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Bacterial Haemoprotein Sensors of NO: H-NOX and NosP

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Abstract

Low concentrations of nitric oxide (NO) modulate varied behaviours in bacteria including biofilm dispersal and quorum sensing-dependent light production. H-NOX (haem-nitric oxide/oxygen binding) is a haem-bound protein domain that has been shown to be involved in mediating these bacterial responses to NO in several organisms. However, many bacteria that respond to nanomolar concentrations of NO do not contain an annotated H-NOX domain. Nitric oxide sensing protein (NosP), a newly discovered bacterial NO-sensing haemoprotein, may fill this role. The focus of this review is to discuss structure, ligand binding, and activation of H-NOX proteins, as well as to discuss the early evidence for NO sensing and regulation by NosP domains. Further, these findings are connected to the regulation of bacterial biofilm phenotypes and symbiotic relationships.

1. Physiological Functions of Nitric Oxide

Nitric oxide (NO) is a diatomic, free radical gas molecule. It can readily diffuse into cells (Barraud, Kelso, Rice, & Kjelleberg, 2015) due to its lipophilicity (Shaw & Vosper, 1977) and its high diffusion coefficient of $3300 \text{ Mm}^2 \text{ s}^{-1}$ (Malinski et al., 1993). As such it is capable of rapid intercellular signalling. In eukaryotes, NO signalling plays many important and well-studied physiological roles. For example, NO engages in paracrine signalling (Thomas, Liu, Kantrow, & Lancaster, 2001). It is produced in a generator cell by an NO synthase from L-arginine (Denninger & Marletta, 1999), after which it subsequently diffuses across the lipid bilayer of an adjacent cell and activates the mammalian NO sensor protein sGC (Cary, Winger, Derbyshire, & Marletta, 2006).

NO also plays many roles in bacterial physiology. For example, when bacteria are engaged in infecting eukaryotic cells, immune cells produce high concentrations of NO upon stimulation by pathogen-associated molecular patterns, such as flagellin and lipopolysaccharides (Bellota-Anton et al., 2011). Additionally, bacteria may encounter NO endogenously produced as an intermediate in respiratory denitrification (Bellota-Anton et al., 2011). At elevated concentrations, NO causes nitrosative stress that hinders normal bacterial cell function and leads to cell death. Consequently, to ensure their survival, bacteria have evolved various mechanisms to detect and detoxify NO. Such mechanisms include the detection of NO by various transcriptional regulators including FNR-like transcription factors (fumarate and nitrate regulatory proteins) (Cruz-Ramos et al., 2002), the NO-

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responsive transcriptional activator NorR (regulator of NO reductase) (D'Autreaux, Tucker, Dixon, & Spiro, 2005), and the NO-sensitive repressor NsrR (repressor of nitrosative stress) (Bodenmiller & Spiro, 2006). Bacteria detoxify NO using NO-binding enzymes such as flavohaemoglobins, flavorubredoxin NO reductases, respiratory NO reductases, and cytochrome *c* nitrite reductases, each of which converts NO into less toxic molecules such as ammonia, nitrate, and nitrous oxide (Gardner, Helmick, & Gardner, 2002; Mills, Rowley, Spiro, Hinton, & Richardson, 2008; Poole & Hughes, 2000; Stevanin, Moir, & Read, 2005; Stevanin, Poole, Demoncheaux, & Read, 2002). While NO detoxification has generally been documented to occur at micromolar concentrations of NO (Gardner et al., 2002; Mills et al., 2008; Poole & Hughes, 2000; Stevanin et al., 2005, 2002), in some bacteria NO detoxification pathways are known to be stimulated by nanomolar amounts of NO (Hassan, Bergaust, Molstad, de Vries, & Bakken, 2016).

Recently, many studies have also demonstrated that bacteria, like eukaryotes, can also detect NO at low concentrations as a sensitive and specific signalling molecule (Arora, Hossain, Xu, & Boon, 2015). In particular, low concentrations of NO are frequently implicated in regulating bacterial biofilm formation (Barraud et al., 2015; McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2011). Biofilms occur when bacteria accumulate on a surface in a moist environment within a self-secreted exopolysaccharide matrix (Bhinu, 2005). Biofilms are problematic, as when in this state, bacteria not only exhibit up to 1000-fold higher tolerance to immune responses and antibiotics (Hoiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Stewart & Costerton, 2001), but have also been demonstrated to be the leading cause of nosocomial infections (Lindsay & von Holy, 2006; Wenzel, 2007).

Currently, there are two known bacterial haem-based sensors of NO used in this type of signal transduction: the relatively well-studied H-NOX (haem-nitric oxide/oxygen-binding domain) family and the newly discovered NosP (nitric oxide sensing protein) family. Bacterial H-NOX domains are members of the same superfamily of haem sensors that includes soluble guanylate cyclase (sGC), the eukaryotic sensor of NO (Iyer, Anantharaman, & Aravind, 2003). In fact, the large majority of H-NOX proteins are animal proteins. NosP, on the other hand, is almost exclusively coded for in bacterial genomes. This paper focuses on reviewing what is currently known about NO sensing by H-NOX and NosP in bacteria.

2. sGC: The Animal no Sensing Protein

Bacterial H-NOX proteins were discovered based on homology to sGCs. Therefore, a brief overview of sGC is presented here. sGC is a haem-based sensor. It is a heterodimeric enzyme composed of $\alpha 1$ (mass ranging from 73 to 88 kDa) and $\beta 1$ (mass of ~80 kDa) subunits that bind one protoporphyrin IX group per dimer (Hobbs & Ignarro, 1996). The haem-binding domain of sGC has been localized to the first 194 amino acids of the $\beta 1$ subunit (Karow et al., 2005) with histidine 105 as the proximal ligand (Wedel et al., 1994). In its resting state, the haem iron of sGC is in the reduced, unligated ferrous state [Fe(II)-unligated], and is available for NO detection. sGC binds picomolar NO at the haem iron, forming the ferrous-NO [Fe(II)-NO] complex, which activates the enzyme to convert GTP (guanosine triphosphate) to cGMP (cyclic guanosine monophosphate) (Stone & Marletta, 1996), ultimately mediating downstream events such as vasodilation, platelet aggregation,

neurotransmission, and myocardial function via calcium channels, phosphodiesterases, and kinases (Garthwaite, 2008; Lucas et al., 2000).

On a molecular level, NO ligation to the haem iron causes cleavage of the Fe–His bond, which is believed to result in a conformational change that ultimately leads to the activation of the catalytic domain (Cary, Winger, & Marletta, 2005; Fernhoff, Derbyshire, & Marletta, 2009; Russwurm & Koesling, 2004). Much of what is known about the activation of sGC comes from studies of bacterial H-NOX proteins, however, which will be detailed later.

