CHEMICAL REVIEWS

Review

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# Gaseous $O_2$, NO, and CO in Signal Transduction: Structure and Function Relationships of Heme-Based Gas Sensors and Heme-Redox Sensors

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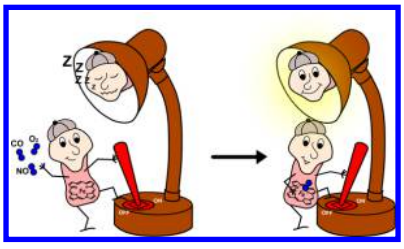

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## 1. INTRODUCTION

The heme iron complex is one of the most important cofactors in biological systems (Figure 1). The major functions of the heme iron complex are to serve as the $O_2$ binding site for hemoglobin ($O_2$ carrier protein), myoglobin ($O_2$ storage protein), and cytochrome P450 ($O_2$ activating enzyme); the peroxide activation site for peroxidase; and the electron transfer site for cytochromes.[1−8] The heme iron complex in well-known heme proteins acts as the functional active center by virtue of its binding to an $O_2$ molecule forming a heme iron−$O_2$ complex, which carries the $O_2$ molecule to peripheral tissues as hemoglobin, stores the $O_2$ molecule in muscle as myoglobin, and helps activate the $O_2$ molecule by cleaving the O−O bond in cytochrome P450 monooxygenase. Importantly, the $O_2$ molecule binds only to heme Fe(II) complexes and not to heme Fe(III) complexes. For cytochromes, electrons are transferred to/from the partner protein via the heme iron complex, whereas for peroxidases, the active heme iron species required for function, compound I and compound II, are formed by hydrogen peroxide, reinforcing the idea that the heme iron complex is the important active center for these heme proteins.[6]

The heme iron complex also functions as the binding site for toxic gaseous molecules, such as NO and CO. The binding of these non-$O_2$ gaseous molecules to the heme iron complex regulates various important physiological and pathological functions associated with $O_2$ binding, such as mitochondrial respiration, primarily inhibiting these functions.[9−22]

The heme iron complex of hemoglobin and myoglobin also functions to eliminate the toxicity of NO[23,24] and generate NO from nitrate and nitrite anions.[25−27] In contrast to $O_2$ and CO, which bind exclusively to the heme Fe(II) complex, the NO molecule binds to both the heme Fe(II) complex and the heme Fe(III) complex. Intriguingly, peroxynitrite, the reactive nitrogen species formed by NO and hydrogen peroxide under various pathological conditions, has various functional impacts on NO.[28,29] In addition to interacting with the heme iron complex, NO binds to cysteine thiolate and forms −SNO on the protein surface, thereby regulating numerous important functions;[30−32] it also binds to nonheme iron complexes.[33]

In contrast to NO and $O_2$ (as well as reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical), CO is stable and inert, and thus binds exclusively to metal complexes, including heme iron and nonheme iron complexes, to inhibit their function, but it does not interact with nonmetal cofactors or amino acid residues on the protein surface.[13,16−19]

Noteworthy among the important novel and emergent physiological functions of the heme iron complex is its role as a gas-sensing site (Tables 1−3). Most heme-based gas sensors are composed of an N-terminal heme iron-bound gas-sensing domain and a C-terminal functional domain (Figure 2).[7,9,11,34−55] The ubiquitous and physiologically important gaseous molecule, $O_2$, plays an additional role as the signaling molecule for heme-based oxygen sensors. The notorious toxic gaseous molecules, NO and CO, have also come to be known as signaling molecules that regulate numerous important physiological functions. For heme-based gas sensors, a gaseous molecule, the first signal, associates with (or dissociates from) the heme iron complex in the N-terminal sensing domain, causing changes in the heme iron coordination structure that lead to concomitant changes in the surrounding protein. These changes in the protein structure within the sensing domain constitute the second signal, which is transduced to the functional domain, resulting in switching on/off various crucial physiological functions, such as catalytic activity (e.g., phosphodiesterase [PDE], diguanylate cyclase [DGC]), methyl-accepting chemotaxis, or transcription, which are mediated by the C-terminal functional domain (Figure 2). Many bacterial NO sensors are stand-alone heme-based NO sensors, in that the isolated NO sensing domain interacts with the isolated/separated functional domain and switches its functions on and off (Table 2).[56] In some gas sensors, the heme-bound gas-sensing domain is located in the central region between the N- and C-terminal domains.[57] Numerous heme-based gas sensors have been identified in archaea and bacteria, whereas among eukaryotes, the only heme-based NO sensor that has been reported is soluble guanylate cyclase (sGC).[36] The mechanisms by which the heme iron complex in heme-based gas sensors discriminates among these gaseous ligand molecules have been discussed.[47,48,55,58,59] Notably, the structure of the iron−sulfur cluster in gas-sensing nonheme iron proteins such FNR (fumarate and nitrate reduction regulator), which senses both $O_2$ and NO, is strikingly changed after these gaseous molecules bind to the cluster. Thus, the gas-sensing mechanisms of heme-based gas sensors, in which the heme iron complex structure is essentially retained after sensing the gaseous molecules, are substantially different from those of nonheme iron proteins.[33]

The emergent, novel role of gas sensing in various important physiological functions in bacteria and eukaryotes, as well as their involvement in disease processes, provides a compelling and timely rationale for presenting a summary of structure and function relationships of heme-based gas sensors. In this Review, we comprehensively summarize current knowledge of heme-based gas ($O_2$, NO, and CO) sensors and heme-redox sensors and delineate the structure/function relationships and common characteristics of the bacterial, archaeal, and mammalian sensors. We try to cite mainly new papers published after the year 2000 rather than citing numerous classical papers (even well-known ones). In this way, we are able to present many of the most recent papers.

## 2. HEME-BASED $O_2$ SENSORS

The $O_2$ molecule is the most important gaseous molecule for metabolism and the mitochondrial energy transfer system, playing a conserved role in nearly all organisms. Notably, additional important signaling roles of $O_2$ in bacterial and archaeal physiology are also emerging. Among all the gas sensors, the heme-based oxygen sensors of bacterial or archaeal origin are the best characterized (Table 1).[34,35,38−46,50,53−55]

### 2.1. Heme-Bound PAS Domain-Containing $O_2$ Sensors

The PAS (Per, ARNT, and Sim proteins) domain is characterized by its three-dimensional structure in which several parallel $\beta$-sheets are aligned and an $\alpha$-helix crosses


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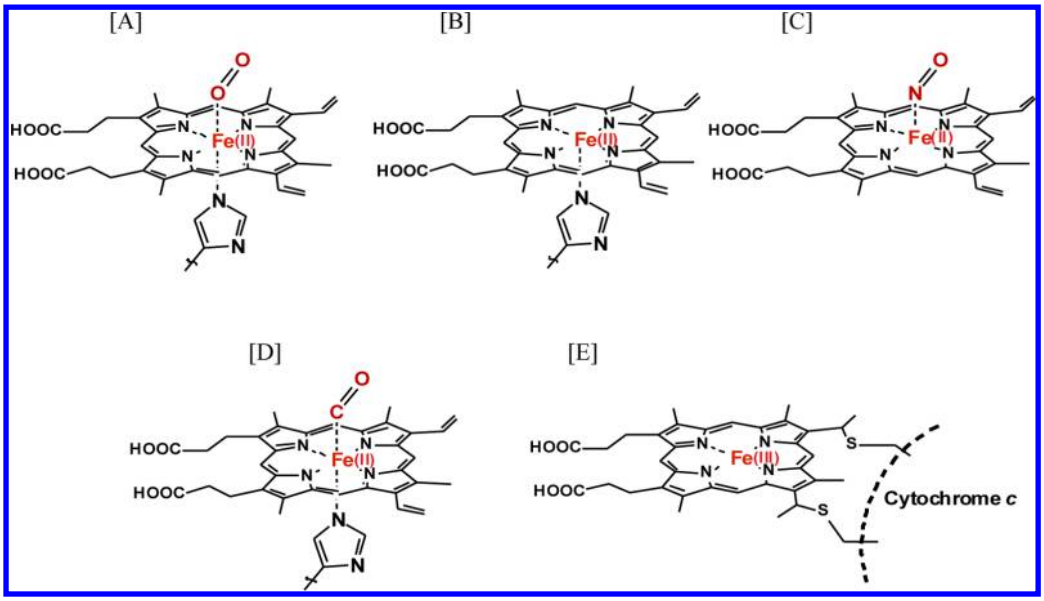


**Figure 1.** Heme iron/protoporphyrin IX iron complex. In hemoglobin, myoglobin, and many heme-based oxygen sensors, the heme Fe(II) complex is ionically bound to the imidazole of a histidine residue on the proximal side trans to the $O_2$-binding distal side [A].[1,6,8,50,51,81−83,85,190] The active form of FixL, a heme-based oxygen sensor containing a heme-bound PAS domain, is the $O_2$-free 5-coordinated Fe(II) complex [B],[41−43,66] whereas that of *Af*GcHK, a heme-based oxygen sensor containing a heme-bound globin domain, is the $O_2$-bound 6-coordinated Fe(II) complex [A].[73] For the heme-based NO sensor guanylate cyclase, the 5-coordinated Fe(II)-NO complex is the catalytically active form [C].[9,36] For the heme-based CO sensor CooA, the 6-coodinated Fe(II)-CO complex is the transcriptionally active form [D].[34,35,52,296] Cytochrome *c* contains a *c*-type heme in which the porphyrin ring is covalently bound to a cysteine residue of the protein [E].

from one end to the other, forming a shape similar to that of a fruit basket (Figure 3A).[49,51] The PAS domain often serves as the signaling site for protein−protein interactions as well as a signaling-accepting site for inter- and intramolecular signal transduction.[49,60−65] The PAS domain in heme-based $O_2$ sensors functions as the $O_2$ sensing/binding site, which is important for intramolecular signal transduction (Table 1).[35,41−43,50,53,55,60]

**2.1.1. FixL.** FixL is a heme protein kinase involved in nitrogen fixation. The discovery of FixL from *Rhizobium meliloti* (or *Sinorhizobium meliloti*), the first identified heme-based oxygen sensor, substantially changed our view of the role of the heme iron complex, showing that it can be an oxygen-sensing site for regulating histidine kinase function.[41−43,66] The structure−function relationships of FixLs from *R. meliloti* (*Rm*FixL), *S. meliloti* (*Sm*FixL), and *Bradyrhizobium japonicum* (*Bj*FixL) have been well characterized, partly because FixLs are suitable for overexpressing in *Escherichia coli* and can be readily purified as stable proteins.

*2.1.1.1. Two-Component Signal Transduction Systems.* FixL is an example of a two-component signal transduction system in which autophosphorylation at a histidine residue in the functional domain within the same FixL molecule is triggered by input from the external first signal and the phosphorylated group is then transferred to an aspartate residue in the response regulator (Figure 4).[67,68] Once this aspartate residue is phosphorylated, the output signal is manifested and transcription is switched on, resulting in enhanced nitrogen fixation in the plant root bacteria.[42,43,66] In its heme Fe(II)−$O_2$ complex form, FixL is inactive. Dissociation of the $O_2$ molecule from the heme Fe(II) complex switches on phosphorylation of the histidine residue. This oxygen-sensing system, in which $O_2$ dissociation acts as an "on" switch, is in contrast with other heme-based oxygen sensors such as *Ec*DOS and globin-coupled oxygen sensors, in which $O_2$ binding to the heme Fe(II) complex switches on their catalytic functions.[38,39,42,43,50] It has been proposed that the sensory/signaling module of the FixL network has been conserved such that its transcriptional output is adapted to the unique physiology of *Caulobacter crescentus* and nitrogen-fixing rhizobia.[69]

Note that there are additional types of two-component bacterial signal transduction systems (described in detail below), including (a) other heme-based oxygen sensors, such as DosS (DevS)/DosR and DosT/DosR from *Mycobacterium tuberculosis*,[70−72] and *Af*GcHK from *Anaeromyxobacter* sp. Fw109-5;[73] (b) stand-alone heme-based NO sensors, such as Hnox from *Shewanella oneidensis* (*So*H-NOX), *Vibrio cholera* (*Vc*H-NOX), *Vibrio harveyi* (*Vh*H-NOX), and *Vibrio fischeri* (*Vf*H-NOX);[56] and (c) the heme-redox sensor NtrY/NtrX from *Brucella abortus*.[74]

*2.1.1.2. Catalytic Regulation.* FixL with a heme Fe(II)−$O_2$ complex is inactive, whereas that with the $O_2$-free heme Fe(II) complex is active. FixL with a heme Fe(II)−CO or heme Fe(II)−NO complex is also inactive. In the absence of FixJ, a cognate response regulator, auto histidine kinase (autophosphorylation) activity of FixL is very low, but it becomes high in the presence of this partner protein.[42,43,75−78] However, this partner-protein-induced catalytic enhancement is not as obvious for the two-component signal transduction system *Af*GcHK, a globin-coupled, heme-based oxygen-sensing histidine kinase from soil bacteria.[73] Notably, turnover numbers for FixL, as well as those for other heme-based oxygen sensors and signaling enzymes, are relatively low as compared to those for metabolic enzymes. This is because, unlike the case for metabolic enzymes, the natural concentrations of their substrates (e.g., proteins to be phosphorylated) are usually comparable to the concentrations of the enzyme.[42,43] For signaling enzymes, the effective concentration of product


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depends on downstream signal transduction steps; thus, a vast excess of substrate is often unnecessary.[42,43]

