in phosphotransfer with an appropriate response regulator protein (52). H-NOX proteins have also been shown to regulate the activity of hybrid histidine kinases and their associated phosphorelay signaling pathways, specifically those that have been implicated in quorum sensing and symbioses with eukaryotes.

Two H-NOX associated signaling pathways, those from *Vibrio harveyi* (39) and *V. parahaemolyticus* (Ueno *et al.*,

unpublished) (Fig. 6A), have thus far been implicated in quorum sensing. Quorum sensing is a process that governs population-wide bacterial group behaviors, including virulence gene production, biofilm formation, and bioluminescence, in response to the production, secretion, and detection of autoinducers in a cell density-dependent manner (69). In both *V. harveyi* and *V. parahaemolyticus*, the HqsK proteins (H-NOX-associated quorum-sensing histidine kinase), as expected, exhibit ATP-dependent autophosphorylation in a time-dependent manner that is inhibited in the presence of the Fe(II)-NO complex of H-NOX. What makes these systems most interesting is that HqsK signaling merges with the kinase pathways regulated by quorum-sensing autoinducers. Thus, NO/H-NOX participates in quorum-sensing signaling pathways, with NO acting analogously to an autoinducer in these bacteria.

In addition to regulating quorum sensing, NO/H-NOX-regulated hybrid-histidine kinases have also been implicated in regulating microbial symbiotic relationships with various hosts, including insects, nematodes, and marine invertebrates (106). Specifically, *V. fischeri* and its partner the Hawaiian bobtail squid *Euprymna scolopes*, as well as *Silicibacter sp. strain Trich4B* and *Trichodesmium erythraeum*, have been demonstrated to be involved in symbiotic relationships that are dependent on H-NOX (80, 105) (Fig. 6B). *Silicibacter* (*Sili*)-HahK has definitively been shown to exhibit ATP-dependent autophosphorylation in a time-dependent manner, activity that is inhibited in the presence of NO-bound *Sili*-H-NOX (80) (Fig. 6A). Further biochemical characterization of the *Sili*-HahK signaling pathway revealed that *Sili*-HahK can engage in phosphorelay with a Hpt, *Sili*-Hpt, which further engages in phosphotransfer to regulate a DGC, ultimately regulating symbiosis with *T. erythraeum* (80).

Discovery of NosP, an Alternative NO Sensor in Bacteria

Although H-NOX has been shown to be the primary NO sensor in many bacteria, there are many more bacteria that lack an H-NOX domain but are still able to respond to low concentrations of NO to regulate processes such as quorum sensing and biofilm formation, phenotypes that are reminiscent of NO/H-NOX-mediated pathways. For example, NO regulation of biofilm dispersal in *P. aeruginosa*, a principal pathogen in cystic fibrosis (25) and hospital-acquired

infections (18), is well documented (4, 6), but the primary NO sensor is unknown; *P. aeruginosa* does not encode an H-NOX domain. Thus, our laboratory has hypothesized the existence of a bacterial NO sensor alternative to H-NOX.

We recently discovered a novel NO-binding protein, a domain we have named NosP. NosP domains belong to a family of uncharacterized proteins currently annotated as FIST (F-box intracellular signal transduction protein) domains due to their predicted secondary structure (13). NosP domains, such as H-NOX domains, are predicted to be co-cistronic or fused to MCPs, two-component signaling histidine kinases, and c-di-GMP synthase and/or phosphodiesterase enzymes. Interestingly, like the H-NOX-associated enzymes already discussed, these enzymes do not encode for sensory domains, suggesting that NosP domains may function as such *in trans*.

NosP signaling in *P. aeruginosa*

In *P. aeruginosa*, we have demonstrated that a mutant strain lacking components of the NosP pathway loses the ability to disperse biofilms in response to NO (45). *Pseudomonas aeruginosa* (*Pa*)-NosP is co-cistronic with a hybrid histidine kinase (that we have named NosP-associated histidine kinase or NahK) that has been previously implicated in biofilm formation in this bacterial species (Fig. 7A) (46). Using purified proteins, we demonstrated that *Pa*-NosP is a hemoprotein that binds to NO and CO, but does not form a stable ferrous-oxy complex (45), consistent with the ligand binding properties expected of a dedicated NO sensor. In addition, resonance Raman spectroscopy of ferrous NosP from *P. aeruginosa* demonstrated that it is a histidine-ligated 6-coordinate low-spin complex that on binding NO forms a 5-coordinate high-

spin complex (Bacon *et al.* submitted for publication). Further, although the NO association rate constant of this protein has not yet been measured, the NO dissociation rate constant has been measured and it is $1.8 \times 10^{-4} \text{ s}^{-1}$ (45). Moreover, we found that NO-bound NosP inhibited phosphorelay from NahK to the phosphotransfer protein HptB (Pa3345) (45), which ultimately results in a reduction in *Pseudomonas aeruginosa* biofilms (Fig. 7A) (45, 46). These data collectively suggest that *Pa*-NosP is an NO-sensitive hemoprotein (45).