3. Discovery of a Bacterial no Sensing Protein: H-Nox

In 2003, a family of haem proteins in bacteria with 15%–40% identity to the haem domain from rat sGC was identified (Iyer et al., 2003) (Fig. 1). The family includes the eukaryotic sGCs as well as several hundred predicted open-reading frames (ORFs) from bacterial genomes. Based on their high homology with sGC, these domains were initially named HNOBs for haem-nitric oxide-binding domain.

These bacterial haem domains are all about 190 amino acids long. At the time of discovery, the structure of this domain was unknown, but it would later become clear that these bacterial proteins have particularly high identity to sGC in the haem-binding region (Schmidt, Schramm, Schroder, Wunder, & Stasch, 2004). In particular, several amino acids are absolutely conserved and can be considered signatures of the family. Besides the histidine haem ligand (H105 in rat sGC β 1), a YxS/TxR motif is conserved (residues 135, 137, and 139, respectively, in rat β 1), as is a proline residue about 10 amino acids towards the C-terminus from the haem-binding histidine residue (residue 118 in rat β 1) (Fig. 2). The YxS/TxR has now been identified as important for coordinating the haem propionate side chains (Pellicena, Karow, Boon, Marletta, & Kuriyan, 2004) (Fig. 3). This motif, along with the proline residue, is now known to be important for maintaining a unique haem structure in this family (see Section 5.2).

In 2004, the Marletta group recombinantly expressed and purified two HNOB domains, one cloned from *Vibrio cholerae* (Vc_0270) and one from *Caldanaerobacter subterraneus* (at the time known as *Thermoanaerobacter tengcongensis*; TTE0680 basepairs 1–564) (Karow et al., 2004). They found that, as expected, these domains are histidine-ligated protoporphyrin IX haemoproteins that bind gaseous ligands at the ferrous iron atom of the haem 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]. Based on this discovery, this family of haem sensors was renamed H-NOX, for haem-nitric oxide and/or oxygen-binding domain.

In facultative aerobic bacteria, in general, most H-NOX domains are encoded in a histidine kinase-containing operon; some are encoded in a cyclic-di-GMP synthase and/or phosphodiesterase-containing operon (Iyer et al., 2003) (Fig. 1). H-NOX domains from obligate anaerobes are encoded as the N- or C-terminal domain of methyl-accepting chemotaxis proteins (MCPs) (Cary et al., 2006; Iyer et al., 2003). Based on homology to sGC and genomic association with signalling proteins, it was hypothesized that bacterial H-

NOX proteins would also serve as sensors of NO (or possibly O₂ in the case of obligate aerobic organisms), regulating signal transduction of the associated signalling proteins. Many subsequent studies have confirmed the role of the interaction of H-NOX with NO in modulating the activities of these proteins (Arora & Boon, 2012; Henares, Higgins, & Boon, 2012; Henares, Xu, & Boon, 2013; Liu et al., 2012; Muralidharan & Boon, 2012); these studies will be described later in this review (see Section 6).

4. Ligand-Binding Properties of H-Nox Domains

Many bacterial H-NOX domains have now been cloned, expressed, purified, and spectroscopically characterized (Arora & Boon, 2012; Boon et al., 2006; Dai, Farquhar, Arora, & Boon, 2012; Henares et al., 2012; Karow et al., 2004; Liu et al., 2012; Ma, Sayed, Beuve, & van den Akker, 2007; Mukhopadhyay, Sudasinghe, Schaub, & Yukl, 2016; Price, Chao, & Marletta, 2007; Tsai, Berka, Martin, & Olson, 2012; Tsai et al., 2010; Wang et al., 2010; Wu, Liu, Berka, & Tsai, 2015). UV/vis spectroscopy, paired with resonance Raman (rR) spectroscopy, has demonstrated that ferrous H-NOX domains from facultative aerobes form high-spin 5-coordinate Fe(II)-unligated complexes (Boon et al., 2006; Karow et al., 2004). Upon addition of carbon monoxide (CO), these proteins form low-spin 6-coordinate Fe(II)-CO complexes, while upon ligation to NO the proteins form high-spin 5-coordinate Fe(II)-NO complexes (Karow et al., 2004). Thus, H-NOX domains from facultative aerobes, like sGC, form 5-coordinate NO complexes and rigorously exclude O₂ as a ligand (Karow et al., 2004; Price et al., 2007). 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 (Cary et al., 2006; Karow et al., 2004; Tran, Boon, Marletta, & Mathies, 2009).

To date, 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 *Sw*-H-NOX, $21 \times 10^{-4} \text{ s}^{-1}$ for *Vf*-H-NOX, $3.6 \times 10^{-4} \text{ s}^{-1}$ for rat sGC, 0.05 s^{-1} for *Ns*-H-NOX, 0.3 s^{-1} for *Vc*-H-NOX) (Liu et al., 2012; Stone & Marletta, 1994, 1996; Tsai et al., 2010; Wang et al., 2010; Wu, Liu, Berka, & Tsai, 2013; Zhao, Brandish, Ballou, & Marletta, 1999) with an assumed diffusion-limited association rate of $\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$. Consequently, these domains are thought to possess nanomolar to picomolar affinity for NO, underscoring a role for them in NO sensing and signalling in bacteria (see Section 6).

5. Structure and the Molecular Basis for Function in H-Nox Domains

Thus far, H-NOX domains from *C. subterraneus*, *Nostoc* sp., and *Shewanella oneidensis* have been characterized with molecular resolution. In this section of the review, we will discuss how key observations from these structural studies provide possible insight into the molecular mechanisms by which H-NOX proteins initiate downstream signal transduction events, most notably haem flattening and Fe-His bond cleavage.

The first H-NOX domain to be crystallized was from the thermophilic anaerobe *C. subterraneus*. The structure of the Fe(II)-O₂ complex of the *Cs*-H-NOX domain (at the time known as *Tt*-H-NOX) was solved to 1.7 Å resolution (Pellicena et al., 2004), revealing that

the H-NOX family has a novel protein fold that consists of seven α -helices and a four-stranded anti-parallel β -sheet. The N-terminal region consists of five helices, α A- α D and α G, which are situated on the distal side of the haem. The C-terminal region, however, is on the proximal side of the haem and consists of the β -sheet, the α F helix (the signalling helix where the histidine proximal ligand is located), and a one-turn helix, α E.

Further analysis of the ferrous-oxy *Cs*-H-NOX structure reveals that a distal pocket hydrogen-bonding network between the iron-bound O₂ and three distal pocket amino acids (Trp9, Asn74, and Tyr140 in *Cs*-H-NOX) is present (Pellicena et al., 2004) (Fig. 4). This distal pocket H-bonding network has been shown to play a role in O₂ binding by this H-NOX (Boon, Huang, & Marletta, 2005; Hespen, Bruegger, Phillips-Piro, & Marletta, 2016) and is hypothesized to contribute to NO/O₂ ligand discrimination in the H-NOX family (Boon et al., 2005; Boon & Marletta, 2005a, 2005b), as will be further discussed in Section 5.2.