A study of the role of FixL−FixJ complexation in catalytic regulation[78] showed that *Sm*FixL−*Sm*FixJ complexation precedes phosphorylation for a two-component regulatory system. An investigation of the effect of $O_2$ on formation of the *Rm*FixL−*Rm*FixJ complex by fluorescence polarization[77] further found that $O_2$ blocks the reaction of the FixL−FixJ complex with ATP, but does not influence binding of FixJ or ATP to FixL. These results suggest mechanistic roles for the signal and regulatory partner in this signal transduction system. The novel concept, termed "memory of oxygen binding", was introduced to explain the $O_2$ dose response of FixL.[79] A crucial role for the autophosphorylation domain of FixL in regulating coupling between $O_2$ binding and kinase activity has been suggested.[76] It has also been suggested that $\alpha$4 and $\alpha$5 helices not only serve as a dimerization interface, but are also involved in transferring the postphosphorylation/dimerization signal that unveils the DNA-binding activity of the C-terminal domain. It has been found that ADP reduces the $O_2$-binding affinity of FixL, suggesting the possibility of reciprocal enhancement of the kinase reaction.[80]

**Table 1. Functions of Heme-Based $O_2$ Sensors[a]**

| | functions | refs |
|---|---|---|
| Heme-Bound PAS Domain | | |
| FixL | HK, TCS with FixJ | 66, 84, 107 |
| *Ec*DOS (or *Ec*DosP) | PDE | 117, 119, 121 |
| *Ax*PDEA1 | PDE | 131 |
| Aer2 | MCP | 165, 167 |
| Gyc-88E | DGC | 168 |
| ThkA | HK, TCS with TrrA | 169, 170 |
| Heme-Bound GAF Domain | | |
| DosS (or DevS) | HK, TCS with DosR | 70−72, 172, 176 |
| DosT | HK, TCS with DosR | 71, 72, 174 |
| Heme-Bound Globin Domain: GCS | | |
| HemAT | MCP | 188, 189, 192, 195 |
| YddV (or *Ec*DosC) | DGC | 115, 116 |
| *Af*GcHK | HK, TCS with RR | 73 |
| HemDGC | DGC | 200 |
| *Av*GReg | putative DGC | 201 |
| *Bpe*GReg | DGC | 202 |
| *Gs*GCS | unknown | 203 |
| HemAC-*Lm* | adenylate cyclase | 132, 133 |
| Heme-Bound SCHIC Fold at the Central Region | | |
| AppA | transcriptional regulation with PpsR | 57 |

[a]HK, histidine kinase; TCS, two-component system; PDE, phosphodiesterase; MCP, methyl-accepting chemotaxis; DGC, diguanylate cyclase; SCHIC, sensor containing heme instead of cobalamin.

*2.1.1.3. Ligand-Induced Global Protein Structural Changes Revealed by X-ray Crystal Structure and EXAFS (Extended X-ray Absorption Fine Structure) Studies.* Similar to other heme-based oxygen sensors, the heme-binding domain is separated from the functional domain in FixL. The molecular mechanism underlying transduction of an intramolecular signal from the heme-binding domain to the functional domain is intriguing and thus worthy of exploration. Although only the crystal structure of the isolated heme-bound domain has been determined, efforts to resolve the crystal structure of the entire protein have not yet succeeded. Nevertheless, the crystal structure of the isolated heme-bound domain of FixL provides insight into the molecular mechanism of the oxygen-sensing system.[47,81−84]

**Table 2. Functions of Heme-Based NO Sensors[a]**

| | functions | refs |
|---|---|---|
| Mammalian | | |
| sGC | guanylate cyclase | 9, 36, 232, 233 |
| Insect | | |
| E75 | transcriptional regulator | 227, 375, 377 |
| Bacterial: Stand-Alone Type | | |
| *Tt*H-NOX, TT-SONO | putative MCP: oxygen sensor? | 245, 246 |
| CB-SONO | putative MCP: oxygen sensor? | 245 |
| *Ns*H-NOX | putative guanylate cyclase | 243, 244, 263, 265 |
| *So*H-NOX | MCS: HnoX/HnoK/HnoD/PDE: biofilm | 242, 266−268, 272 |
| | MCS: HnoX/HnoK/HnoC: transcription | |
| *Vc*H-NOX | MCS: HnoX/HnoK/HnoD/PDE: biofilm | 272, 273 |
| | MCS: HnoX/HnoK/HnoC: transcription | |
| *Sw*H-NOX | HnoX/DGC/PDE: biofilm | 274, 275 |
| *Pa*H-NOX | TCS: H-NOX/HarK/HarR/PDE: biofilm | 277 |
| *Vf*H-NOX | TCS: HnoX/HnoK/Hpt?/Hur? heme uptake | 278 |
| *Vh*H-NOX | TCS: HnoX/HqaK/LuxU: quorum sensing | 279, 280 |
| Bacterial: Fused Type | | |
| DNR | transcriptional regulator | 282−284 |
| *Bs*YybT | PDE | 285 |
| *Gt*YybT | PDE | 285, 286 |

[a]*Bs*YybT, YybT PDE with a heme-bound PAS domain from *Bacillus subtilis*; CB-SONO, sensor of NO from *Clostridium botulinum*; DNR, dissimilative nitrate respiration regulator NO sensor from *Pseudomonas aeruginosa*; E75, nuclear receptor from *Oncopeltus fasciatus*; *Gt*YybT, YybT PDE with a heme-bound PAS domain from *Geobacillus thermodenitrificans*; MCS, multicomponent system; *Ns*H-NOX, H-NOX from *Nostoc punctiforme*; *Pa*H-NOX, H-NOX from *Pseudoalteromonas atlantica*; sGC, soluble guanylate cyclase; *So*H-NOX, H-NOX or NnoX from *Shewanella oneidensis*; *Sw*H-NOX, H-NOX from *Shewanella woodyi*; TCS, two-component system; *Tt*H-NOX, H-NOX from *Thermoanaerobacter tengcongensis*; TT-SONO, sensor of NO from *Thermoanaerobacter tengcongensis*; *Vc*H-NOX, H-NOX from *Vibrio cholera*; *Vf*H-NOX, H-NOX or HnoX from *Vibrio fischeri*; *Vh*H-NOX, H-NOX from *Vibrio harveyi*.

Global protein structural changes at the heme distal side are caused by association/dissociation of external axial ligands, including $O_2$, at the heme iron complex (Figure 5A).[81−83] Specifically, binding of $O_2$ to Arg220 in the heme Fe(II) complex in FixL results in flattening of the heme porphyrin and a shift of a critical loop, the FG loop, away from the heme pocket (Figure 6).[83] This type of profound protein structural change is unprecedented and had not been reported for prototypical heme proteins. The global changes in protein structure caused by $O_2$ are able to fully account for intramolecular signal transduction from the heme-bound domain to the functional domain. Similar global protein structural changes are induced by heme redox changes in *Ec*DOS, another heme-based oxygen sensor with the heme-bound PAS fold (Figure 5B).[51,85]



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Table 3. Functions of Heme-Based CO Sensors[a]

| | functions | refs |
|---|---|---|
| Bacterial | | |
| *Rr*CooA | transcription regulator | 52, 296, 314, 317 |
| *Ch*CooA | transcription regulator | 325, 332, 334 |
| $RcoM_{Bx}$-1 and $RcoM_{Bx}$-2 | transcription regulator | 294, 338, 339, 341 |
| Mammalian | | |
| CBS | cystathionine, $H_2S$, thiol ester synthesis | 146, 147, 287, 289, 291 |
| Slo1/BK | $K^+$ channel | 351, 352, 354, 355, 361 |
| Kv2.1 | $K^+$ channel | 357 |
| Nav1.5 | $Na^+$ channel | 358 |
| connexin hemichannel | ATP, glutathione, $Ca^{2+}$ channel | 359 |
| NPAS2 | transcription regulator: circadian rhythms | 363, 364, 366−368 |
| CLOCK | transcription regulator: circadian rhythms | 369, 370 |
| Per1 | transcription regulator: circadian rhythms | 378 |
| Per2 | transcription regulator: circadian rhythms | 380, 382, 384 |
| Rev-erb$\alpha$ | transcription regulator: circadian rhythms | 371, 373 |
| Rev-erb$\beta$ | transcription regulator: circadian rhythms | 371, 374−376 |
| Insect | | |
| E75 | transcription regulator | 227, 375, 377 |

[a]CBS, mammalian cystathionine $\beta$-synthase; *Ch*CooA, CooA from *Carboxydothermus hydrogenoformans*; $RcoM_{Bx}$-1 and $RcoM_{Bx}$-2, RcoMs from *Burkholderia xenonorans*; *Rr*CooA, CooA from *Rhodospirillum rubrum*; Slo1/BK, large-conductance $Ca^{2+}$- and voltage-activated $K^+$ channel.

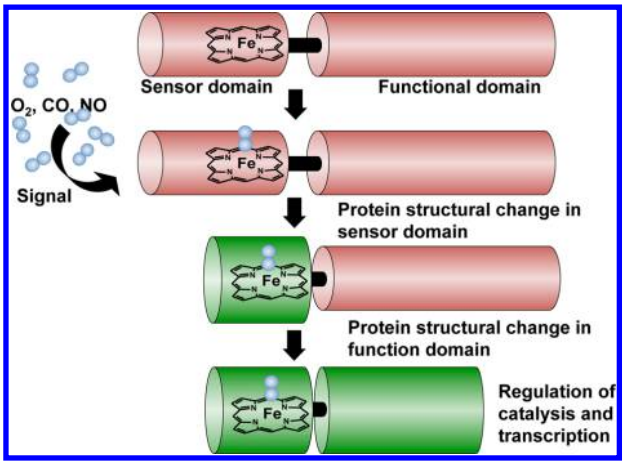


**Figure 2.** Heme-based gas sensor. The sensor is composed of an N-terminal heme-bound gas-sensing domain and a C-terminal functional domain.[41−43,50,66] For gas sensors, a gaseous molecule is the first signal. Binding of a gaseous molecule to the heme Fe(II) complex in the N-terminal sensing domain induces a structural change in the protein. This change in protein structure represents the second signal. This signal is transduced to the functional domain, switching on/off function in response to the association/dissociation of the gaseous molecule. For the heme-based oxygen sensors FixL and *Ax*PDEA1, dissociation of the $O_2$ molecule from the heme Fe(II) complex initiates the function, whereas for *Ec*DOS (or *Ec*DosP) and globin-coupled oxygen sensors, function is switched on by binding of the $O_2$ molecule to the heme Fe(II) complex.[50] For all heme-based NO and CO sensors reported to date, binding of gaseous molecules to the heme Fe(II) complex triggers the function.

The FG loop has been proposed to play an important role in domain−domain interactions.[84,86] Interactions between heme propionates and arginine side chains of the protein that occur upon $O_2$ binding[81,82,86] have been suggested as an explanation for intramolecular signal transduction from the heme-bound domain to the histidine kinase domain. Time-resolved crystallographic studies suggest that ligand (CO) binding causes structural changes in the FG loop and in the $\beta$-sheet distal to the heme, which are coupled to the movement of the main chain atoms of Leu236 in the I strand.[87] Conformational changes were also apparent at propionate 6 and in the FG loop residues Ile215 to Ile218. The sensing mechanism was proposed such that successive reactions would take place, including doming of the heme and subsequent propionate 6 movement and ligand shape sensed by steric clash of Leu236 with the bound ligand.[88]

Differences in the environment surrounding the heme structure between FixLs from *B. japonicum* (*Bj*FixL) and *R. meliloti* (*Rm*FixL) have been proposed on the basis of Fe K-edge EXAFS studies, crystal structures, and resonance Raman (RR) spectroscopic findings.[84,89] It was suggested that profound conformational changes in the FG loop are induced by steric repulsion between the bent, bound $O_2$ and Ile209 side chain, and that these changes are transmitted to the histidine kinase domain. The same research group also found a correlation between the heme-to-nitrogen (proximal His194) distance in the heme domain and the phosphorylation activity of the kinase domain.[89]

Heme redox-dependent substitution of the axial ligands of FixL was not observed, in contrast to *Ec*DOS, another PAS oxygen sensor, in which the axial ligands, hydroxide anion and histidine imidazole, of the heme Fe(III) complex are converted to methionine thiolate and histidine imidazole upon heme reduction to the heme Fe(II) complex.[51]

The Arg220 residue in the heme distal side is characteristic and important for $O_2$ binding (Figures 5A and 6[1]).[81−83] This is in contrast to hemoglobin and myoglobin, where a His residue in the distal side is important for $O_2$ binding and helps stabilize the heme Fe(II)−$O_2$ complex and thus should be beneficial for $O_2$ binding or storage.[1,6,8] This is also unlike P450, in which the threonine residue of the $O_2$-interacting site of the heme Fe(II)−$O_2$ complex serves to help activate the $O_2$ molecule or cleave the O−O bond of the heme Fe(II) complex-bound $O_2$ molecule with the aid of the cysteine residue of the proximal axial ligand by pushing electrons to the $O_2$ molecule.[2−6] Arg220 is reported to be involved in $O_2$-coupled structural changes[90] involved in caging $O_2$ near the heme[91−94] and in interactions with heme propionate 7.[95] Furthermore, the involvement of Arg226 in the formation of salt bridges[96] and contributions of Arg200, Arg208, Ile209, Ile210, and Arg214 to signal transduction between the heme and the kinase domain[86,97] have been proposed.

The importance of the heme proximal Arg206 in signal transduction has also been emphasized.[98] Crystal structure and oxygen kinetic data for an Arg206Ala mutant of *Bj*FixL suggest that the interaction between Arg206 and the heme propionate is important for the planarity of the porphyrin ring, tension of His214, and, ultimately, transmission during signal transduction.


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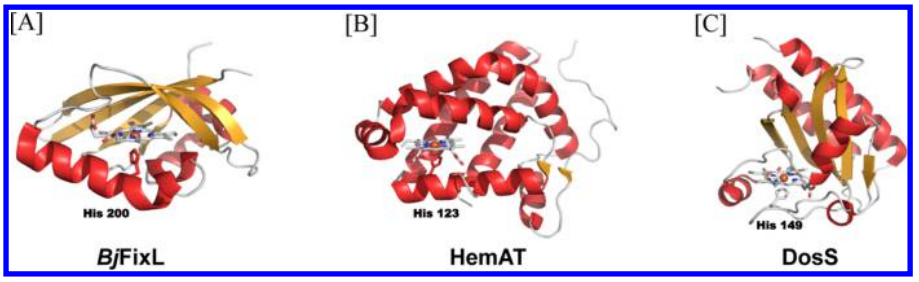


**Figure 3.** Typical protein structures of the heme-bound PAS [A],[81,82] globin [B],[190] and GAF [C][174] domains. The heme iron complex is bound to these domains, forming the gas-sensing site. FixL, *Ec*DOS, *Ax*PDEA1, and Aer2 are heme-based oxygen sensors containing a heme-bound PAS domain.