NosP signaling in *V. cholerae*

In *V. cholerae*, a stand-alone NosP domain is predicted to be co-cistronic with a histidine kinase that has recently been implicated in both quorum sensing and biofilm formation (Fig. 7B) (48, 49). Using purified protein, we have observed that NO-bound *Vc*-NosP inhibits the autokinase activity of its co-cistronic histidine kinase (Hossain and Heckler *et al.*, submitted for publication). These findings, thus, suggest that NO may regulate quorum sensing and therefore biofilm formation in this bacterium through ligation to *Vc*-NosP.

NosP signaling in *L. pneumophila*

In *L. pneumophila*, *Lpg*-NosP is encoded in the same operon as a histidine kinase and a response regulator receiver protein containing c-di-GMP synthase and phosphodiesterase domains (Fig. 7C). Since a recent publication revealed that the deletion of this response regulator protein in the *L. pneumophila* Lens strain (lpg1054) results in a hyper-biofilm phenotype (14), we hypothesized that *Lpg*-NosP may be involved in regulating biofilm regulation in this bacterium. We have found that purified NO-ligated *Lpg*-NosP

FIG. 7. NosP-associated signal transduction pathways in bacteria. (A) In *P. aeruginosa*, NO/NosP inhibits NahK autokinase activity and therefore phosphate flux through the associated signaling pathway. This leads to a change in biofilm formation in *P. aeruginosa*. (B) In *V. cholerae*, NO/NosP inhibits *Vc*-NqsK autokinase activity and, therefore, quorum sensing. (C) In *L. pneumophila*, NO/NosP inhibits NahK activity, which, in turn, decreases phosphate flux through the associated signaling pathway and may result in the regulation of biofilm formation in this bacterium. (D) In *S. oneidensis*, NO/NosP inhibits NahK autokinase activity, which decreases phosphate flux to three response regulator proteins: EAL, HtH, and HD-GYP. ?, uncharacterized pathway; NahK, NosP-associated histidine kinase; NosP, NO-sensing protein; NqsK, NosP-associated quorum-sensing histidine kinase; *Vc*, *Vibrio cholerae*.

inhibits the autokinase activity of its co-cistronic histidine kinase, which, subsequently, affects phosphate flux to the downstream response regulator (Hossain and Fischer *et al.*, unpublished data). This biochemistry should result in altering the intracellular concentration of c-di-GMP, and therefore biofilm formation, in this bacterium in an NO-dependent manner.

NosP signaling in *S. oneidensis*

Recently, our lab has also characterized an NosP signaling pathway in *S. oneidensis*. Like *Pa-NosP*, *So-NosP* is a hemoprotein that binds NO and CO but does not form a stable complex with O₂ (Binnenkade and Nisbett *et al.*, submitted for publication). In addition, *So-NosP* is predicted to be co-cistronic with a histidine kinase known to be involved in the *S. oneidensis* NO-regulated c-di-GMP signaling network (Fig. 7D) (72). Using biofilm analysis of NosP and H-NOX mutants of *S. oneidensis*, we discovered that NosP regulates biofilm formation upstream of H-NOX. Consequently, these preliminary findings indicate that *So-NosP* may directly participate in regulating biofilm formation in *S. oneidensis* by way of regulating the activity of its co-cistronic histidine kinase.

Other Heme-Containing NO-Binding Proteins

YybT

Although the H-NOX family are, by far, the most well-studied and characterized of heme-based NO sensors to date, several other heme proteins have also been identified as possible NO-responsive proteins. YybT family proteins (COG3887, GdpP) are heme-containing proteins that are involved in c-di-AMP signaling and are widely distributed among bacterial phylum Firmicutes, including the opportunistic human pathogen *Staphylococcus aureus* (79). These proteins respond to NO to catalyze the hydrolysis of c-di-AMP to 5'-phosphoadenylyl-(3'→5')-adenosine (79). The secondary messenger c-di-AMP is suggested to be involved in stress sensing and cellular responses, as well as in biofilm formation and virulence (19, 71, 77).