The structure of ferrous-oxy *Cs*-H-NOX also led to the identification of a unique structural feature of the H-NOX family: a highly distorted haem cofactor, as well as the suggestion that a proline residue [Pro115 in *Cs*-H-NOX; this proline is absolutely conserved in the H-NOX family (Karow et al., 2004)] is vitally important in maintaining this haem distortion (Dai & Boon, 2011; Erbil, Price, Wemmer, & Marletta, 2009; Olea, Boon, Pellicena, Kuriyan, & Marletta, 2008; Olea, Kuriyan, & Marletta, 2010; Sun et al., 2016). To assess the significance of Pro115 in haem distortion in *Cs*-H-NOX, the proline to alanine mutant was crystallized and solved to 2.1 Å (Olea et al., 2008). This structure demonstrated that, in addition to haem flattening in the absence of steric crowding from Pro115, this change in haem structure is accompanied by an N-terminal subdomain shift. The connection between the overall protein structure and haem flattening suggests that haem flattening may play a vital role in the H-NOX signalling process. Subsequent structures of *Cs*-H-NOX, as well as other H-NOX domains in a variety of ligand-bound complexes have provided support for this hypothesis and will be discussed further in Section 5.2.

An additional interesting feature revealed by this initial structure of *Cs*-H-NOX, is that the haem is buried in the protein fold (Pellicena et al., 2004). This has raised the question of gas ligand migration within the H-NOX fold. Computational models predict that gas migration to the haem site is guided by molecular tunnels (Zhang, Lu, Cheng, & Li, 2010). Confirming this prediction, *Cs*-H-NOX and *Ns*-H-NOX (H-NOX from *Nos-toc* sp.) were crystallized under pressure with xenon gas (Winter, Herzik, Kuriyan, & Marletta, 2011). In comparison to *Cs*-H-NOX (which is not predicted to contain gas tunnel networks), clear continuous tunnels containing xenon were observed in *Ns*-H-NOX.

In order to gain insight into the structural basis of signal transduction, H-NOX constructs representative of pre- and post-NO binding have been obtained and studied. To this end, high-resolution structures of *S. oneidensis* H-NOX were solved by both nuclear magnetic resonance (NMR) and crystallography. *So*-H-NOX in 5-coordinate Fe(II)-NO, 6-coordinate Mn(II)-NO, and Fe(II)-unligated states have been solved to 1.65, 2.45, and 2.00 Å resolution, respectively (Herzik et al., 2014). While *So*-H-NOX usually forms a 5-coordinate complex with NO (due to Fe-His bond cleavage), a mimic of the 6-coordinate intermediate

(with metal–His bond intact) was generated by substituting manganese protoporphyrin IX for haem. NMR solution structures of *S*-H-NOX with and without an intact Fe–His bond have also been obtained (Erbil et al., 2009) by comparing the Fe(II)-CO complexes of wild-type *S*-H-NOX and the haem ligand mutant H103G. These studies provide molecular insight to the role of histidine–iron cleavage in H-NOX signalling transduction. Further analysis of the Fe–His interaction and its relation to signalling is discussed in Section 5.3.

5.1 Ligand Discrimination in H-NOX Domains

sGC and the H-NOX domains from facultative anaerobes have been demonstrated to exclusively bind NO and CO, but not molecular O₂ (Karow et al., 2004; Liu et al., 2012; Price et al., 2007; Stone & Marletta, 1994; Tsai et al., 2010; Wang et al., 2010). 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 (Pellicena et al., 2004) revealed that the dioxygen ligand is bent with respect to the plane of the porphyrin, as expected from studies of the globin family. Furthermore, the O₂ molecule was found to interact with a distal pocket hydrogen-bonding network. A primary H-bond is present between the iron-bound O₂ and the distal pocket tyrosine 140. Tyr140 is further part of a distal pocket hydrogen-bonding network with tryptophan 9 and asparagine 74. It appears that Tyr140 is held in the optimal position for H-bonding with iron ligands through stabilizing H-bonds from Trp9 and Asn74 (Fig. 4). Sequence alignments reveal that this hydrogen-bonding network is conserved only in H-NOX proteins from obligate anaerobes (Pellicena et al., 2004) (i.e., they bind O₂, NO, and CO), 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 (Boon et al., 2005) was found to increase the O₂ dissociation rate constant by 20-fold, while the NO dissociation rate constant remained unchanged. Furthermore, introduction of a mutant tyrosine residue into the hydrophobic distal pockets of the rat sGC H-NOX domain (β1 1–194) or the *Legionella pneumophila* L2-H-NOX domain enabled these domains to gain O₂-binding function (Boon et al., 2005). 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, while the rate of NO dissociation remains unchanged, thus providing discrimination (Boon et al., 2005).

Studies of the Pro115 mutant of *Cs*-H-NOX (haem-flattened mutant) have bolstered this hypothesis (Dai & Boon, 2011; Olea et al., 2008; Sun et al., 2016). The *Cs*-H-NOX P115A

mutant was shown to have a significantly slower O₂ dissociation rate constant in comparison to wild-type *Cs*-H-NOX, but the O₂ association rate constant remained unchanged (Olea et al., 2008). As a result, the authors reasoned that the absence of the proline residue causes an increase in the affinity for dioxygen, leading to a more stable Fe(II)-O₂ complex. This was elaborated in vibrational correlation spectroscopy experiments (Sun et al., 2016). Photolysis of the Fe-O₂ bond in wild-type and P115A *Cs*-H-NOX constructs revealed that the wild-type Fe-O₂ bond is more resistant to photolysis and has a longer O₂-rebinding time in comparison to the P115A mutant. Consequently, these data suggest that there is an overall stronger iron-O₂ bond in the distorted haem of wild type, but a greater degree of ligand trapping in the nondistorted mutant, resulting from a more tightly packed distal pocket and a stronger H-bond between Tyr140 and O₂.

Mutational studies introducing a Tyr into the distal pocket of full-length sGC, however, seem 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 distal tyrosine (I145Y) was introduced into the distal pocket of full-length sGC, as opposed to the haem-binding domain of sGC only (β1 1–194) (Boon et al., 2005), researchers did not find evidence for O₂-binding (Martin, Berka, Bogatenkova, Murad, & Tsai, 2006). Moreover, characterization of the H-NOX domain from the obligate anaerobic bacterium *Clostridium botulinum* revealed that, although *Cb*-H-NOX contains a distal pocket tyrosine, it has no measurable affinity for O₂ (Nioche et al., 2004). Consequently, these findings suggest 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, called the sliding-scale hypothesis (Tsai, Berka, et al., 2012). This hypothesis is based on the observation that it is consistently true that gas-binding haem 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 (Tsai, Berka, et al., 2012). According to the sliding-scale hypothesis, in order to compensate for the much lower affinity haem iron would normally 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 nonpolar residues, the sliding-scale ratios are restored (Tsai, Berka, et al., 2012). This hypothesis predicts, therefore, that the H-bonding network in H-NOX from anaerobic bacteria functions to favour O₂-binding with respect to NO- and CO-binding, by lowering the binding rate constants and distorting the sliding scale, while H-NOX from aerobic bacteria conform to the sliding scale (Wu et al., 2013). It follows from this hypothesis that sGC also conforms to the sliding-scale rule and simply does not have the affinity to bind O₂ under physiologically relevant conditions, and that the introduction of a single tyrosine residue is insufficient to lower the O₂-binding affinity to measurable levels.