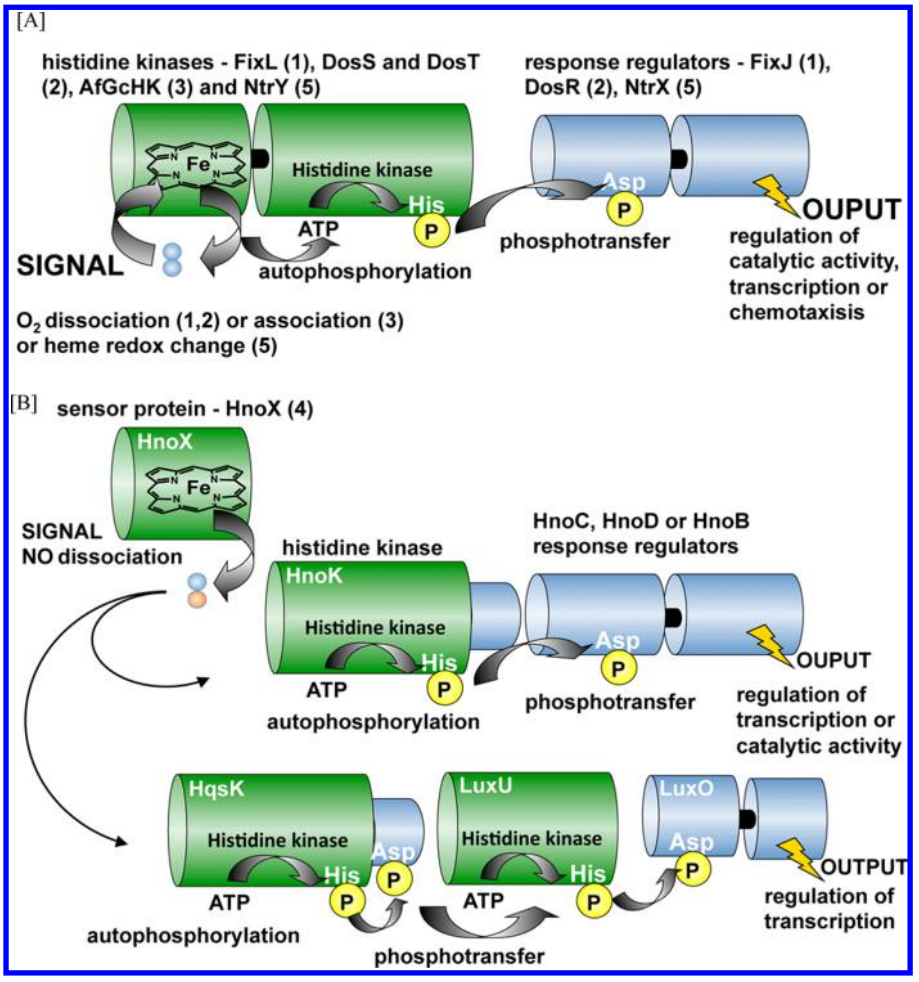


**Figure 4.** Bacterial two-component system of [A] fused type and [B] stand-alone type. Physical stimuli, such as light illumination or oxygen association/dissociation, interact with the sensing site of a signal-sensing protein, initiating an autophosphorylation reaction at a histidine residue in the functional domain. The self-added phosphate group is ultimately transferred to an aspartate residue of the response regulator, triggering a functional response, such as DNA binding and initiation of transcription of an important protein.[67,68] (1) FixL, a signal-sensing protein containing a heme-bound PAS domain, adopts a heme Fe(II)−$O_2$ complex form under resting conditions. Dissociation of $O_2$ from the heme Fe(II) complex triggers the autophosphorylation reaction and subsequent phosphotransfer reaction to FixJ, a response regulator.[41−43,66] (2) DosS and DosT are signaling proteins containing a heme-bound GAF domain.[70−72,171] Their response regulator is DosR. Similar to FixL, $O_2$ dissociation from the heme Fe(II) complex drives the autophosphorylation and phosphotransfer reaction to DosR. (3) *Af*GcHK is a signal-sensing protein containing a heme-bound globin domain.[73] Unlike the case for FixL and DosS/DosT, $O_2$ association with the heme Fe(II) complex significantly enhances autophosphorylation and phosphotransfer reactions to the response regulator. (4) HnoX is a stand-alone heme-based NO sensor.[56] NO association with or dissociation from the heme Fe(II) complex in HnoX triggers phosphorylation of HnoK or HqsK, which are not covalently bound to HnoX. The phosphotransfer reaction occurs from HnoK or HqsK to a response regulator or to another site within the same protein. Note that HnoK/HahK of *Pseudoalteromonas atlantica*,[277] HnoX/HnoK of *V. fischeri*,[278] and HnoX/HqsK of *V. harveyi*[279] also regulate NO-associated histidine kinase in a two-component signal transduction system. (5) NtrY is a heme redox sensor.[74] The heme Fe(II) complex form of NtrY autophosphorylates a histidine kinase domain at its C-terminus and stimulates phosphotransfer to NtrX, the response regulator, to control gene expression, whereas the heme Fe(III) complex form of NtrY does not.



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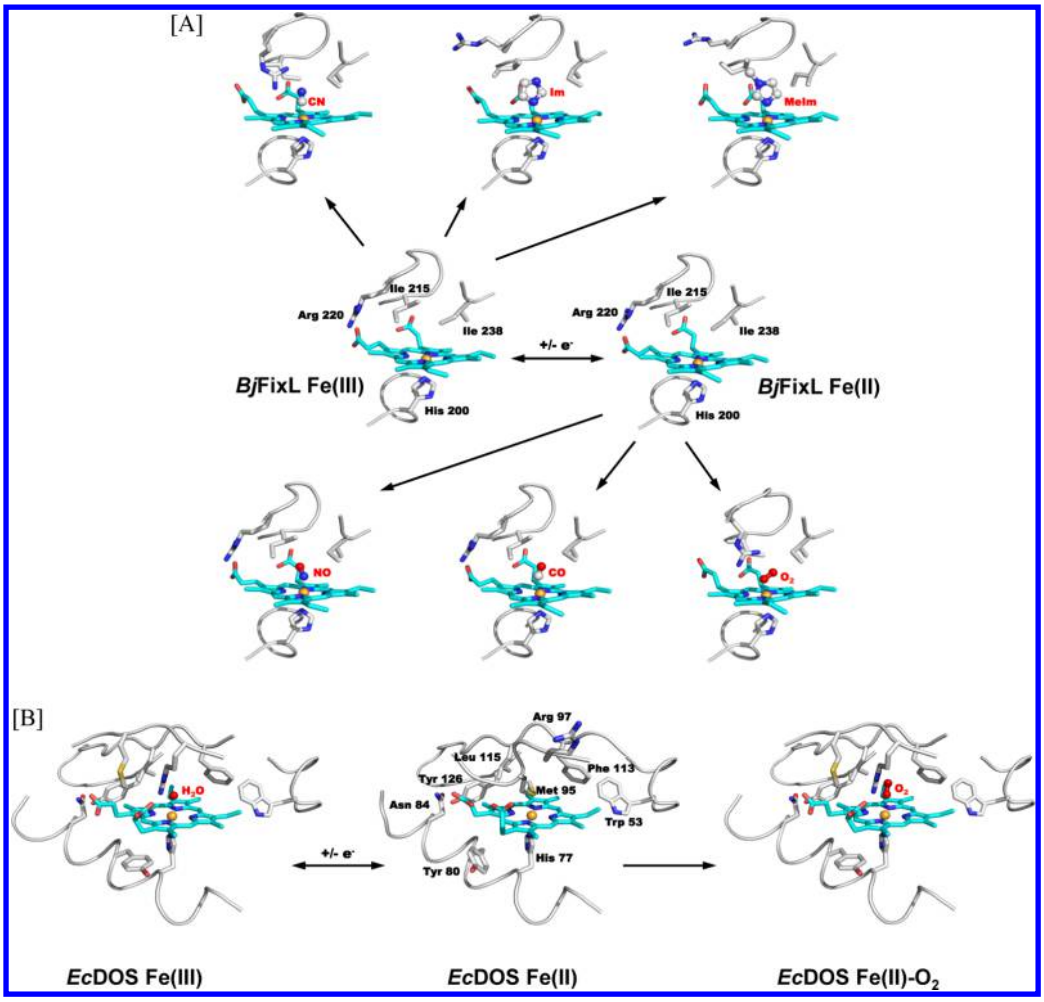


**Figure 5.** Ligand binding to the heme iron complex in *Bj*FixL induces global changes in protein structure [A][81−83] and in *Ec*DOS (or *Ec*DosP) [B].[51,85] The protein structure of the heme Fe(III) complex form of *Bj*FixL is virtually identical to that of the heme Fe(II) complex form. Binding of an external $CN^-$ to the heme Fe(III) complex induces profound changes in protein structure involving both the FG loop and the heme axial ligands. The heme distal structure of inactive complexes, such as heme Fe(III)−CN, Fe(III)−imidazole, and Fe(II)−$O_2$, differs substantially from that of active complexes, such as heme Fe(III), Fe(II), and Fe(II)−CO. Unlike the case for *Bj*FixL, the protein structure of the heme Fe(III) complex in *Ec*DOS is substantially different from that of the heme Fe(II) complex. Here, $O_2$-binding to the heme Fe(II) complex induces changes in the protein structure of the distal side such that Arg97 interacts with $O_2$ bound to the heme Fe(II) complex. Amino acid residues that were discussed in the ultraviolet and time-resolved RR spectra of *Ec*DOS are shown in [B].[136,137,142,143]

Reduction of the heme Fe(III) complex by dithiothreitol was shown to be more effective in catalytic regulation by $O_2$ than reduction by treatment with sodium dithionite.[99] This suggests a significant role for a free cysteine thiol, or a relevant reduced state, in the catalysis of FixL.

Several recombinant, isolated, heme-bound domains of *Bj*FixL have been characterized by MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) mass spectrometry.[100] These studies included an assessment of the temporal mass stabilities of these domains, which revealed that as much as ∼25% of the starting mass was not observed, a finding that could explain the "missing" terminal amino acids in published crystal structures. This phenomenon could be attributable to sample sterilization techniques and protease inhibitors usage, primary sequence variations, the presence/absence of a heme Fe(III) complex ligand, and the presence/absence of $O_2$.

*2.1.1.4. $O_2$ Association/Dissociation Kinetics.* Extensive studies have been conducted to examine the $O_2$ binding properties of FixL. Interestingly, these studies have shown that the equilibrium dissociation constant ($K_d$ value) of $O_2$ from the heme Fe(II) complex in FixL, estimated at 29−140 $\mu$M, is unusually high as compared to that for other heme proteins.[66,81,98] By comparison, the well-known heme protein, myoglobin, has a $K_d$ value of 0.88 $\mu$M.[50,101,102] The important role of the arginine residue in $O_2$ binding to FixL has been well characterized. The kinetic parameters for $O_2$ and CO binding to the heme Fe(II) complex of FixL are significantly changed by mutations at Arg220, the distal $O_2$ binding site, as well as by mutations at other hydrophobic amino acids and arginine residues that interact with the heme on the proximal side and with the heme propionate.[93,95,98,103] Different laboratories have reported different values for some kinetic parameters. Methods for determining these values appear critical for obtaining solid and reliable results.

A comparison of the mechanism of FixL autoxidation with that of *Aplysia* myoglobin[104] suggested a role for water interaction with the heme Fe(II) complex in determining the autoxidation rate of FixL, based on the dependence of this rate on $O_2$ concentration.