YybT is a transmembrane protein that consists of two N-terminal transmembrane helices, a heme-binding PAS domain, a degenerate diguanylate cyclase (GGDEF) domain, and a C-terminal DHH/DHHA1 phosphodiesterase domain (Fig. 8). The heme-binding domain of YybT from *Geobacillus thermodenitrificans* has been resolved by NMR in the absence of heme (99). The structure revealed a characteristic,

but compact, PAS-like fold consisting of distorted α -helices and a five-stranded antiparallel β -sheet. Spectroscopic characterization of *Geobacillus thermodenitrificans* (Gt)-YybT and *Bacillus subtilis* YybT, after heme reconstitution, demonstrated that both proteins are able to bind b-type heme with 1:1 stoichiometry (78). In addition, sequence analysis of YybT led to the proposal that YybT proteins might coordinate heme by means of a hydrophobic pocket, since YybT lacks a conserved histidine or cysteine residue to facilitate heme coordination (78). Further spectroscopic characterization demonstrated that the Fe(II) heme cofactor is a low-spin 6-coordinate heme complex, and on CO binding, the 6-coordinate heme complex is conserved and phosphodiesterase activity of YybT is unchanged (78). Notably, YybT has no measurable affinity for O₂. However, NO binding to the unligated 6-coordinate Fe(II) heme results in a 5-coordinate Fe(II)-NO complex and presented a threefold increase in the initial velocity of phosphodiesterase activity at a saturating substrate concentration (78). Further, the activation of phosphodiesterase activity was shown to be dose dependent with NO concentration.

Interestingly, however, additional studies have suggested that YybT may act as a heme-dependent NO sensor (78). The *holo*-form (with heme) of the protein was shown to have 276-fold less activity than the *apo*-form (without heme) of the protein (78). This inhibitory effect of heme binding has also been demonstrated for other heme-based sensors, such as the oxygen sensors EcDOS and YddV (90, 111). Another study, investigating the influence of YybT on heme resistance, showed that a YybT knockout strain of *Lactococcus lactis* was less equipped to deal with heme treatment (99). These observations, heme resistance and phosphodiesterase inhibition by heme, are consistent with a role for YybT in responding to heme toxicity through the modulation of c-di-AMP hydrolysis.

Dissimilative nitrate respiration regulator

DNR is an NO-responsive, heme-containing transcriptional regulator that activates the expression of genes involved in denitrification. This anaerobic metabolic process involves the reduction of nitrate to dinitrogen gas. Denitrification is essential for *P. aeruginosa* survival at low oxygen tension and has been associated with growth, virulence, and biofilm formation during persistent infections (1, 38, 88, 101).

Figure 9 shows a general scheme of anaerobic denitrification. First, the transcriptional regulator ANR (anaerobic regulation of arginine deiminase and nitrate reduction), an Fe-S cluster-containing protein, is responsible for the expression of DNR and is activated under hypoxic conditions. NO, a key reaction intermediate in the denitrification, is responsible for activating DNR. Lastly, activated DNR is responsible for the expression of the enzymes, nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, in the denitrification process (1).

DNR belongs to the cAMP receptor protein/fumarate and nitrate reductase regulator (CRP-FNR) family of transcription regulators and is composed of two domains, a C-terminal DNA binding domain and an N-terminal heme-containing sensing domain. Similar to other proteins in the CRP-FNR family, the DNA-binding domain contains an EXXSX (X = any amino acid) motif in a helix-turn-helix that is responsible for DNA recognition and binding (32). Unlike most characterized

FIG. 8. The domain architecture of YybT proteins. YybT is composed of two N-terminal TM helices, a heme-binding PAS domain, a degenerate diguanylate cyclase (GGDEF) domain, and a C-terminal DHH/DHHA1 phosphodiesterase domain. In the presence of NO, phosphodiesterase activity increases threefold compared with unligated-heme-bound YybT. c-di-AMP, bis-(3',5')-cyclic dimeric adenosine monophosphate; pApA, 5'-phosphoadenylyl-(3'→5')-adenosine; TM, transmembrane.