5.2 Haem Distortion and Its Role in Signal Transduction

As mentioned earlier, a particularly striking feature of the H-NOX fold is the severely distorted nature of the haem cofactor. Normal-coordinate structural decomposition (NCS) analysis (Senge et al., 1997) revealed that the *Cs*-H-NOX haem has displacements of -1.0 Å saddling and -1.2 Å ruffling. It is not uncommon for haem proteins to cause distortion of the haem cofactor, but the degree of distortion in H-NOX proteins is significantly higher than that of other known haem proteins. For example, cytochrome *c3* and nitrophorin (protein data bank codes 2CDV and 1ERX), both of which are considered to have severely distorted haem groups, exhibit about 1 and 0.4 Å saddling and -0.4 and -0.8 Å ruffling, respectively (Pellicena et al., 2004).

This large degree of nonplanarity can largely be attributed to two factors specific to H-NOX. First, the haem in H-NOX is buried within the protein, where the propionate groups are constrained by interactions with the highly conserved YxS/TxR motif (Pellicena et al., 2004) (Fig. 3). The arginine has been shown to contribute H-bonds to the haem propionate groups on both pyrrole A and D, while the tyrosine and serine have both been demonstrated to contribute to hydrogen bonding with only the propionate on pyrrole A. Secondly, Van der Waals interactions between the absolutely conserved H-NOX proline residue and one of the pyrroles of the haem causes the pyrrole to shift out of plane (Olea et al., 2008; Pellicena et al., 2004).

Interestingly, the structure of the *Cs*-H-NOX Fe(II)-O₂ complex was solved in two different crystal forms, each with two monomers per asymmetric unit, which yielded four independent views of the protein structure (Pellicena et al., 2004). Variation in the structure of the haem group between these structures suggests that there is conformational flexibility in the H-NOX haem structure. This observation has led to the hypothesis that alterations in the degree of haem distortion could be coupled to surface changes in H-NOX, which could ultimately lead to changes in inter-molecular interactions with cocistronic partner proteins and subsequent changes in signal transduction downstream.

Crystal structures of H-NOX domains bound to various 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, reveals that ligand binding causes haem pivoting and bending to occur concomitantly with a shift in the N-terminal helices of the protein (Ma et al., 2007).

The structure of *Cs*-H-NOX in the absence of O₂ was recently solved to 2.3 Å and provides further evidence for this theory (Hespen et al., 2016). In this structure, the researchers found that in the absence of O₂, the W9/N74/Y140 hydrogen-bonding network is displaced from the face of haem, leading to an overall shift of the helix containing Trp9. This helix also contains isoleucine 5, which is a major contributor to haem distortion (Olea, Herzik, Kuriyan, & Marletta, 2010; Weinert, Phillips-Piro, Tran, Mathies, & Marletta, 2011). Consequently, movement of this residue, and overall shifts in the protein that displace Pro115, results in haem relaxation compared to the ferrous-oxy complex. Another helix that undergoes a major shift upon oxygen binding contains the H-bonding network member

Asn74 as well as glycine 71. Gly71 is an absolutely conserved H-NOX family residue that is thought to be vital in helix mobility.

5.3 Histidine Dislocation and Its Role in Signal Transduction

Spectroscopic characterization of sGC and facultative anaerobic bacterial H-NOX family members indicates that the haem-iron bond to the histidine ligand (H105 in rat sGC) is broken upon NO ligation to H-NOX (Zhao et al., 1999). 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 signalling, although as discussed later, the details of these structural changes are not yet agreed upon.

For many years, a structure of the Fe(II)-unligated complex of an H-NOX domain was unavailable, making it difficult to compare the structural changes that might take place upon NO ligation and Fe-His bond cleavage. Thus, in order to evaluate the role of the Fe-His bond, *So*-H-NOX Fe(II)-NO, Mn(II)-unligated, and Mn(II)-NO structures were compared (Herzik et al., 2014). The Mn(II)-NO complex remains 6-coordinate, making it possible to evaluate any structural changes that take place upon NO binding independently from those that take place due to histidine dissociation. In the Mn structures, the Mn atom moves towards the distal side of the porphyrin in Mn(II)-NO compared to the Mn(II)-unligated state. No other appreciable structural changes resulted from NO ligation, however, which supports the hypothesis that cleavage of the histidine bond is indeed an important effector of NO-based signalling.

NMR studies have also contributed to our understanding of the role of Fe-His bond breakage, as well as haem structure, in H-NOX function. NMR structures of *S. oneidensis* 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 (Erbil et al., 2009). 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 revealed that the distorted haem cofactor relaxes upon Fe-His bond cleavage, which further supports the earlier findings about haem relaxation and signal transduction mechanism of H-NOX (Erbil et al., 2009). Moreover, a crystal (solved to 2.0 Å) of the homologous proximal histidine mutant of *Cs*-H-NOX (H102G) has corroborated the NMR studies (Olea, Herzik, et al., 2010). As expected, one of the molecular features evident in this crystal structure is loss of haem distortion as a result of His-Fe cleavage. The protein conformational changes associated with haem relaxation are also present. Therefore, it appears that Fe-His bond cleavage may lead to haem 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 5- or 6-coordinate complexes with NO were determined (Dai et al., 2012). *Shewanella woodyi* and *Pseudoalteromonas atlantica* H-NOX proteins are known to form 5-

coordinates complexes with NO (Arora & Boon, 2012; Liu et al., 2012), which is consistent with dissociation of the proximal histidine. Yet, 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 (Dai et al., 2012). 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 (Herzik et al., 2014) (Fig. 3B). This structure demonstrates that upon NO binding, the proximal histidine dissociates, resulting in a 5-coordinate complex with NO bound to the proximal side of haem. The authors acknowledge, however, that this might be an artefact 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*. Here, multiple rate constants were apparent in NO-binding reactions in the presence of a large excess of NO, but not at stoichiometric NO concentrations (Tsai et al., 2010; Tsai, Berka, et al., 2012; Wu et al., 2013). The authors explain the results by comparing them to studies performed on cytochrome *c'* (Tsai, Martin, Berka, & Olson, 2012). In cytochrome *c'*, weak binding of NO to the distal side of haem 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 haem pocket (Tsai, Berka, et al., 2012; Tsai, Martin, et al., 2012). 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 (see Section 5.1), as O₂ would not be able to sufficiently weaken the Fe–His bond to allow for a second binding and displacement reaction to occur (Tsai et al., 2010; Tsai, Berka, et al., 2012; Tsai, Martin, et al., 2012; Wu et al., 2013).