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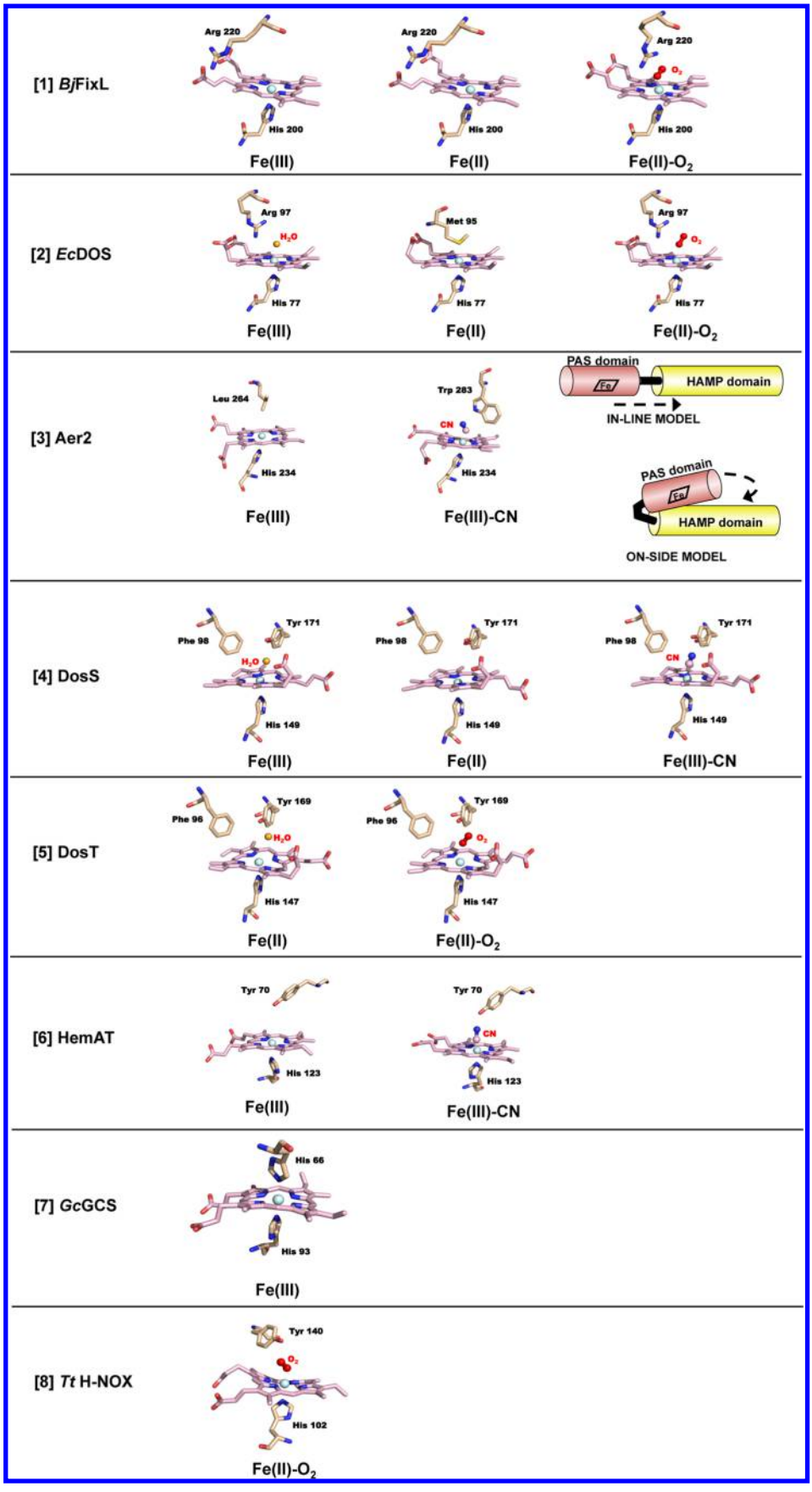


**Figure 6.** continued



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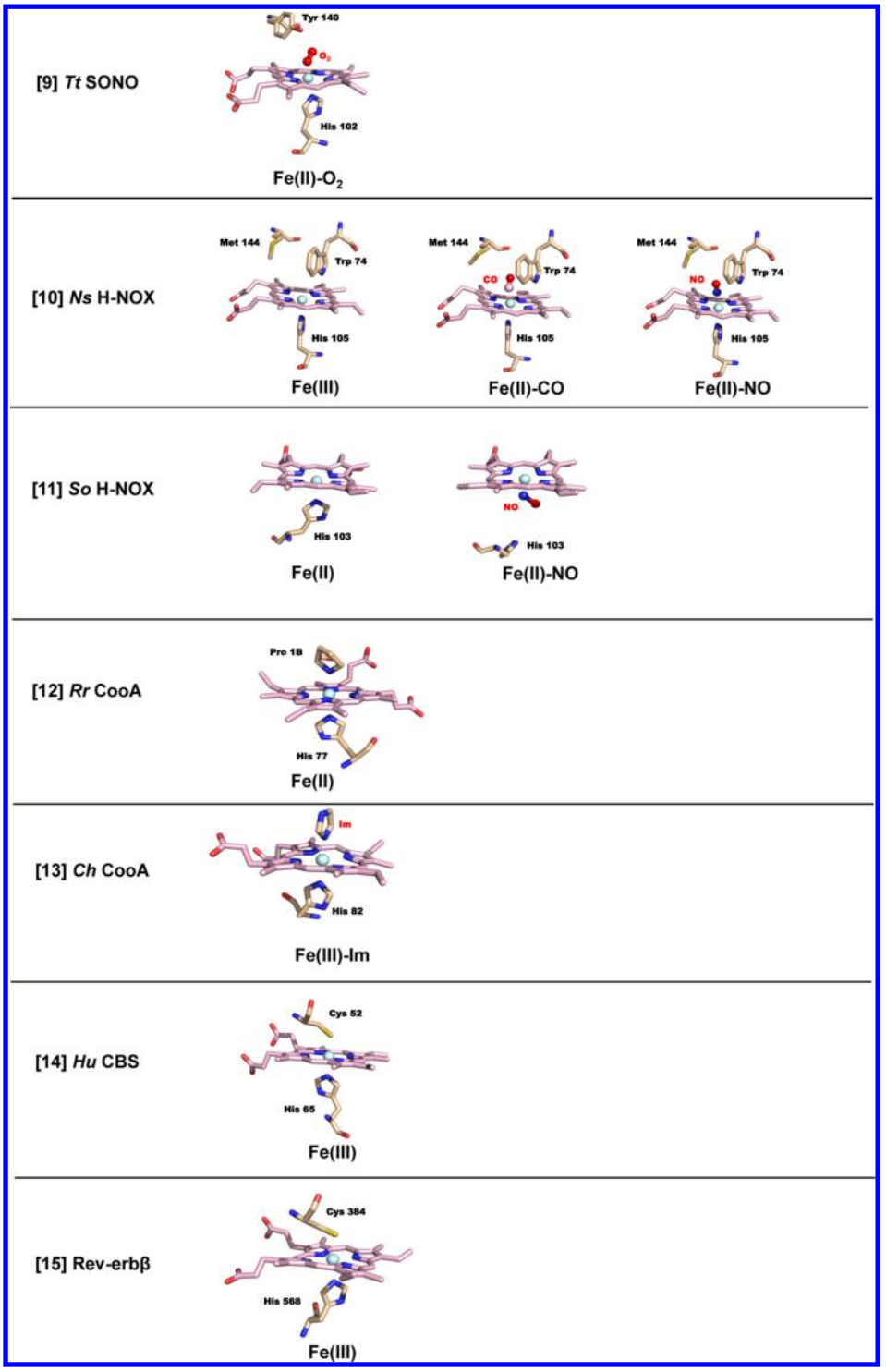


**Figure 6.** Heme-surrounding structures of heme Fe(III), Fe(II), and their ligand-bound complexes in heme-based gas sensors determined by X-ray crystallography. $O_2$ sensors: PAS fold, [1] FixL,[83] [2] *Ec*DOS (or *Ec*DosP),[51,85] and [3] Aer2;[165,167] GAF fold, [4] DosS (or DevS)[172] and [5] DosT;[174] globin fold, [6] HemAT[190] and [7] *Gs*GCS.[203] The in-line and on-side models of Aer2 are shown in [3]. NO sensors: [8] H-NOX from *T. tengcongensis*,[246] [9] SONO from *T. tengcongensis*,[245] [10] H-NOX from *Nostoc* sp.,[243,264] and [11] H-NOX from *S. oneidensis*.[242] CO sensors: [12] *Rr*CooA from *R. rubrum*,[312] [13] *Ch*CooA from *C. hydrogenoformans*,[333] and human [14] CBS[344,348,400] and [15] Rev-erb$\beta$.[376]

Photodissociation of $O_2$ from the heme Fe(II) complex of *Sm*FixL has been studied by time-resolved RR spectroscopy.[90] On the basis of a comparison of the three steps in the dynamic structural changes that result from $O_2$ dissociation, the authors of this study proposed an FG loop switching mechanism to explain intramolecular signal transduction in this enzyme, in that the interactions of the heme propionates with the distal residues, including the FG loop region, change after $O_2$ dissociation.

*2.1.1.5. CO Complex.* Although CO is not a physiological axial ligand, extensive CO-binding kinetic studies have been conducted to explore the binding mechanism and access channel for this ligand.[96,105] CO-binding kinetic data suggest that interdomain signal transmission is coupled to heme ligand


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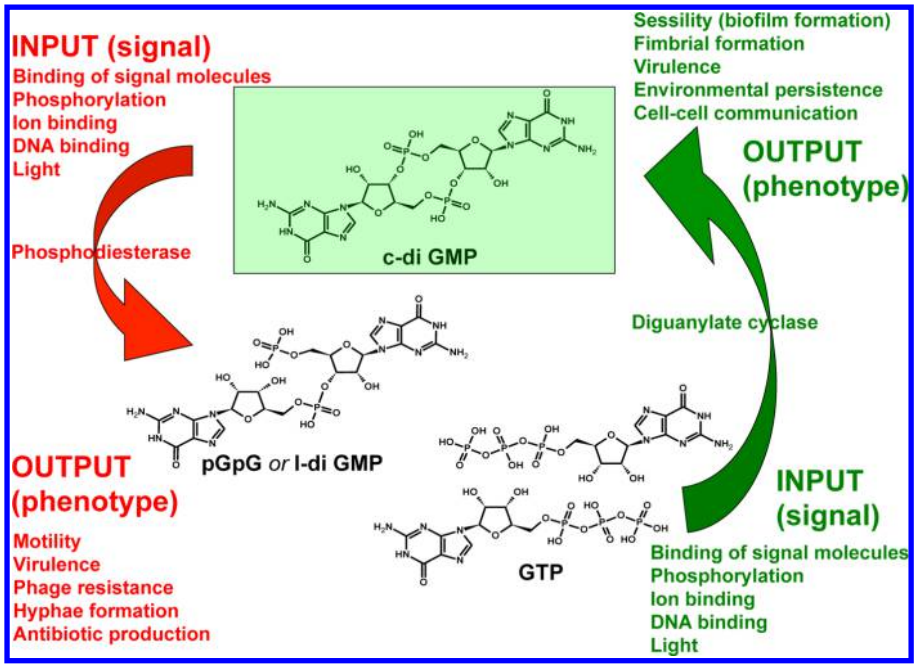


**Figure 7.** Cyclic-di-GMP is an important bacterial second messenger.[110−114] *Ec*DOS is a heme-based oxygen sensor PDE that linearizes c-di-GMP into l-di-GMP.[117,119] YddV is a heme-based oxygen sensor DGC that synthesizes c-di-GMP from GTP.[115,116] *Ec*DOS and YddV are *E. coli* enzymes that synergistically regulate the concentration of c-di-GMP to maintain intracellular c-di-GMP homeostasis.

release and that ligand-coupled protein oligomerization is physiologically relevant.[106] An ultrafast infrared spectroscopic analysis applied to the heme Fe(II)−CO complex of FixL[94] suggested that CO rotates toward the heme plane upon photodissociation in the heme pocket. Remarkably, CO bound to the heme iron is tilted by ∼30° with respect to the heme normal.

*2.1.1.6. NO Complex.* NO also is not a physiological axial ligand, but valuable information regarding ligand binding to the heme Fe(II) complex of FixL has been obtained on the basis of RR spectral data for the heme Fe(II)−NO complex of FixL. These studies suggested that the kinase domain influences the heme structure and that interdomain interactions between the heme and kinase domains are associated with ligand-binding-mediated kinase activity.[107]

*2.1.1.7. pH Dependence and Tyrosine Residues.* The pH-dependent behavior of the heme Fe(III) forms of both full-length Fix and the isolated heme-bound domain of FixLs was studied to ascertain how the spin state and ligation state of the heme iron complex respond to changes in pH by UV−visible, RR, and EPR spectroscopy.[108] The hemes of both FixL proteins were not fully ligated by hydroxide ion under strongly alkaline conditions, thus differing from the hemes of both myoglobin and hemoglobin. Both FixL proteins undergo additional alkaline transitions that involve the deprotonation of tyrosine residues, considering the p$K_a$ values of tyrosine residues. Spectroscopic evidence indicates that the loss of activity at elevated pH was not due solely to the generation of a low-spin heme hydroxide. Rather, one or more tyrosines may be involved in signal transduction between the heme iron and kinase domain of FixL.

*2.1.1.8. Chimeric Sensory Kinases.* Chimeric sensory kinases composed of the heme-bound oxygen-sensor domain of FixL and the histidine kinase domain from the hyperthermophile, *Thermotoga maritima*, were constructed, and their molecular properties were characterized. An analysis of these chimeric kinases suggested that the interaction with the physiological sensor histidine kinase is strictly and finely controlled to mediate the signal ligand-dependent autophosphorylation activity in the histidine kinase domain.[109]

**2.1.2. *Ec*DOS (or *Ec*DosP).** Cyclic-di-GMP (c-di-GMP) is an important second messenger for bacteria, regulating numerous important physiological functions including cell motility, differentiation, development, virulence, biofilm formation, cell−cell communications, environmental persistence, photosynthesis, phage resistance, heavy metal resistance, and many others (Figure 7).[110−114] Homeostasis of c-di-GMP, regulated by diguanylate cyclase (DGC)-mediated synthesis and phosphodiesterase (PDE)-mediated degradation/linearization of c-di-GMP, is crucial for bacterial survival. These enzymes are controlled by phosphorylation, heavy metal binding, gas ($O_2$, NO) binding, DNA binding, and light illumination, among many other external stimuli. In *E. coli*, two heme-based oxygen sensors, *E. coli* direct oxygen sensor (*Ec*DOS, also known as *Ec*DosP) and YddV, with PDE and DGC activity, respectively, as well as numerous other enzymes with the same functions, work together in response to various external environmental stimuli such as changes in $O_2$ concentration.[115,116]

*Ec*DOS is a heme-based $O_2$-sensing PDE that catalyzes the conversion of c-di-GMP to linear di-GMP (pGpG) (Figure 7).[117] Similar to FixL, *Ec*DOS is composed of an N-terminal heme-bound $O_2$-sensing PAS domain, in which heme is bound to one of two tandem PAS domains, and a C-terminal PDE catalytic domain (Figure 2).[42,43,46,53] However, in contrast to FixL, where $O_2$ dissociation stimulates catalysis, the activity of *Ec*DOS is markedly enhanced by $O_2$ binding to the heme Fe(II) complex.[118−120] The molecular mechanism responsible for $O_2$-stimulated *Ec*DOS PDE activity has been characterized in numerous studies.