FIG. 9. A general scheme of anaerobic denitrification. The transcriptional regulator ANR activates DNR under hypoxic conditions. NO, a product of nitrite (NO_2^-) reduction by NIR, activates DNR. Activated DNR induces the expression of the enzymes nitrate (NO_3^-) NAR, nitrite (NO_2^-) NIR, nitric oxide (NO) NOR, and nitrous oxide (N_2O) reductase (N_2OR). ANR, anaerobic regulation of arginine deiminase and nitrate reduction; DNR, dissimilative nitrate respiration regulator; NAR, nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase.

CRP-FNR proteins, the sensing domain contains a 6-coordinate deoxy Fe(II) heme complex coordinated by a proximal histidine residue and an unidentified amino acid residue in the distal position (21, 81). The only other characterized CRP-FNR protein that contains a heme cofactor is the CO sensor transcription factor CooA in *Rhodospirillum rubrum* and *Carboxydotherrmus hydrogenoformans* (32). Another common characteristic of transcription regulators in the CRP-FNR family is structural flexibility (75), which allows allosteric control of protein function and regulation on NO binding to the sensing domain of DNR (81).

The crystal structure of DNR (purified without heme or NO bound and in a transcriptionally inactive conformation) from *P. aeruginosa* was characterized at a resolution of 3.6 Å (33).

Comparison to other known structures of the CRP-FNR family and the structure of a DNR variant (truncated DNR lacking the DNA binding domain) (34) have revealed an unusual 55° – 66° rotation of the sensing domain (33). Thus, compared with the activation mechanism of other CRP-FNR proteins, an uncommon and large conformational rearrangement would need to occur to produce biologically active DNR (33). This observation presented another possible transcriptional activation mechanism involving an initial heme binding step to drive the first conformational rearrangement before activation by NO (33).

Both *in vivo* and *in vitro* biochemical analyses of DNR mutants and wild-type and spectroscopic analysis of heme-bound DNR have unveiled the ligand discrimination strategy and NO activation mechanism of DNR (17, 21, 34, 55, 81). Early characterization revealed that the unligated Fe(II) heme cofactor of DNR presented a transcriptionally inactive 6-coordinate heme complex (34). Upon NO binding, a 5-coordinate Fe(II)-NO complex forms due to the dissociation of both the proximal and distal amino acid ligands (21, 81). This NO-dependent change from a 6- to 5-coordinate heme complex induces a conformational rearrangement within the protein to produce transcriptionally active DNR (32). However, unlike NO, CO binding to heme results in the conservation of the 6-coordinate complex due to displacement of one of the amino acid ligands (83). Thus, vital for NO-specific signaling, CO is unable to produce biologically active DNR and, therefore, transactivate the genes involved in the *P. aeruginosa* denitrification pathway (17, 55).

Perspectives and Conclusions

The field of NO-specific signaling pathways in bacteria, especially the study of bacterial H-NOX domains, has grown considerably. In this review, we have presented a summary of known bacterial heme-based NO sensors (Table 1). H-NOX

TABLE 1. CHARACTERIZED BACTERIAL NITRIC OXIDE-SIGNALING HEMOPROTEINS

Sensor or species	Functional domains (NO effect)	Regulation	References
H-NOX			
<i>S. Woodyi</i>	DGC (↓)-PDE (↑)	Biofilm	Liu <i>et al.</i> (54)
<i>A. vitis</i>	DGC (–)-PDE (↑)	Biofilm	^a
<i>L. pneumophila-L1</i>	DGC (↓)-PDE (×)	Biofilm	Carlson <i>et al.</i> (14)
<i>S. oneidensis</i> and <i>V. cholerae</i>	HK (↓)	Biofilm	Plate and Marletta (72)
<i>Patl</i>	HK (↓)	Biofilm	Arora and Boon (2)
<i>V. harveyi</i>	HK (↓)	QS	Henares <i>et al.</i> (39)
<i>V. parahaemolyticus</i>	HK (↓)	QS	^a
<i>Silicibacter</i>	HK (↓)	Symbiosis	Rao <i>et al.</i> (80)
NosP			
<i>P. aeruginosa</i>	HK (↓)	Biofilm	Hossain and Boon (45)
<i>S. oneidensis</i> and <i>L. pneumophila-L2</i>	HK (↓)	Biofilm	^a
<i>V. cholerae</i>	HK (↓)	QS	^a
Other sensors			
YybT	PDE (↑)	Heme resistance	Rao <i>et al.</i> (78)
DNR	Transcriptional regulator (↑)	Denitrification	Giardina <i>et al.</i> (32)

↑, increase; ↓, decrease; –, unchanged; ×, inactive domain; DGC, diguanylate cyclase; DNR, dissimilative nitrate respiration regulator; HK, histidine kinase; H-NOX, heme-NO/oxygen binding; NO, nitric oxide; NosP, NO sensing protein; PDE, phosphodiesterase; QS, quorum sensing.