There is also evidence against the proximal pocket NO-binding model, however. Interestingly, the H-NOX domains from *Nostoc punctiforme* (Np-H-NOX) (Boon et al., 2006), *L. pneumophila* (L2-H-NOX) (Boon et al., 2006), and *Vibrio parahaemolyticus* (Vp-H-NOX) (Ueno, Fischer, & Boon, unpublished data) form mixtures of 5- and 6-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, while 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 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 haem pocket of the H-NOX fold.

Finally, two additional studies speak to an important role for the proximal histidine ligand in H-NOX structure and function: a molecular modelling study of sGC and X-ray spectroscopy studies of Cs-H-NOX. From structural studies, it has been shown that a bound water molecule makes several interactions with the protein backbone and side chains, as well as with the proximal histidine ligand (Fig. 3A). Modelling of sGC (based on the H-NOX crystal structure from *Nostoc* sp.) (Baskaran, Heckler, van den Akker, & Beuve, 2011) has

shown that various mutations of residues in this water-network resulted in loss of haem binding and/or loss of sGC activation. The water molecule and its coordinating residues were therefore concluded to be needed for accomplishing the protein conformational changes necessary for signalling. In support of this, the *So*-H-NOX structure showed that this water is lost upon dissociation of the proximal histidine ligand, as the contacts between the histidine and other portions of the protein are disrupted (Herzik et al., 2014).

Cs-H-NOX has also been studied by X-ray absorption near edge spectroscopy (XANES) (Dai & Boon, 2011). The excitation energy of the edge of the iron K-shell, the innermost electron shell, is useful for understanding the coordination, oxidation, and ligand-binding properties of haem iron. An interesting observation from this study was that, while it is normally assumed that forming an iron—ligand complex will lower the K-shell excitation energy, the opposite is true in H-NOX due to a commensurate increase in shielding as the remaining valence electrons get pulled to the iron core. By comparing the K-shell energies of wild-type and P115A complexes of *Cs*-H-NOX, it was concluded that haem distortion is important for the energy of ligand binding (as discussed in Section 5.2). Another H-NOX structural detail extracted from these XANES measurements is that the haem iron is displaced, with respect to the plane of the porphyrin nitrogen atoms, in the Fe(II)-unligated state, while upon ligand binding, iron moves away from the histidine towards the distal side of haem. This corresponds well to the NO-dependent conformational changes that are dependent on loss of histidine coordination.

6. Biochemical Functions of H-Nox Proteins

First documented in *Nitrosomonas europaea* (Schmidt, Steenbakkens, op den Camp, Schmidt, & Jetten, 2004) and the cystic fibrosis-associated pathogen *Pseudomonas aeruginosa* (Barraud et al., 2006, 2009), regulation of bio-film formation by nanomolar levels of NO has now been documented in numerous bacteria (Arora et al., 2015; Barraud et al., 2015, 2009; McDougald et al., 2011). In several bacteria, NO/H-NOX signalling through cyclic-di-GMP- and kinase-mediated pathways has been shown to be responsible for these biofilm phenotypes, as well as other bacterial group behaviours such as quorum sensing and symbiosis (Arora & Boon, 2012; Carlson, Vance, & Marletta, 2010; Henares et al., 2012, 2013; Liu et al., 2012; Plate & Marletta, 2012; Rao, Smith, & Marletta, 2015; Wang et al., 2010).

As previously noted, in the genomes of facultative anaerobic bacteria, stand-alone H-NOX domains are commonly encoded in the same operon with two-component signalling histidine kinases and cyclic-di-GMP processing enzymes (Fig. 1). It is noteworthy that the histidine kinase and cyclic-di-GMP enzymes in these H-NOX operons lack sensory domains. Consequently, it is hypothesized that H-NOX functions as an NO sensor to regulate the activities of these enzymes *in trans*. In this section of the review, the effect of H-NOX on the activity of cocistronic partner proteins is briefly discussed. For more extensive details on the molecular mechanisms by which H-NOX proteins explicitly regulate their associated signalling pathways, the following reviews are available (Arora et al., 2015; Nisbett & Boon, 2016; Plate & Marletta, 2013).

6.1 H-NOX and HaCE Signalling

Although they are in the minority, some facultative aerobic bacteria code for an H-NOX domain in the same operon as a cyclic-di-GMP synthase and/or phosphodiesterase. We have recently termed these enzymes, collectively, HaCEs for H-NOX-associated cyclic-di-GMP processing enzymes. This category of H-NOX domains is of particular interest as they directly implicate NO/H-NOX signalling in the regulation of cyclic-di-GMP, a secondary messenger molecule in bacteria that regulates biofilm formation (Ross et al., 1987).

HaCE enzymes generally have one or both of two enzymatic domains that directly participate in cyclic-di-GMP regulation: a diguanylate cyclase domain that synthesizes cyclic-di-GMP from two molecules of GTP and/or a cyclic-di-GMP phosphodiesterase domain that hydrolyzes cyclic-di-GMP into pGpG (5'-phosphoguanylyl-(3'-5')-guanosine) or GMP (guanosine-5'-monophosphate). H-NOX/HaCE complexes have been shown to link NO detection with regulation of HaCE activity, leading to intracellular changes in the concentration of cyclic-di-GMP and biofilm formation.

H-NOX regulation of HaCE activity has been biochemically observed in the bacteria *L. pneumophila* (Carlson et al., 2010), *S. woodyi* (Liu et al., 2012), and more recently, *Agrobacterium vitis* (Nesbitt et al., unpublished data) (Fig. 5). In *L. pneumophila*, *Lpg*-HaCE has only cyclic-di-GMP synthase activity (although it contains a phosphodiesterase domain, it is inactive) (Carlson et al., 2010). In both *S. woodyi* and *A. vitis*, however, *Sw*-HaCE (Liu et al., 2012) and *Av*-HaCE (Nesbitt et al., unpublished data) proteins were found to exhibit both cyclic-di-GMP synthase and phosphodiesterase activities in vitro. In *L. pneumophila*, *Lpg*-HaCE cyclic-di-GMP synthase activity is unaffected in the presence of Fe(II)-unligated *L1*-H-NOX (Carlson et al., 2010), while in *S. woodyi*, *Sw*-HaCE cyclic-di-GMP synthase activity is upregulated (by ~ 10-fold) in the presence of Fe(II)-unligated *Sw*-H-NOX and phosphodiesterase activity remains unchanged (Liu et al., 2012). Thus, it appears that NO-free H-NOX variably affects HaCE activity; NO-bound H-NOX, however, universally results in changes in HaCE activity. In *L. pneumophila*, NO-bound *L1*-H-NOX causes a decrease in *Lpg*-HaCE cyclic-di-GMP synthase activity, leading to a decrease in cyclic-di-GMP concentration in vitro (Carlson et al., 2010). In *S. woodyi*, *Sw*-HaCE cyclic-di-GMP production is also downregulated in the presence of Fe(II)-NO bound *Sw*-H-NOX, but by a slightly different mechanism. Here, in addition to decreasing HaCE cyclic-di-GMP synthase activity, the phosphodiesterase activity of *Sw*-HaCE is increased (Liu et al., 2012), leading to a dramatic decrease in c-di-GMP concentration.