*2.1.2.1. Catalytic Properties.* The C-terminal catalytic domain of *Ec*DOS has the EAL subdomain typical for PDE,


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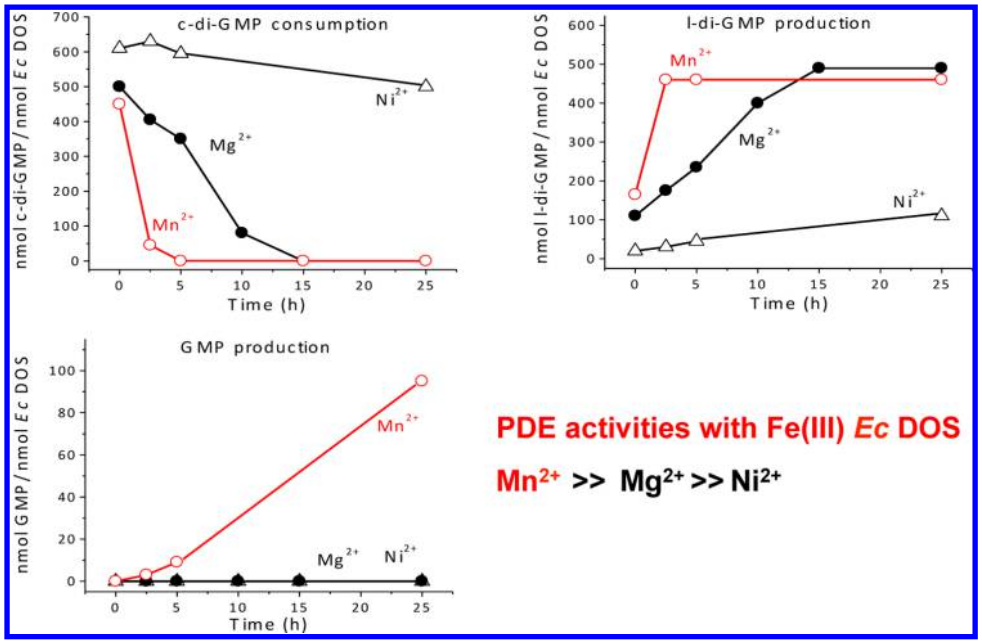


**Figure 8.** Role of $Mn^{2+}$ in *Ec*DOS catalysis is different from that of $Mg^{2+}$. The heme Fe(III) complex form of *Ec*DOS in the presence of $Mn^{2+}$ instead of $Mg^{2+}$ exhibits robust PDE activity toward c-di-GMP in the absence of external ligands, such as $O_2$, CO, NO, cyanide, and imidazole.[129] This reaction also mediates the two-step hydrolysis of c-di-GMP all of the way to GMP via the linear di-GMP intermediate. Adapted from Honjo et al.[129] Adapted with permission from ref 129. Copyright 2012 Nova Science: Hauppaug.

which converts c-di-GMP into linear di-GMP (l-di-GMP); although it also possesses the GGDEF subdomain characteristic of DGC, which synthesizes c-di-GMP from GTP, DGC activity has not been reported for *Ec*DOS.[118−120] The C-terminal PDE activity is substantially enhanced by $O_2$ binding to the heme Fe(II) complex in the N-terminal sensing domain. Interesting features of ligand-induced catalytic stimulation include the fact that enhancement of catalysis by NO or CO binding to the heme Fe(II) complex is similar to that of $O_2$ and the observation that cyanide or imidazole binding to the Fe(III) complex stimulates catalysis to an extent similar to that of these gaseous molecules.[120] These axial ligand-induced increases in catalytic activity appear to be associated with the upward movement of the heme distal amino acids caused by ligand binding to the heme iron complex. As shown by the crystal structures of *Ec*DOS, catalytic enhancement of the heme Fe(II) complex by $O_2$, NO, and CO is achieved by dissociation of Met95;[51,85] in this scenario, the axial ligand Met95 on the heme distal side dissociates from the heme Fe(II) complex, and these gaseous molecules subsequently bind to the heme iron complex (Figures 5B and 6[2]). Therefore, it is reasonable to suppose that the activity of mutants containing a substitution at Met95, the axial ligand for the heme Fe(II) complex, is already high enough without those gaseous molecules, reinforcing the suggestion that the upward movement of the distal amino acids impelled by gaseous ligands is significantly involved in the resulting catalytic enhancement. The protein structure of the heme distal side is not clear from a study of the X-ray crystal structure of the heme Fe(III) complex of *Ec*DOS (Figure 6[2]).[51] However, it appears that similar movement of the protein in the heme distal side occurs upon binding of cyanide and imidazole, leading to catalytic enhancement via intramolecular signal transduction from the N-terminal heme-bound domain to the C-terminal catalytic domain.[120] The protein surface of the catalytic domain may interact, directly or indirectly, with the surface of the heme distal side in the heme-binding domain. Protein movement at the heme distal side may form an optimal structure at the protein surface of the heme-bound domain, thus stimulating catalysis in the functional domain.

YddV (or *Ec*DosC) is the heme-based oxygen-sensing DGC from *E. coli*,[115,116] as described in greater detail below (YddV section), whereas *Ec*DOS is the corresponding heme-based oxygen-sensing PDE toward c-di-GMP (Figure 7). Notably, the reactions catalyzed by YddV and *Ec*DOS leading to the synthesis and degradation of c-di-GMP in vitro are coupled, suggesting that both oxygen sensors function synergistically to regulate c-di-GMP concentration in response to external stimuli or $O_2$ concentration.[115,116]

*Ec*DOS also acts on the alternative substrate cyclic AMP (cAMP),[53,121−123] although its PDE activity toward this substrate is much lower than that of the native PDE enzyme in *E. coli*.[124] Intracellular cAMP concentrations in *Ec*DOS-knockout *E. coli* cells were ∼26-fold higher than those in wild-type cells,[125] and the morphologies of *Ec*DOS-knockout and wild-type *E. coli* cells were significantly different.[125] These results suggest that cAMP is also a native substrate of *Ec*DOS under physiological conditions. In its heme Fe(II) complex form, *Ec*DOS shows PDE activity toward cAMP, whereas the heme Fe(III) complex form of *Ec*DOS lacks PDE activity toward this substrate. It has been suggested that these heme redox-dependent catalytic differences in *Ec*DOS are partly caused by a heme redox-dependent domain−domain interaction at the C-terminal subdomain and protein flexibility (see next subsection for more detail).[51]

$Mg^{2+}$ is essential for facilitation of the PDE activity of *Ec*DOS; it is also essential for kinase, phosphatase, PDE, guanylate cyclase, and numerous other nucleotide-associated reactions.[8,126−128] It is well-known that $Mn^{2+}$ assists these nucleotide-associated reactions instead of $Mg^{2+}$.[8,126−128] A comparison of the effects of $Mn^{2+}$ and $Mg^{2+}$ on the PDE reaction of *Ec*DOS[129] showed that, in the presence of $Mn^{2+}$ and


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in the absence of external ligands (cyanide, imidazole), the heme Fe(III) complex form of *Ec*DOS exhibited full PDE activity that was significantly faster than that in the presence of $Mg^{2+}$ (Figure 8). This finding is in marked contrast to the case for the heme Fe(II) complex form of *Ec*DOS, in which binding of an external ligand ($O_2$, NO, or CO) is needed to stimulate PDE activity in the presence of $Mg^{2+}$.[118,119] Furthermore, in the presence of $Mn^{2+}$, the heme Fe(III) complex form of *Ec*DOS was found to mediate the two-step hydrolysis of c-di-GMP all of the way to GMP via the l-di-GMP intermediate. It was speculated that $Mn^{2+}$ may be situated or coordinated at an active-site position that, even in *Ec*DOS heme Fe(III), is suitable for promoting optimal catalytic activity comparable to that observed for the heme Fe(II)−$O_2$ form with $Mg^{2+}$. Thus, one plausible explanation for the gas-sensing function of *Ec*DOS is that $O_2$ (or NO/CO) binding enhances $Mg^{2+}$ affinity for the active site and/or creates a $Mg^{2+}$ coordination structure that is optimal for efficient PDE catalysis.

*2.1.2.2. Heme Redox-Induced and Ligand-Induced Changes in Global Protein Structure.* Heme redox-dependent axial ligand substitution of *Ec*DOS should be a specific characteristic of *Ec*DOS distinct from that of FixL. This is because the axial ligand, hydroxide anion, on the distal side of the heme Fe(III) complex of *Ec*DOS is changed to Met95 upon the reduction of heme to the heme Fe(II) complex (Figures 5B and 6[2]).[51,85] Global protein rearrangements, including an ionic interaction between the heme propionate and protein arginine residue, are reported to occur upon heme reduction in *Ec*DOS. These heme redox-dependent axial ligand substitutions and global protein structural changes are not observed for FixL.

Heme redox-dependent changes in protein flexibility have also been reported. For the heme Fe(III) complex, the protein structure on the heme distal side cannot be definitely determined due to protein flexibility; in contrast, the corresponding structure of the heme Fe(II) complex is resolvable. Because it is assumed that the heme Fe(III) complex is inactive toward the alternative substrate, cAMP, as well as c-di-GMP, and that the heme Fe(II) complex is partially active toward both substrates, as described above,[53,118−123] the protein flexibility near the heme surrounding of *Ec*DOS should significantly contribute to the catalytic regulation of this enzyme. A high-pressure study revealed similar relationships between protein flexibility and the heme redox state of *Ec*DOS with respect to protein compressibility.[130]

Furthermore, upon $O_2$ binding to the heme Fe(II) complex, the heme distal side undergoes profound structurally changes, with Arg97 moving toward and eventually interacting with the $O_2$ molecule bound to the heme Fe(II) complex (Figures 5B and 6[2]).[85] This profound structural change induced by ligand binding and the interaction of Arg97 with the heme-bound $O_2$ molecule are similar to those observed for the heme-bound PAS domain of FixL.[81−83] For heme-based oxygen sensors, only limited $O_2$-free and $O_2$-bound structures have been determined for FixL and *Ec*DOS. However, on the basis of available information, it is reasonable to suggest that, in general, binding of the $O_2$ molecule to the heme Fe(II) complex in heme-based oxygen sensors profoundly changes the protein structure surrounding the heme, and these structural rearrangements, in turn, switch on intramolecular signal transduction to the C-terminal functional domain. It has also been suggested that the protein surface of the heme distal side and the functional domain face each other and directly or indirectly interact, as described above.

Notably, removal of the *Ec*DOS heme iron complex or truncation of the heme-bound PAS domain does not affect the catalytic activity toward c-di-GMP; in both cases, the catalytic activity of the heme-free form is as high as that of the $O_2$-stimulated form.[120] This finding suggests that the heme iron complex serves to suppress catalysis, and $O_2$ association with the heme Fe(II) complex releases this catalytic suppression. Similar findings have also been reported for the heme-based oxygen sensors, YddV and *Af*GcHK,[73,116] suggesting that the role of the heme iron complex in catalytic regulation is generally common among heme-based oxygen sensors. However, the heme-free forms of two other heme-based oxygen sensors, *Ax*PDEA1 and HemAC-*Lm*, have low activity.[131−133] Thus, one possibility for these oxygen sensors is that binding of the heme iron complex to the sensor domain is critical for maintaining an active site structure suitable for optimal catalysis. Further studies will be needed to elucidate the role of the heme iron complex in heme-based oxygen sensors.

*2.1.2.3. $O_2$ Association/Dissociation Kinetics.* The equilibrium dissociation constant ($K_d$) values of *Ec*DOS for $O_2$ are 13−21 $\mu$M for the isolated heme-bound domain, and 74−340 $\mu$M for the full-length protein.[50,117,134,135] As noted above, these values, like those for FixL (29−140 $\mu$M),[50] are substantially higher than that of myoglobin (0.88 $\mu$M). This suggests that both FixL and *Ec*DOS function under aerobic conditions, sensing slight differences in $O_2$ concentration and then increasing/decreasing their catalytic activity in response. Because the $K_d$ value of the c-di-GMP synthetic enzyme YddV for $O_2$ (14 $\mu$M)[116] is lower than that of the c-di-GMP degradative enzyme *Ec*DOS, both YddV and *Ec*DOS would function synergistically to regulate c-di-GMP concentration in response to the stress imposed by changes in external $O_2$ concentration.

Not surprisingly, mutations at the heme distal side-localized Arg97, which interacts with the $O_2$ molecule bound to the heme Fe(II) complex, substantially change various $O_2$ binding kinetics, in particular, $O_2$ dissociation and auto-oxidation rate constants.[134] These observations suggest that Arg97 is important for the stability of the heme Fe(II)−$O_2$ complex in *Ec*DOS and for anchoring the $O_2$ molecule, thus preventing $O_2$ dissociation from the heme Fe(II) complex and/or rapid auto-oxidation. Furthermore, mutations at Met95, the axial ligand to the heme Fe(II) complex, cause more drastic changes in the $O_2$ association rate constant, suggesting that prior dissociation of Met95 from the heme iron plays a more crucial role than subsequent association of the $O_2$ molecule with the heme iron.[135]

*2.1.2.4. CO and Cyanide Association Kinetics.* CO association rate constants, unlike the case for $O_2$ association rate constants, are slightly increased in Arg97 mutants as compared to the wild-type protein.[134] This suggests that Arg97 does not have a significant direct effect on the CO access/escape channel in heme Fe(II) complex-bound *Ec*DOS. However, the loss of specific interactions between Arg97 and the heme 6-propionate in the mutant proteins may contribute to an increase in the CO association rate, as suggested by RR spectroscopic findings.[136,137] On the other hand, mutations at Met95 do not significantly change CO association kinetic values, suggesting that Met95 plays a different role in CO association than in $O_2$ association.