^aUnpublished data from the Boon's lab.

proteins have generally been accepted as bacterial NO sensors. They are found in operons adjacent to genes encoding for histidine kinases and/or c-di-GMP-metabolizing enzymes and have been shown to regulate the activities of these proteins in an NO-dependent manner (Figs. 4–7). Several key structural features have been identified in NO activation of H-NOX, including the dissociation of a proximal histidine ligand (23, 27, 41, 65) and flattening of a heme cofactor (22, 27, 64, 66, 97) due to a shift in the highly conserved proline residue (Fig. 3). The latter ultimately causes large-scale conformational changes in H-NOX that relay the signal to downstream signaling proteins. This detailed understanding of the structure of H-NOX has, in fact, contributed to the development of drug agonists for the eukaryotic homolog sGC (35).

NosP domains are far less well characterized at present, but they appear to be functionally homologous to H-NOX domains; we expect them to be established as primary NO sensors in bacteria. Further understanding of the function of H-NOX and NosP domains may lead to strategies to alleviate or control bacterial biofilm formation or persistent biofilm-related infections. Interestingly, there are still many bacteria that are NO responsive but do not code for either H-NOX or NosP domains in their genomes. Consequently, the identities of the putative primary NO sensor and NO-responsive signaling pathway(s), as well as the molecular mechanism of NO regulation in these bacteria, are all currently unknown.

Both the transcriptional regulator DNR and the c-di-AMP-specific phosphodiesterase YybT (Fig. 8) feature a heme-binding domain that is fused to a functional domain, a DNA-binding domain and phosphodiesterase domain for DNR and YybT, respectively. DNR is a transcriptional regulator that, in the presence of NO, activates the expression of enzymes involved in the denitrification pathway. Recent work on YybT proteins focuses on its role in modulating c-di-AMP and its consequent role in affecting bacterial stress response and other downstream signaling pathways. However, the biological relevance of the heme-binding ability of YybT has not yet been elucidated.

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Abbreviations Used

ADP = adenosine diphosphate
 ANR = anaerobic regulation of arginine deiminase and nitrate reduction
 ATP = adenosine triphosphate
 Av = *Agrobacterium vitis*
 c-di-AMP = bis-(3',5')-cyclic dimeric adenosine monophosphate
 c-di-GMP = cyclic diguanylate monophosphate
 cGMP = cyclic guanosine monophosphate
 CO = carbon monoxide
 CRP-FNR = cAMP receptor protein/fumarate and nitrate reductase regulator

Abbreviations Used (Cont.)

Cs = *Caldanaerobacter subterraneus*
 DGC = diguanylate cyclase
 DNR = dissimilative nitrate respiration regulator
 EAL = conserved amino acids in the catalytic site
 of some phosphodiesterases
 EXAFS = extended X-ray absorption fine structure
 FNR = fumarate and nitrate regulatory proteins
 GGDEF = conserved amino acids in the
 catalytic sites of diguanylate cyclases
 GMP = guanosine-5'-monophosphate
 GTP = guanosine triphosphate
 HaCE = H-NOX-associated c-di-GMP processing
 enzymes
 HahK = H-NOX-associated histidine kinase
 HD-GYP = conserved amino acids in the catalytic
 site of some phosphodiesterases
 HK = histidine kinase
 H-NOX = heme-NO/oxygen binding
 Hpt = histidine-containing phosphotransfer
 protein

HqsK = H-NOX-associated quorum-sensing
 histidine kinase
Lpg = *Legionella pneumophila*
 MCPs = methyl-accepting chemotaxis proteins
 NahK = NosP-associated histidine kinase
NAR = *Nitrate reductase*
NIR = *Nitrite reductase*
 NO = nitric oxide
NOR = *nitric oxide reductase*
 NosP = NO-sensing protein
Pa = *Pseudomonas aeruginosa*
 pApA = 5'-phosphoadenylyl-(3' → 5')-adenosine
 PDE = phosphodiesterase
 pGpG = 5'-phosphoguanylyl-(3'-5')-guanosine
 QS = quorum sensing
 sGC = soluble guanylyl cyclase
Sili = *Silicibacter*
So = *Shewanella oneidensis*
Sw = *Shewanella woodyi*
 TM = transmembrane
Vc = *Vibrio cholerae*