Finally, in light of the *S. oneidensis* H-NOX NMR solution structures and the *C. subterraneus* crystal structures revealing that NO-haem binding may induce haem flattening and subsequent protein conformational changes (Erbil et al., 2009; Olea et al., 2008; Olea, Kuriyan, et al., 2010), it has been hypothesized that changes in both haem and protein conformation may directly translate into changes in downstream signalling events in H-NOX signalling pathways (as discussed in Section 5). Since there was no direct evidence in support of this hypothesis, however, our lab investigated the role of the H-NOX haem structure in the H-NOX/HaCE signalling pathway from *S. woodyi*. In this study, as expected, the relaxed haem proline mutant of *Sw*-H-NOX led to upregulation of the phospho-diesterase activity of *Sw*-HaCE (Muralidharan & Boon, 2012), which is the very

same effect that NO-bound H-NOX has on HaCE activity (Liu et al., 2012). This study, therefore, provided the first direct evidence for the role of haem relaxation in H-NOX signal transduction.

6.2 H-NOX and Two-Component Signalling

H-NOX genes are cocistronic with two-component signalling histidine kinase (HaHK; H-NOX-associated histidine kinase) genes instead of HaCE genes in most facultative aerobic bacteria (Fig. 1). Two-component signalling networks are signalling systems that bacteria use to sense and respond to various environmental stimuli including nutrient availability, pH, osmolarity, and host factors (Beier & Gross, 2006). Interestingly, thus far, most H-NOX two-component signalling systems have been implicated in c-di-GMP metabolism and biofilm formation in many bacteria, which is functionally consistent with H-NOX/HaCE signalling systems (Henares et al., 2013; Plate & Marletta, 2012; Rao et al., 2015).

Simple two-component signalling networks are comprised of a sensor histidine kinase and a response regulator protein. The sensor histidine kinase detects the environmental stimulus via its sensory domain, and responds by utilizing adenosine triphosphate (ATP) as a phosphodonator to catalyse 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 then results in activation of the appropriate cellular response (Laub, Biondi, & Skerker, 2007). A common variation in these signalling systems, sometimes called three-component signalling (Elsen, Duche, & Colbeau, 2003; Ortiz de Orue Lucana & Groves, 2009; Szurmant, Bu, Brooks, & Hoch, 2008), 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 (Elsen et al., 2003; Szurmant et al., 2008).

Histidine kinases (HaHKs) that are cocistronic with H-NOX genes 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 signalling systems have been characterized in *S. oneidensis* (Plate & Marletta, 2012), *V. cholerae* (Mukhopadhyay et al., 2016; Plate & Marletta, 2012), and *P. atlantica* (Arora & Boon, 2012) (Fig. 6). In all three bacteria, in the absence of H-NOX, the HaHK proteins exhibit ATP-dependent autophosphorylation activity (Arora & Boon, 2012; Plate & Marletta, 2012; Price et al., 2007) that is strongly inhibited in the presence of NO-bound H-NOX.

Another common deviation from simple two-component histidine kinase-response regulator architecture is found in hybrid histidine kinase signalling. Here the histidine kinase has a receiver domain within the same polypeptide (Laub et al., 2007). These hybrid kinase proteins (in response to a stimulus) autophosphorylate their conserved histidine residues and then transfer the phosphate intramolecularly to a conserved aspartic acid residue contained within their receiver domains. The phosphate is then subsequently transferred to a conserved histidine residue in a Hpt (histidine-containing phosphotransfer protein) which then further engages in phosphotransfer with an appropriate response regulator protein (Laub et al.,

2007). H-NOX proteins have also been shown to regulate the activity of hybrid histidine kinases and their associated phosphorelay signalling pathways, specifically, those that have been implicated in quorum sensing and symbioses with eukaryotes.

Two H-NOX-associated signalling pathways, those from *Vibrio harveyi* and *V. parahaemolyticus*, have thus far been implicated in quorum sensing (Henares et al., 2012; Ueno et al., unpublished data) (Fig. 7). Quorum sensing is a process that governs population-wide bacterial group behaviours, including virulence gene production, biofilm formation, and bioluminescence, in response to the production, secretion, and detection of autoinducers in a cell density-dependent manner (Papenfort & Bassler, 2016). 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 (Henares et al., 2012; Ueno et al., unpublished data). What makes these systems most interesting is that HqsK signalling merges with the kinase pathways regulated by quorum sensing autoinducers. Thus, NO/H-NOX participates in quorum sensing signalling 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 (Wang & Ruby, 2011) (Fig. 8). Specifically, *Vibrio 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 (Rao et al., 2015; Wang et al., 2010). *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. Further biochemical characterization of the *Sili*-HaHK signalling pathway revealed that *Sili*-HaHK can engage in phosphorelay with a Hpt, *Sili*-Hpt, which further engages in phosphotransfer to regulate a cyclic-di-GMP synthase, ultimately regulating symbiosis with *T. erythraeum* (Rao et al., 2015). The *Vf*-H-NOX/HaHK pathway has not been biochemically characterized. However, since the *Vf*-H-NOX/HaHK signalling pathway is architecturally similar to other H-NOX/HaHK signalling pathways, we can hypothesize that *Vf*-H-NOX will also regulate downstream signalling, in this case to help establish symbiosis with *E. scolopes*, in a NO-dependent manner.

6.3 H-NOX and Methyl Accepting Chemotaxis Signalling

For obligate anaerobes, O₂ is toxic. In these bacteria, H-NOX domains are found fused to MCPs (Fig. 1). MCPs typically mediate bacterial movement to either avoid toxins or seek nutrients (Dahl, Boos, & Manson, 1989). MCP receptors are usually transmembrane proteins that regulate a histidine kinase-signalling pathway based on the methylation state of the receptor (Roberts, Papachristodoulou, & Armitage, 2010; Williams & Stewart, 1999).

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. Thus, in order to determine whether the function of H-NOX in *C. subterraneus* is to sense NO or O₂, and thus regulate a biochemical output, an orthogonal assay was developed. Here, *Cs*-H-NOX

regulation of an orthogonal HaHK cloned from *V. cholerae* (Hespen et al., 2016) was determined. This study showed that Fe(II)-unligated *Cs*-H-NOX-inhibited HaHK autophosphorylation, and the O₂-bound, but not the CO- or NO-bound, complex was able to relieve *Cs*-H-NOX inhibition of *Vc*-HaHK. By extension, the data indicate that O₂ is a likely regulator of chemotaxis in many anaerobic bacteria, a finding that is consistent with the earlier structural findings that this class of H-NOX proteins undergoes ligand induced conformational changes in the presence of molecular oxygen (Hespen et al., 2016).