Association rate constants for cyanide binding to the heme Fe(III) complex are significantly lower for Arg97 mutants than for the wild type; as a result, the $K_d$ values of cyanide for Arg97


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mutants are substantially higher.[120,134] This suggests that Arg97 also plays a role in the association of cyanide with the heme Fe(III) complex. Furthermore, Ala and Ile mutations at Met95 increase cyanide association with the heme Fe(III) complex, suggesting that Met95 is also involved in negatively regulating the association of cyanide to the heme iron. A study of the pH dependence of cyanide binding to the heme Fe(III) complex of *Ec*DOS provided interesting insights into the protein structural dynamics associated with alkaline denaturation.[138] Cyanide binding to heme Fe(III) complex of *Ec*DOS was found to be strongly affected by the apolar nature of the heme pocket, indicating that cyanide binding to this protein is distinct from cyanide binding to metmyoglobin and other heme enzymes.[138]

*2.1.2.5. Reduction Potential.* The reduction potential value of the isolated heme-bound domain of *Ec*DOS, at approximately 45−70 mV versus a standard hydrogen electrode (SHE), is similar to that of myoglobin.[54] This implies that the heme Fe(II) complex is more stable than the heme Fe(III) complex, enabling it to exert its oxygen-sensing function. Mutations at heme distal and proximal sides invariably change the reduction potential value to a certain extent.[54,139,140] Notably, mutations at Met95 drastically decrease reduction potential values from −1 to −122 mV versus a SHE.[54,139] Therefore, Met95 appears to be important in keeping the reduction potential in a range suitable for physiological function as an oxygen sensor under aerobic conditions.

*2.1.2.6. Resonance Raman (RR) Spectroscopy.* RR spectroscopy is a powerful tool for elucidating the structure and ionic characteristics of the environment of the heme iron complex and aromatic amino acid residues of heme proteins.[55,136,137,141−143] It has been successfully used to dissect the structure and function relationships of *Ec*DOS involved in intramolecular signal transduction from the heme-bound domain to the functional domain.

The spin states and coordination structures of the heme Fe(III) and Fe(II) complexes of *Ec*DOS, determined by RR spectra, revealed that complexes without any external ligand form 6-coordinated low-spin complexes.[143,144] The RR spectrum of the heme Fe(II)−CO complex suggested that the Fe−C−O geometry is nearly linear and that the CO environment has a hydrophobic character. RR spectra of the heme Fe(II) complex of His77 and Met95 mutants suggested that these amino acid residues are axial ligands for the heme Fe(II) complex of *Ec*DOS.[143] A His77Ala mutant showed no directing binding between the heme Fe(II) and the proximal side in *Ec*DOS, but time-resolved RR spectra revealed that exogenous imidazole was inserted between the heme Fe(II) complex and the protein surface of the mutant protein.[143]

Ultraviolet and time-resolved RR spectra of *Ec*DOS suggested the following structural features:[136,137,141,142] (1) Upon binding of $O_2$ to the heme Fe(II) complex, replacement of the distal axial ligand from Met95 with $O_2$ influences the heme 7-propionate hydrogen-bond network, resulting in profound conformational changes in the FG loop. (2) Interactions of Arg97 with the heme 7-propionate and steric interaction of Phe113 with $O_2$ are crucial for regulation of competitive axial ligand (Met95 and $O_2$) binding. (3) The hydrogen-bonding network from the heme 6-propionate to Tyr126 through Asn84 is important in transducing the signal from the PAS domain to the functional domain. (4) Trp53, Trp34, Tyr126, and Tyr80 are located near the 2-vinyl, 4-vinyl, and propionate side changes of heme, respectively. These amino acids then communicate structural changes in the heme side change induced by heme redox changes to the PAS protein surface, resulting in global protein conformational changes that are associated with the redox-dependent catalytic simulation of *Ec*DOS (Figure 5B).

*2.1.2.7. Interactions with $H_2S$.* $H_2S$ (hydrogen sulfide) is the fourth most important signal gas molecule after $O_2$, NO, and CO.[17,145−153] Numerous studies have investigated the role of $H_2S$ in physiology and pathology demonstrating several major mechanisms by which $H_2S$ could affect physiological functions, including (1) reduction/breakage of the S−S bond, (2) formation of persulfide and polysulfide, and (3) interaction with metal cations.[17,145−153] Interactions of $H_2S$ with heme proteins have been extensively studied,[154−157] and the formation of sulfhemoglobin and sulfmyoglobin containing sulfheme, a sulfur-incorporated protoporphyrin IX−iron complex, in the presence of both $H_2S$ and $H_2O_2$ has been examined.[155−157]

Given that *Ec*DOS accepts NO and CO in addition to $O_2$ as the catalysis-stimulating gas molecule, it is not surprising that the effects of $H_2S$ on the catalytic function of *Ec*DOS have also been explored. These studies showed that the PDE activity of *Ec*DOS is substantially enhanced by adding $H_2S$ under aerobic conditions.[158] Mutagenesis and pharmacological inhibitor approaches were used to address the possibility that cysteine residue(s) are involved in this catalytic enhancement. Simultaneously mutating all eight cysteine residues to alanine or blocking them by adding *N*-ethylmaleimide completely abolished catalytic activity. However, stepwise mutation of each cysteine residue to alanine failed to yield an individual mutation that completely inhibited *Ec*DOS catalytic activity. Therefore, although one or more cysteine residue(s) clearly is involved in the catalytic enhancement of *Ec*DOS by added $H_2S$, it was not possible to specifically identify the cysteine residue(s) directly involved in this stimulatory effect.

Changes in the coordination structure of the heme Fe(III) complex induced by adding $H_2S$ under aerobic conditions are complicated.[158] The heme Fe(III) complex is first changed to the heme Fe(III)−SH intermediate, and then the heme iron is reduced and becomes a heme Fe(II) intermediate, which quickly binds $O_2$. The heme Fe(II)−$O_2$ complex is autoxidized to yield the heme Fe(III) complex, which is further reduced to the heme Fe(II) intermediate. Absorption spectroscopy revealed that these complicated changes in coordination structure occur within several minutes.

The Arg97Ile and Arg97Ala mutants of *Ec*DOS form verdoheme, an oxygen-incorporated protoporphyrin IX iron complex, after $H_2S$ treatment.[159,160] Mutations at Arg97, the $O_2$-interacting site in the heme Fe(II)−$O_2$ complex of *Ec*DOS, would generate considerable amounts of $H_2O_2$ due to the instability of the heme Fe(II)−$O_2$ complex in the presence of $H_2S$. $H_2O_2$ would likely form a heme Fe(III)−OOH complex that, with the aid of the water molecule near the heme environment, would help to catalyze the verdoheme-formation reaction.[161−163] Contrary to expectations, sulfheme was not formed under these conditions.

**2.1.3. *Ax*PDEA1.** The phosphodiesterase A1 protein of *Acetobacter xylinum*, *Ax*PDEA1, is a key regulator of bacterial cellulose synthesis.[131] *Ax*PDEA1 linearizes c-di-GMP, an allosteric activator of the bacterial cellulose synthase, to the inactive l-di-GMP (pGpG). This PDE function toward c-di-GMP is the same as that of *Ec*DOS. The domain structure of *Ax*PDEA1, composed of an N-terminal heme-bound PAS domain and a C-terminal PDE domain, is also homologous to


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that of *Ec*DOS, although *Ec*DOS has two heme-bound PAS domains, whereas *Ax*PDEA1 has only one.

Characteristic functional features of *Ax*PDEA1 that distinguish it from *Ec*DOS include (1) stimulation of catalysis by dissociation of $O_2$ from the heme Fe(II) complex in *Ax*PDEA1, in contrast to *Ec*DOS, where $O_2$ binding to the heme Fe(II) complex stimulates catalysis;[119] (2) loss of activity of the heme-free form, apo-*Ax*PDEA1, in contrast to *Ec*DOS, where the heme-free form, apo-*Ec*DOS, has the same high activity as the $O_2$-stimulated enzyme;[120,123] and (3) substantial stability of the heme Fe(II)−$O_2$ complex in *Ax*PDEA1, with an autoxidation rate constant less than 0.001 $min^{-1}$, which is much lower than that of *Ec*DOS (0.022−0.005 $min^{-1}$).[134] These specific molecular characteristics would abet the bacterial survival-promoting functions of *Ax*PDEA1. Regarding point (2) above, the heme-free form of the heme-based oxygen sensor adenyl cyclase, HemAC-*Lm*, is inactive, similar to AxPDEA1,[132,133] whereas the heme-free form of another heme-based oxygen sensor, the globin-coupled oxygen sensor YddV, is also active, similar to *Ec*DOS.[116]

**2.1.4. Aer2.** Aer2 from *Pseudomonas aeruginosa* is a heme-based oxygen sensor containing a heme-bound PAS fold that acts in a microbial aerotaxis control system. This oxygen-sensing system helps bacteria survive by enabling them to move to environments with a high $O_2$ concentration or away from low $O_2$ environments.[164,165] The oxygen-sensing characteristics together with methyl-accepting chemotaxis are similar to those observed in the heme-bound globin fold-containing HemAT from *Halobacterium salinarum* and *Bacillus subtilis*, as described in a later section. The crystal structure of the heme Fe(III)−CN complex of a truncated Aer2 consisting of the PAS and di-HAMP domains was determined (Figure 6).[165] The PAS domain is composed of two $\alpha$-helices and a five-stranded $\beta$-sheet. The heme iron complex is accommodated by a hydrophobic pocket in which His234 is the proximal axial ligand, and heme-bound $CN^-$ and Trp283 interact. This arrangement suggests that a hydrogen bond will form between the heme-bound $O_2$ and Trp283 that stabilizes the heme Fe(II)−$O_2$ complex. It also revealed that in HAMP (histidine kinases, adenylate cyclases, methyl accepting proteins, and phosphatases), a linker domain composed of a nearly 50-amino-acid $\alpha$-helical region[166] is disordered for the most part.

Consistent with observations based on the crystal structure of the heme Fe(III)−CN complex, RR spectral data suggest that His251 forms a salt bridge with the 7-propionate in the heme Fe(II)−$O_2$ complex, similar to the heme Fe(III)−CN complex, implying that His251 is not the direct $O_2$-binding site in the heme Fe(II)−$O_2$ complex. The absorption spectrum of the heme Fe(II)−$O_2$ complex in the His251Ala mutant was the same as that in the wild type, although the autoxidation rate constant of this mutant was 10-fold higher. No stable heme Fe(II)−$O_2$ complex was observed for a Trp283Leu mutant.

On the basis of these findings, it was proposed[165] that hydrogen-bond formation between Trp283 and $O_2$ in the heme Fe(II)−$O_2$ complex causes conformational changes in the $\beta$5 strand upon $O_2$ binding, because Trp283 is located in the C-terminal region of the $\beta$5 strand. Once the PAS domain senses $O_2$ through binding to the heme Fe(II) complex, the $O_2$-sensing signal is transduced to the C-terminus of the MCP domain. Because the $\beta$5 strand is directly linked to the $\alpha$4 helix, consisting of a linker between the PAS and di-HAMP domains, a conformational change in the di-HAMP domain results in a conformation change in the MCP domain. Unfortunately, however, the predicted $O_2$-regulated catalytic behavior of Aer2 has not been experimentally confirmed.

A comparison of the crystal structure of the heme-binding PAS domain in the ligand-free heme Fe(III) complex with the previously determined heme Fe(III)−CN complex identified conformational changes induced by ligand binding that are likely essential for the signaling mechanism.[167] Heme pocket alterations share some similarities with those of the heme-based PAS sensors FixL and *Ec*DOS, but propagate to the I$\beta$ strand in a manner predicted to alter PAS-PAS associations and the downstream HAMP junction within full-length Aer2. It was suggested that the Aer2 signaling mechanism differs from that of the *E. coli* Aer paradigm, where direct (side-on) PAS-HAMP contacts are key. An in-line model of Aer2 signaling was proposed in which ligand binding induces alterations in PAS domain structure and subunit association that are relayed through the poly-HAMP junction to downstream domains. The PAS and HAMP domains do not interact directly in the in-line model of Aer2 signaling (Figure 6 [3]).[167]

**2.1.5. Gyc-88E.** sGC is an NO sensor whose catalysis is activated several hundred fold by binding of NO to the heme Fe(II) complex, as described in detail in a subsequent sGC section. The heme Fe(II) complex of sGC does not bind $O_2$, thereby allowing sGC to selectively bind NO despite micromolar cellular concentrations of $O_2$. However, Gyc-88E from *Drosophila* is a DGC containing an $O_2$-bound heme iron complex in the PAS domain.[168] Gyc-88E is active as a homodimer (5600 nmol $min^{-1}$ $mg^{-1}$) and is inhibited 3.2-, 2.9-, and 2-fold by $O_2$, CO, and NO, respectively. Accordingly, absorption spectra revealed that $O_2$, CO, and NO bind to the heme Fe(II) complex. The $K_m$ for GTP was estimated to be 0.66 mM in air (273 mM $O_2$) and 0.82 mM under anaerobic conditions, and the $K_i$ for $O_2$ was calculated to be 51 mM. Thus, it was suggested that Gyc-88E is a DGC that potentially functions as an oxygen sensor in which $O_2$ dissociation from the heme Fe(II) complex stimulates catalysis, similar to FixL and DosS/DosT.

**2.1.6. ThkA.** The structure of a histidine kinase (ThkA) complexed with a response regulator (TrrA) in the two-component regulatory system from the hyperthermophile *Thermotoga maritima* was determined by a combination of X-ray crystallography, small-angle X-ray scattering (SAXS), and nuclear magnetic resonance (NMR) (Table 1).[169,170] The PAS sensor domain of ThkA interacts with both the catalytic domain of the same polypeptide chain, through formation of an interdomain $\beta$-sheet, and the TrrA molecule. The association interface between ThkA and TrrA contains the phosphotransferring histidine residue in ThkA, indicative of efficient receipt of the phosphoryl group. SAXS experiments also confirmed this association state in solution and showed specific binding between ThkA and TrrA ($K_a$ = 8.2 × $10^{-11}$ $M^{-2}$). NMR confirmed the specific interaction between ThkA and TrrA and demonstrated that the interaction would be suitable for the intermolecular phosphate group transfer reaction. The observed interdomain and intermolecular interactions clearly reveal a signal transduction pathway in the kinase/regulator complex.