6.4 H-NOX as a Redox Sensor

It should be noted that there is some disagreement that H-NOX is strictly a family of NO/O₂ sensing proteins. For example, there is evidence that in addition to, or alternatively to, NO sensing, H-NOX proteins may be redox sensors. This was first suggested based on findings that *Ns*-H-NOX undergoes conformational changes between the ferrous and ferric oxidation states (Tsai et al., 2010). This argument is supported by the fact that *Ns*-H-NOX forms a 6-coordinate complex upon NO binding, with the Fe–His bond, therefore, remaining intact. However, as discussed earlier, it is not clear that Fe–His bond cleavage is absolutely necessary to activate downstream signalling. It has been difficult to conclusively test the hypothesis that *Ns*-H-NOX is a redox sensor because downstream partners have not been identified for *Ns*-H-NOX, making a biochemical assay of downstream signalling prohibitive.

Stronger evidence of redox sensing has manifested in gammaproteobacteria, however. Several cysteine residues are conserved among roughly half of H-NOX domains from gammaproteobacteria (Fig. 2). The crystal structure of *So*-H-NOX revealed that these residues function to coordinate a zinc atom (Herzik et al., 2014) (Fig. 9). Furthermore, it was shown that *Vc*-H-NOX, which contains the conserved cysteines, binds stoichiometric zinc, while *Cs*-H-NOX, which does not contain the conserved cysteines, does not bind zinc (Herzik et al., 2014). When the predicted zinc-binding residues were mutated in *So*-H-NOX, the protein was insoluble, so it was originally concluded that the role of zinc is to maintain structural integrity.

More recently, however, it has been suggested that these cysteine residues may serve as redox-dependent mediators of signalling upon the formation and breakage of disulfide bonds (Mukhopadhyay et al., 2016). Using *Vc*-H-NOX and *Vc*-HaHK, it was demonstrated that *Vc*-HaHK activity, in addition to being inhibited by the Fe(II)-NO complex of *Vc*-H-NOX, is also inhibited by apo- (haem-free) *Vc*-H-NOX, but only when the conserved cysteines are oxidized. This redox inhibition of *Vc*-HaHK activity is attributed to disulfide bonds crosslinking *Vc*-H-NOX and *Vc*-HaHK. In this model, the purpose of zinc is to stabilize the thiolate groups until oxidizing conditions allow these to form disulfide bonds with *Vc*-HaHK.

Therefore, unlike other characterized simple H-NOX/HaHK signalling pathways (*S. oneidensis* and *P. atlantica*), *Vc*-H-NOX appears to have dual functionality and is able regulate to *Vc*-HaHK in both a haem-dependent (upon NO binding) and a haem-independent (upon cysteine oxidation) manner (Mukhopadhyay et al., 2016). Future studies need to be conducted, however, to determine if this dual functionality of *Vc*-H-NOX is physiologically relevant in regulating biofilm formation and pathogenicity in *V. cholerae*.

7. A Novel no Sensing Protein in Bacteria: Nosp

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 like 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 (Driscoll, Brody, & Kollef, 2007) and hospital-acquired infections (Castiglione et al., 2011), is well documented (Barraud et al., 2006, 2009), 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 (Borziak & Zhulin, 2007). NosP domains, like H-NOX domains, are predicted to be cocistronic or fused to MCPs, two-component signalling histidine kinases, and cyclic-di-GMP synthase and/or phosphodiesterase enzymes (Fig. 1). Interestingly, like the H-NOX-associated enzymes discussed earlier, these enzymes do not encode for sensory domains, suggesting NosP domains may function as such *in trans*.

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 (Hossain et al., unpublished data), confirming NosP as a bacterial NO sensor. *Pa*-NosP is cocistronic with a hybrid histidine kinase (that we have named NosP-associated histidine kinase; NaHK) that has been previously implicated in biofilm formation in this bacterial species (Hsu, Chen, Peng, & Chang, 2008). Using purified proteins, we have shown that *Pa*-NosP is a haemoprotein that binds to NO and CO, but does not form a stable ferrous-oxy complex (Hossain et al., unpublished data), consistent with the ligand-binding properties expected of a dedicated NO sensor. Additionally, preliminary results from rR spectroscopy of ferrous NosP from *P. aeruginosa* demonstrate that it is a histidine-ligated 6-coordinate low-spin complex, which upon binding NO, forms a 5-coordinate high-spin complex (Bacon et al., unpublished data). Furthermore, 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 $1.8 \times 10^{-4} \text{ s}^{-1}$ (Hossain et al., unpublished data). Moreover, we found that NO-bound NosP is able to regulate the phosphorelay activity of NaHK in *P. aeruginosa*. These data collectively suggest *Pa*-NosP is a NO-sensitive haemoprotein (Hossain et al., unpublished data).

Recently, our lab has also characterized a NosP signalling pathway in *S. oneidensis*. Like *Pa*-NosP, *So*-NosP is also a haemoprotein that binds NO and CO but does not form a stable complex with O₂ (Binnenkade and Nisbett et al., unpublished data). Additionally, *So*-NosP is predicted to be cocistronic with a histidine kinase known to be involved in the *S. oneidensis* NO-regulated c-di-GMP signalling network (Plate & Marletta, 2012). Using biofilm analysis of NosP and H-NOX mutants of *S. oneidensis*, we show 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 cocistronic histidine kinase.

8. Perspectives and Conclusions

The field of NO signalling in bacteria, including the identification and characterization of the targets of NO in bacteria, is rapidly expanding. In this review, we have presented a summary of the structural information available on select H-NOX domains, the ligand-binding properties of H-NOX domains, how NO/H-NOX domain interactions biochemically result in the regulation of downstream H-NOX-associated signalling pathways, and the discovery of novel NO-sensing mechanisms in bacteria that lack H-NOX domains.

It is now clear that H-NOX proteins, irrespective of whether they are from facultative or obligate anaerobes, are predicted to have approximately picomolar affinity for NO, and H-NOX-associated signalling pathways are governed at nanomolar concentrations of NO (Arora et al., 2015; Nisbett & Boon, 2016; Plate & Marletta, 2013). Several key structural features have been identified in NO-activation of H-NOX, including the dissociation of a proximal histidine ligand (Dai et al., 2012; Erbil et al., 2009; Herzik et al., 2014; Olea, Herzik, et al., 2010) and flattening of a haem cofactor (Dai & Boon, 2011; Erbil et al., 2009; Olea et al., 2008; Olea, Kuriyan et al., 2010; Sun et al., 2016) that is otherwise exceedingly distorted. Features specific to some H-NOX proteins have also been distinguished, including a hydrogen-bonding network that stabilizes O₂ binding in H-NOX from obligate anaerobes (Boon et al., 2005; Hespen et al., 2016) and a zinc-binding feature that may be connected to redox sensing in H-NOX domains from some gammaproteobacteria (Mukhopadhyay et al., 2016). This detailed understanding of the structure of H-NOX has, in fact, allowed for rational engineering of H-NOX domains to bind specific ligands (Dai & Boon, 2010; Olea, Kuriyan, et al., 2010; Weinert, Phillips-Piro, & Marletta, 2013) and for development of optimized drug agonists for sGC (Kumar et al., 2013; Martin et al., 2010; von Wantoch Rekowski et al., 2013).