## 2.2. Heme-Bound GAF Domain-Containing $O_2$ Sensors

*Mycobacterium tuberculosis* DosS (DevS) and DosT are oxygen sensor histidine kinases of a two-component signal transduction system that function together with the response regulator, DosR (DevR), which acts to up-regulate downstream genes essential for bacterial survival under hypoxic conditions (Table


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1).[37,70−72,171] This two-component signal transduction system is similar to that which includes FixL in that DosS or DosT first undergoes autophosphorylation in response to environmental stimuli. The phosphoryl group of phosphorylated DosS or DosT then is transferred to DosR, a DNA-binding protein that regulates the dormant state of the bacteria (Figure 4A). Similar to FixL, both DosS and DosT are composed of an N-terminal heme-bound domain and C-terminal kinase and ATPase domains. The heme iron complex of both DosS and DosT is bound to one of two tandemly repeated GAF (cGMP-specific and stimulated phosphodiesterases, adenylate cyclases, and *E. coli* formate hydrogen lyase transcriptional activator) domains, forming the oxygen-sensing site.[171] The GAF domains of DosS and DosT are structurally similar to one another.[172−174]

The GAF domain, like the PAS domain, is a ubiquitous cytoplasmic signaling unit in prokaryotic and eukaryotic organisms.[171,172,174,175] The overall topology of the GAF domain resembles that of the PAS domain, consisting of one five-stranded antiparallel $\beta$-sheet and four $\alpha$-helices (Figure 3[C]).[171,172,174,175] The GAF domain has a ligand-binding pocket that accommodates a variety of small molecule ligands, such as flavin, adenine, cGMP, and heme, also similar to the PAS domain. Although the GAF domain is structurally similar to the PAS domain, its heme-binding mode differs from that of the PAS domain.[171,172,174,175] The role of $O_2$ dissociation from the GAF domain-bound heme Fe(II) complex is in activating the heme-bound histidine kinases. DosS and DosT from *M. tuberculosis* are similar to FixL, an element of the two-component system associated with nitrogen fixation in *B. japonicum* (*Bj*FixL) and *R. meliloti* (*Rm*FixL) described in a previous section.

**2.2.1. DosS (or DevS).** DosS is a member of the DosS−DosR two-component regulatory system that is thought to mediate the cellular response to anaerobiosis.[71,72,176] UV−vis and RR spectroscopic studies have revealed that His149 is the proximal ligand to both heme Fe(III) and heme Fe(II) complexes and that interactions between the two GAF domains within DosS influence the heme distal pocket environment in the heme-bound GAF domain (Figure 6[4]).[176] It has been found that unliganded, CO-bound, and NO-bound heme Fe(II) complexes are active, whereas the $O_2$-bound heme Fe(II) complex is inactive.[72] A specific hydrogen-bonding network near the $O_2$ access channel or the $O_2$-binding site on the heme distal side of DosS is responsible for discriminating $O_2$ from NO and CO to the heme Fe(II) complex.[177] Interactions between the two GAF domains are likely responsible for transducing structural changes at the heme domain that accompany ligand binding/dissociation to modulate activity at the kinase domain.[177]

Although DosS has been claimed to function as a redox sensor, spectral findings and analyses of histidine kinase activity under various conditions have shown no evidence of an oxygen or NO sensor.[37,71] However, other groups have argued that DosS is an oxygen (or NO) sensor, but not a redox sensor, based on its very low autoxidation rate constant and low $O_2$ dissociation rate constant as well as enhancement of the stability of the heme Fe(II)−$O_2$ complex by interdomain interactions.[176] Results based on RR spectra and autophosphorylation assays of the wild-type and Tyr171Phe mutant of DosS suggest that the hydroxyl group of Tyr171 acts as a relatively poor hydrogen-bond donor to the heme Fe(II) complex-bound $O_2$ molecule (Figure 6[4]).[177] An examination of DosS and NO interactions showed that, at low concentrations in a reducing environment, NO interacts with the inactive heme Fe(II)−$O_2$ complex and rapidly converts it into the heme Fe(II)−NO complex. This conversion triggers initiation of dormancy.[178] Ultrafast ligand dynamics of the isolated heme-bound GAF domains of DosS and DosT have also been examined. These studies showed that, despite structural and functional similarities between DosS and DosT, ligand-specific primary dynamics during the initial phases of intraprotein signaling are different between the proteins and predict that the distal tyrosine of both proteins plays an important role in these processes.[179]

Crystal structures of the DosS heme-bound GAF-A domain, the first of the two consecutive N-terminal GAF domains,[172] have been determined (Figure 6[4]). These analyses revealed that the heme is embedded in a hydrophobic cavity in the GAF domain and is roughly perpendicular to the $\beta$-sheet of the GAF domain. The heme Fe(III) complex is a 6-coordinated high-spin complex containing a proximal His149 and a water molecule that interacts with Tyr171 at the distal side. Although the heme is isolated inside the GAF domain, the heme Fe(II)−$O_2$ complex is unstable and rapidly oxidizes to the heme Fe(III) complex, leading to an indeterminable crystal structure of the heme Fe(II)−$O_2$ complex. Electron transferability through a defined hydrogen-bond network in the heme active site is a mechanism that has been put forward to explain the instability of the heme Fe(II)−$O_2$ complex. However, again, two other groups have reported that the DosS heme Fe(II)−$O_2$ complex is very stable,[72,171,176] suggesting that DosS is an oxygen (or NO) sensor. The crystal structure of GAF does not undergo a dramatic conformational change when the inactive heme Fe(III) form is reduced to the active heme Fe(II) form. This heme redox-insensitivity of protein structural changes is in contrast to that of *Ec*DOS, an oxygen sensor containing a heme-bound PAS domain, in which dramatic heme-redox-dependent structural changes occur.[51,85]

The crystal structure of the ATP-binding subdomain of the C-terminal kinase domain of DosS has been determined.[173] This structure suggests that proper positioning of a short ATP "lid" that interacts with a His phosphor-accepting subdomain triggers ATP binding of DosS. Accordingly, ATP binding would function as a structural mechanism for regulating histidine kinase activity.

**2.2.2. DosT.** The entire protein structure, heme-binding mode, and histidine kinase functions of DosT are similar to those of DosS.[71,72,171,174,179] However, DosT uses $Mn^{2+}$ in addition to $Mg^{2+}$ as the divalent ion for autophosphorylation and transfer of the phosphoryl group from phosphorylated DosT to DosR. Moreover, the phosphoryl transfer rate from DosT to DosR is much slower than that from DosS. In DosT, the heme Fe(II) complex-bound $O_2$ molecule is stabilized via a hydrogen bond to the OH group of Tyr169 on the distal side.[174] The entire structure of the heme-bound GAF domain of DosT is also similar to that of other heme-based oxygen sensors containing a PAS domain, such as *Ec*DOS and FixL.[51,81−83,85] However, the heme-binding mode in DosT is similar to that of cyclic nucleotide binding in GAF rather than heme binding in the PAS domain, even though DosT contains a five-stranded antiparallel $\beta$-sheet instead of the six-stranded sheet found in GAF motifs that bind cyclic nucleotides or linear tetrapyrrole chromophores.[174]

The crystal structure of the isolated ATP binding subdomain in the kinase domain of DosT was found to be similar to that of DosS. Thus, as is the case for DosS, a proper structural



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relationship between a short ATP lid and a His phospho-accepting subdomain is crucial for optimal ATP binding.[173]

### 2.3. Heme-Bound Globin Domain-Containing $O_2$ Sensors: Globin-Coupled $O_2$ Sensor (GCS)

The well-known representative heme proteins, myoglobin and hemoglobin, contain a globin fold and are composed of several $\alpha$-helices.[1,6,8,180−186] In bacterial GCSs, the heme-bound globin domain is located at the N-terminus, whereas the functional domain with activities such as histidine kinase, PDE, DGC, and methyl-accepting chemotaxis is located at the C-terminus (Table 1).[38−40,50,60,185,187] However, the globin domain of GCSs lacks the entire D-helix and part of the E-helix of myoglobin and hemoglobin, which have a common genetic ancestry with sensor globins.[50] The shortened globin fold would thus be expected to have special characteristics that are beneficial for heme-based oxygen sensors. The most characteristic feature of GCSs is that $O_2$ binding to the heme Fe(II) complex substantially enhances their catalytic activity.[50] This property of GCSs distinguishes them from other heme-based oxygen sensors containing the PAS and GAF domains (with the exception of *Ec*DOS), in which $O_2$ dissociation from the heme Fe(II) complex enhances catalysis. Another interesting feature of GCSs is that the tyrosine residue at B10 is the $O_2$-interaction site.[50] This feature is also different from most heme-based oxygen sensors containing a heme-bound PAS domain (FixL and *Ec*DOS), in which an arginine residue is the $O_2$-interaction site,[81−83,85] although a tyrosine residue is located near the $O_2$-binding site in DosS and DosT.[174,177]

**2.3.1. HemAT.** HemAT-*Hs* from the archaeon *Halobacterium salinarum* and HemAT-*Bs* from the Gram-positive bacterium *Bacillus subtilis* have a methyl-accepting chemotaxis function that controls the movement direction of archaea and bacteria in an $O_2$ gradient.[188,189] The molecular properties of the heme-bound domain of HemATs have been well characterized. However, because no specific substrate/product of a HemAT-mediated reaction has been identified, it has not been possible to definitively establish functional regulation by $O_2$ association/dissociation and/or heme redox changes in reconstituted systems containing full-length HemATs in vitro. The C-terminal domain interacts with the histidine kinase CheA, which is a component of the CheA/CheY two-component signal transduction system involved in bacterial aerotaxis.

The crystal structures of heme Fe(II) and heme Fe(III)−CN complexes of the isolated heme-bound globin domain of HemAT-*Bs* have been determined (Figures 3[B] and 6[6]).[190] HemAT proteins in crystals were found as dimers, whereas gel filtration chromatography showed that the protein in dilute solution was mostly in a monomeric form. The overall structure of the heme Fe(II)-bound protein was very similar to that of the heme Fe(III)−CN-bound protein, suggesting the absence of profound protein structural changes upon a change in the heme redox state or cyanide binding to the heme Fe(III) complex. Instead, the HemAT sensor is predicted to undergo only small conformational changes within the dimer upon ligand association with or dissociation from the heme iron complex. This relative conformational insensitivity of the HemAT sensor protein structure is in contrast to the global protein structural changes observed in response to the heme redox state and ligand binding in other heme-based oxygen sensors, such as FixL and *Ec*DOS, containing a heme-bound PAS domain.[51,81−83,85] The HemAT sensor domain has low (~15%) sequence homology with myoglobin; however, the proximal His123 (F8) residue is well conserved throughout the globin family.[50]

The cyanide ligand is bound to the heme Fe(III) complex and stabilized by the hydroxyl group of Tyr70 (B10) with a hydrogen bond (Figure 6[6]). Moreover, there is one water molecule within the distal pocket. The distal water molecule is stabilized by a hydrogen bond to the $\lambda$ oxygen atom of the Thr95 side chain (E-helix).

The $O_2$ binding equilibrium and kinetic constants for HemAT have high-affinity ($K_d$ = 1−2 $\mu$M, $k_{O2}$ = 50−80 $s^{-1}$) and low-affinity ($K_d$ = 50−100 $\mu$M, $k_{O2}$ = 2000 $s^{-1}$) components.[191] Replacement of Tyr70 (B10) with phenylalanine, leucine, or tryptophan dramatically increases both high- and low-affinity components of $k_{O2}$, implying that the hydroxyl group of Tyr70 (B10) is the direct/indirect $O_2$-interacting site within the heme Fe(II)−$O_2$ complex of HemAT.[191]

RR and ligand binding studies have been conducted on HemAT-*Bs*. These studies, which compared the structural characteristics of this protein with those of other heme proteins, revealed the presence of a unique hydrogen bond between a distal amino acid residue and the proximal atom of $O_2$ in the heme Fe(II)−$O_2$ complex.[192] RR spectral studies on Tyr70 and Thr95 mutants of HemAT-*Bs* suggest that the $O_2$ interaction can take three forms: a closed form, with an interaction between Thr95 and $O_2$; an open $\alpha$-form, with interactions between Tyr70 and Thr95 and between Thr95 and $O_2$; and an open $\beta$-form, without interactions between the distal amino acid and $O_2$.[193] They further suggest the presence of a hydrogen bond between Thr95 and $O_2$ and between His86 and the heme propionate in the heme Fe(II)−$O_2$ complex.[194] A Fourier transform infrared spectrometry (FTIR) study emphasized the importance of hydrogen bonds, including Tyr70 and Thr95, on the heme distal side for ligand recognition and discrimination.[195]

Time-resolved ultraviolet RR spectra suggest the presence of hydrogen bonds between Thr95 and $O_2$ in the heme Fe(II)−$O_2$ complex and between His86 and the heme 6-propionate,[196] and predict that, upon CO photolysis, a change in heme structure drives displacement of the B- and G-helices.[197]

These spectral studies further suggest that the global protein structural changes caused by association/dissociation of the ligand are critical for signal transduction from the heme-bound domain to the functional domain. Discrepancies in the role of Tyr70 and Thr95 in ligand binding can be partially explained by the involvement of a water molecule in ligand recognition or discrimination.[50]

**2.3.2. YddV (or *Ec*DosC).** As briefly described above in the section on *Ec*DOS, YddV is a heme-based oxygen sensor DGC composed of an N-terminal heme-bound globin domain and a C-terminal functional domain.[115,116] YddV synthesizes c-di-GMP from GTP in response to $O_2$ binding to the heme Fe(II) complex bound to the N-terminal sensing domain. YddV is notable in that c-di-GMP is important for various fundamental bacterial physiological functions,[110−114] as described above.