Finally, although two NO-sensitive bacterial protein families have been identified, H-NOX and NosP, there are still many bacteria, many Gram-positive bacteria in particular, that are NO-responsive but do not code for either H-NOX or NosP domains in their genomes. Consequently, the identity of the putative primary NO sensor and NO-responsive signalling pathway(s), as well as the molecular mechanism of NO regulation in these bacteria, are all currently unknown.

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Abbreviations

ATP	adenosine triphosphate
<i>Cb</i>	<i>Clostridium botulinum</i>
CO	carbon monoxide
<i>Cs</i>	<i>Caldanaerobacter subterraneus</i>
Cyclic-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate

FIST	F-box intracellular signal transduction protein
FNR	fumarate and nitrate regulatory proteins
GMP	guanosine 5' monophosphate
GTP	guanosine triphosphate
HaCE	H-NOX-associated cyclic-di-GMP processing enzymes
HaHK	H-NOX-associated histidine kinase
HNOB	haem-nitric oxide binding domain
H-NOX	haem-nitric oxide/oxygen-binding domain
Hpt	histidine-containing phosphotransfer protein
HqsK	H-NOX-associated quorum sensing histidine kinase
<i>Lpg</i>	<i>Legionella pneumophila</i>
MCP	methyl-accepting chemotaxis protein
NaHK	NosP-associated histidine kinase
NCSD	normal-coordinate structural decomposition
NMR	nuclear magnetic resonance
NO	nitric oxide
NorR	regulator of NO reductase
NosP	nitric oxide sensing protein
<i>Ns</i>	<i>Nostoc</i> sp.
O₂	dioxygen
<i>Pa</i>	<i>Pseudomonas aeruginosa</i>
pGpG	5'-phosphoguanylyl-(3'-5')-guanosine
rR	resonance Raman
sGC	soluble guanylate cyclase
<i>Sili</i>	<i>Silicibacter</i> sp.
<i>So</i>	<i>Shewanella oneidensis</i>
<i>Sw</i>	<i>Shewanella woodyi</i>
<i>Tt</i>	<i>Thermoanaerobacter tengcongensis</i>
Vc	<i>Vibrio cholerae</i>

Vf

Vibrio fischeri

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Fig. 1.

H-NOX, the NO sensing domain from the animal NO sensor sGC, is conserved as a haemoprotein sensor in bacteria. In bacteria, it is found in operons with histidine kinases, cyclic-di-GMP synthases and/or phosphodiesterases, and methyl-accepting chemotaxis proteins (*left*). NosP (NO sensing protein), a newly discovered bacterial NO sensing protein has operon arrangements similar to those of H-NOX (*right*).

Fig. 2.

(A) Sequence alignment of H-NOX proteins discussed in this review. Of note are the conserved histidine haem ligand (His104 by rat $\beta 1$ sGC numbering), the YxSxR motif (Tyr135, Ser137, Arg139) that coordinates the haem propionate groups, and the Pro118 residue that maintains a distorted haem cofactor. (B) Multiple sequence alignments of selected gammaproteobacteria in which the zinc-binding cysteine residues are conserved.

Fig. 3.

(A) The structure of Fe(II)-NO *So*-H-NOX. In *So*-H-NOX, upon NO (*orange*) binding, the histidine proximal ligand (*blue*) dissociates. In fact, NO is bound to the proximal face of haem, a condition possibly brought about by the high NO concentrations that the protein is exposed to during crystallization (Herzik, Jonnalagadda, Kuriyan, & Marletta, 2014). The YxSxR (131, 133, and 135, respectively) motif (*magenta*) is shown coordinating haem propionate groups. The haem in this structure is more relaxed than the haem in the Fe(II)-unliganded state. These data support hypotheses that NO binding triggers Fe–His bond cleavage and haem relaxation that ultimately resulting in signal transduction. Generated in pymol from pdb file 4U9B. (B) The structure of Fe(II)-unligated *So*-H-NOX. Notable features include Pro116-induced (*red*) haem distortion and the bound water molecule coordinating residues His99, Ile118, Pro116, and the proximal haem ligand, His102.

Fig. 4.

The structure of Fe(II)-O₂ *Cs*-H-NOX. Oxygen (*red*) binds haem in a bent configuration and is coordinated by Tyr140 and its hydrogen-bonding (*yellow*) network, consisting of Trp9 and Asn74. His104, the proximal iron ligand (*blue*), remains bound upon O₂ binding, forming a 6-coordinate complex. The high level of haem distortion can be observed. Generated in PYMOL using PDB file 1U55.

Fig. 5.

NO regulates HaCE activity through ligation to H-NOX. (A) NO bound H-NOX only affects cyclic-di-GMP production in *Legionella pneumophila* as *Lpg*-HaCE is only functional as a cyclic-di-GMP synthase. (B) Fe(II)-NO bound H-NOX directly influences both the production and hydrolysis of cyclic-di-GMP in *Shewanella woodyi* and *Agrobacterium vitis*.

Fig. 6. NO/H-NOX regulates HaHK autophosphorylation activity and phosphate flow downstream of HaHK. *Shewanella oneidensis*, *Pseudoalteromonas atlantica*, and *Vibrio cholerae* all have similar H-NOX signalling pathways, but *V. cholerae* does not encode a homologous HTH domain-containing response regulator protein.

Fig. 7. NO/H-NOX regulates HqsK autophosphorylation activity and subsequently influences quorum sensing activity in *Vibrio harveyi* and *Vibrio parahaemolyticus*. In both *V. harveyi* and *V. parahaemolyticus*, Fe(II)-NO bound H-NOX inhibits *Vh*-HqsK autophosphorylation activity which leads to a change in phosphate flow to LuxU/LuxO and this ultimately influences quorum sensing in both bacteria.

Fig. 8.

NO/H-NOX regulates *Vfi*-HaHK and *Sili*-HaHK autophosphorylation activity and their symbiotic relationships with their respective eukaryotic hosts. (A) In *V. fischerii*, Fe(II)-NO bound H-NOX may inhibit *Vfi*-HaHK autophosphorylation activity which leads to a change in phosphate flow and symbiosis with *Euprymna scolopes*, the Hawaiian bobtail squid. (B) In *Silicibacter* sp. *TrichCH4B*, NO bound H-NOX has been shown to decrease *Sili*-HaHK autophosphorylation activity, decrease phosphate flow within the *Sili*-HaHK signalling pathway, and influence its symbiosis with *Trichodesmium erythraeum*, an algal symbiont.

Fig. 9.
So-H-NOX structure with zinc (*purple*) bound by cysteine residues (Cys139, Cys164, Cys172; *cyan*). Generated in pymol from pdb file 4U9B.