At least in *E. coli*, two heme-based oxygen sensors, YddV, a DGC, and *Ec*DOS, a PDE for c-di-GMP, function synergistically to maintain c-di-GMP homeostasis in response to environmental $O_2$ concentration. The synthesis of c-di-GMP, subsequent degradation of c-di-GMP, and production of l-di-GMP have been monitored by chromatography in a semi-reconstituted system in which GTP is mixed with both YddV and *Ec*DOS.[115,116] These studies showed that, in the active


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heme Fe(II)−$O_2$ complex form, the DGC activity (0.022 $min^{-1}$) of YddV is much lower than the PDE activity (61 $min^{-1}$) of *Ec*DOS.[115,116] Thus, the YddV reaction leading to production of the *Ec*DOS PDE substrate c-di-GMP is the rate-determining step for the two coupled reactions. By extension, $O_2$ binding and heme redox switching of YddV appear to be critical factors in the regulation of c-di-GMP homeostasis. Because the $K_d$ values for $O_2$ are significantly different between YddV (14−20 $\mu$M) and *Ec*DOS (74−340 $\mu$M),[50,115−117,135] the function of the two enzymes in maintaining c-di-GMP in vivo would be dependent on the $O_2$ concentration. The redox potential and the autoxidation rate constant of the isolated heme-bound domain of YddV are −17 mV and 0.0076 or 0.04 $min^{-1}$, respectively, whereas the corresponding values for *Ec*DOS are 40−70 mV and 0.0053 $min^{-1}$.[54,115,116] Thus, the redox environments of YddV and *Ec*DOS would also significantly affect the catalytic activity of these enzymes and thus influence c-di-GMP homeostasis.

Mutations at the Tyr43 residue in the putative heme distal side substantially reduce $O_2$ affinities, or markedly increase $O_2$ dissociation rate constants or autoxidation rate constants, suggesting that the hydroxyl group of Tyr43 directly or indirectly interacts with $O_2$ in the heme Fe(II)−$O_2$ complex through hydrogen bonding.[115,116] The RR frequency representing $\nu_{Fe-O2}$ of the heme Fe(II)−$O_2$ complex of Tyr43Phe (559 $cm^{-1}$) differs from that of the wild type (565 $cm^{-1}$), again corroborating the importance of Tyr43 in stabilization of the heme Fe(II)−$O_2$ complex or dissociation of the $O_2$ molecule from the heme Fe(II) complex in YddV.[115,116]

An amino acid sequence alignment of GCSs indicates that Leu65 of YddV is well conserved, and the crystal structure of HemAT suggested that Leu65 is located in the heme distal side.[190,198] Absorption spectra of the heme Fe(III) complex and the pH dependences of Leu65 point mutants are substantially different from those of the wild type, suggesting that a water molecule is the axial ligand of the heme Fe(III) complex in Leu65 mutants. Autoxidation rate constants of the heme Fe(II)−$O_2$ complex are much higher for Leu65 mutants (>0.10 $min^{-1}$) than for the wild type (0.011 $min^{-1}$); $O_2$ affinities for the heme Fe(II) complex are also markedly higher for the Leu65 mutants. These results suggest that the well-conserved Leu65 located in the heme distal side is critical for restricting water access to the heme distal side and preventing rapid autoxidation of YddV; thus, Leu65 is needed for formation of a stable Fe(II)−$O_2$ complex with a low autoxidation rate.[198]

Consistent with the purported role of a water molecule as an axial ligand in Leu65 mutants, overexpression of these mutants in *E. coli* tends to result in generation of verdoheme and CO.[199] In particular, the Leu65Asn mutant has strong tendency to exert heme oxygenase activity. Verdoheme and CO are also produced in a reconstituted system composed of Leu65 mutants and NADPH cytochrome P450 reductase. The fact that Leu65 mutations are assumed to allow introduction of a water molecule into the heme distal side of the isolated heme-bound domain of YddV suggests that the water molecule facilitates heme oxygenase reactions in Leu65 mutants. A significant role for a water molecule in the first reaction of the successive heme oxygenase reactions that yield verdoheme has been proposed.[161,162]

**2.3.3. *Af*GcHK.** *Af*GcHK, a globin-coupled histidine kinase from soil bacterial *Anaeromyxobacter* sp. Fw109-5, functions as part of a two-component signal transduction system.[73] $O_2$ binding to the heme Fe(II) complex in the N-terminal sensing domain substantially enhances the catalytic activity of this enzyme, including autophosphorylation at a histidine residue within the enzyme and subsequent phospho-transfer from this phosphorylated enzyme to a response regulator. Absorption spectra and functional studies of site-directed mutants suggest that [1] His99 is the proximal heme axial ligand, [2] His183 is the autophosphorylation site, and [3] Asp52 and Asp169 in the response regulator are the phosphorylated sites in this two-component system.[73] On the basis of the amino acid sequences and crystal structures of other GCSs, Tyr45 is predicted to be the $O_2$-interacting site at the heme distal side. Consistent with this, the $O_2$ dissociation rate constant (0.10 $s^{-1}$) is substantially increased (to 8.0 $s^{-1}$) in the Tyr45Leu mutant. RR frequencies representing $\nu_{Fe-O2}$ (559 $cm^{-1}$) and $\nu_{O-O}$ (1149 $cm^{-1}$) of the heme Fe(II)−$O_2$ complex of the Tyr45Phe mutant are distinct from the values of $\nu_{Fe-O2}$ (557 $cm^{-1}$) and $\nu_{O-O}$ (1141 $cm^{-1}$) for the wild-type protein. These results suggest that Tyr45 is located at the heme distal side and forms hydrogen bonds with the $O_2$ molecule in the heme Fe(II)−$O_2$ complex. Although oxygen sensor histidine kinases with a heme-bound PAS fold (FixL) or GAF fold (DosS, DosT) have been found and are well characterized, *Af*GcHK is the first oxygen sensor histidine kinase in a two-component system that contains a heme-bound globin fold (i.e., a GCS). The mode of $O_2$-induced catalytic stimulation is also a characteristic feature of *Af*GcHK in that $O_2$ binding to the heme Fe(II) complex enhances *Af*GcHK catalytic activity, in contrast to FixL and DosS/DosT, where $O_2$ dissociation from the heme Fe(II) complex enhances catalysis.

**2.3.4. HemDGC.** Structure and function studies of a heme-containing DGC (HemDGC) from *Desulfotalea psychrophila*[200] have shown that HemDGC has an N-terminal heme-bound sensing domain containing a globin fold and a C-terminal functional domain containing a DDDEF subdomain that is presumed to be involved in DGC activity. $O_2$ binding to the heme Fe(II) complex in the N-terminal sensing domain of HemDGC substantially enhances its catalytic activity (to 6.9 $min^{-1}$). Fe−O ($\nu_{Fe-O}$) and O−O ($\nu_{O-O}$) stretching modes of the heme Fe(II)−$O_2$ complex were observed at 566 and 1138 $cm^{-1}$, respectively. The $\nu_{Fe-O}$ frequency of HemDGC was similar to that of several nonvertebrate hemoglobins, such as *Ascaris suum* Hb (566 $cm^{-1}$), *M. tuberculosis* HbN (562−566 $cm^{-1}$), and *Paramecium caudatum* Hb (563 $cm^{-1}$), suggesting that hydrogen bonds are present between amino acid side chains and both proximal and distal oxygen atoms in the heme Fe(II)−$O_2$ complex. A Tyr55Phe mutant, containing a substitution at the well-conserved Tyr55 (B10) residue in the putative heme distal side, showed no catalytic activity, partly because it failed to form a stable heme Fe(II)−$O_2$ complex. Thus, Tyr55 has been suggested to play an important role in stabilization of the heme Fe(II)−$O_2$ complex.[200]

**2.3.5. *Av*GReg.** *Av*GReg, a putative GCS from *Azotobacter vinelandii*, was studied by characterizing its heme-binding domain (*Av*GReg178) and the full-length protein.[201] These studies showed that the heme Fe(II)−$O_2$ complex of *Av*GReg178 is very stable over many hours. Moreover, the heme Fe(III) complex of *Av*GReg was found to be a bis-His coordinated, low-spin complex. This is substantially different from the coordination structures of GCSs hitherto reported for which His has not be observed to be the distal axial ligand for the heme Fe(III) complex; instead, these GCSs are reported to have a vacant site or $OH^-$ as the distal axial ligand.[73,116] $O_2$ affinities of *Av*GReg178 and *Av*GReg for the heme Fe(II) complex are high, with reported $K_d$ values of 0.025 and 0.12


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$\mu$M, respectively. An examination of the kinetics of CO binding to both proteins was reported. It was suggested that the heme Fe(II)−$O_2$ complexes of *Av*GReg178 and *Av*GReg are involved in $O_2$-mediated NO-detoxification, which yields the heme Fe(III) complex. However, the DGC activity of *Av*GReg was not characterized.

**2.3.6. *Bpe*GReg.** *Bpe*GReg, a *Bordetella pertussis* GCS, possesses DGC activity that is stimulated ∼10-fold by $O_2$ binding to the heme Fe(II) complex in a reconstituted system.[202] This enzyme was inferred to consist of three domains: an N-terminal heme-bound globin domain, a middle domain, and a C-terminal DGC domain. The middle domain was suggested to be important for proper folding of the catalytic domain, but not the heme-bound domain. The catalytic activity of *Bpe*GReg is substantially decreased in the presence of the product, c-di-GMP, further suggesting that c-di-GMP binds to inhibitory sites and causes feedback inhibition of enzymatic activity. An examination of catalytic activities for reaction mixtures containing both *Bpe*GReg and a PDE showed that the catalytic rate of the coupled reaction (DGC and PDE) was substantially higher than that of DGC alone, confirming the presence of feedback inhibition of *Bpe*GReg. *Salmonella typhimurium* cell biofilm formation and swimming motility are altered by overexpression of *Bpe*GReg, corroborating that *Bpe*GReg is important for biofilm formation.[202]

**2.3.7. *Gs*GCS.** *Gs*GCS, a heme-bound globin domain of a GCS from *Geobacter sulfurreducens*, has been crystallized and structurally characterized.[203] The crystal structure of this protein indicates that the heme Fe(III) complex has a bis-His coordinated residue as the axial ligand, consistent with absorption and RR spectral studies. The histidine residue, which was unexpectedly found to be located at the E11 topological site, is the distal axial ligand for the heme Fe(III) complex. This arrangement is distinct from that for the E7 site of Hb, Mb, and other GCSs, although a histidine residue at the F8 site has been observed as a proximal axial ligand for this complex in other GCS and is not unusual.[50] RR and electron paramagnetic resonance (EPR) spectra and ligand ($O_2$, CO, and NO)-binding kinetic studies were additionally conducted to understand the role of amino acid residues in the heme environment in the ligand binding, recognition, and discrimination. The DGC activity of *Gs*GCS was not assessed. Regarding the unusual heme binding structure of this globin protein, further studies remain to be conducted.

**2.3.8. HemAC-*Lm*.** The globin-coupled adenylate cyclase, HemAC-*Lm*, in the unicellular eukaryotic organism trypanosomatid parasite *Leishmania major* is the first adenylate cyclase whose function was found to be up-regulated by $O_2$ binding to the heme Fe(II) complex in the N-terminal sensing domain.[132,133] Gene knockdown and overexpression studies suggest that $O_2$-dependent cAMP signaling via protein kinase A plays a fundamental role in cell survival through suppression of oxidative stress under hypoxic conditions. In addition, the enzyme-dependent cAMP generation exerts both stimulatory and inhibitory effects on the proliferation of *Leishmania* promastigotes during normoxia. Thus, $O_2$-dependent cAMP generation by adenylate cyclase likely plays a role in cellular adaptability to varying $O_2$ tensions.[132]

A homology modeling study suggested that His161 is the axial ligand on the proximal side in the sensing domain.[133] Catalytic studies of H161A revealed that the catalytic activity of the heme-free (apo) form (as confirmed by spectral studies) is very low: 0.5 $min^{-1}$. This value is lower than that for both inactive, $O_2$-free (1.2 $min^{-1}$) and stimulated, $O_2$-bound (20 $min^{-1}$) forms. This finding is noteworthy because the catalytic activities of heme-free forms of other heme-based oxygen sensors, such as *Ec*DOS[120,123] and YddV,[116] are high and comparable to those of the $O_2$-stimulated forms, with the exception of *Ax*PDEA1, in which activity is lost in the heme-free form.[131]

Reconstitution of the heme iron complex with a His161Ala mutant revealed that the dissociation rate constant of $O_2$ from the heme Fe(II) complex for the heme-reconstituted His161Ala mutant (51 $s^{-1}$) is significantly higher than that for the wild type (9.1 $s^{-1}$). The autoxidation rate constant for the heme Fe(II)−$O_2$ complex of the heme-reconstituted His161Ala mutant (6.1 $s^{-1}$) is also substantially higher than that of the wild type (<0.0001 $s^{-1}$). These results suggest that the reconstituted heme iron complex is not properly bound to the heme-binding site in the protein, resulting in characteristics that are different from those of the wild-type protein. Thus, proper binding of the heme iron complex to the protein is essential for $O_2$ binding to the heme Fe(II) complex to produce structural changes in the sensing domain that are optimal for catalytic enhancement.

### 2.4. AppA: Heme Sensor Domain with a Central SCHIC Fold

The AppA−PpsR system of *Rhodobacter sphaeroides* plays a key role in up-regulating the expression of numerous photosynthetic genes.[57] PpsR is a transcriptional repressor and AppA is an antirepressor capable of sensing not only $O_2$ but also light. AppA senses light through a FAD chromophore bound to its N-terminal BLUF (sensors of blue light using FAD) domain. Light causes dissociation of the AppA−PpsR complex, releasing PpsR to repress the transcription of photosynthesis-related genes.[57]

AppA is composed of an N-terminal BLUF domain, a central domain containing a noncovalently attached heme, and a C-terminal cysteine-rich domain. It has been suggested that AppA senses $O_2$ indirectly through its C-terminal cysteine-rich domain, an approximately 50 amino acid long fragment containing six cysteine residues. These residues are proposed to respond to changes in the cellular redox state by reversible formation of disulfide bonds.[57] The heme-binding domain in AppA is distantly related to the vitamin $B_{12}$-binding domain and thus has been designated SCHIC, for sensor containing heme instead of cobalamin.[57]

Binding of $O_2$ to the heme Fe(II) complex in AppA discoordinates the heme, and subsequent $O_2$ dissociation fully restores heme coordination. In vitro, the extent of heme discoordination increases gradually with rising $O_2$ levels over a broad concentration range. This response correlates well with the graded decrease in the transcription of photosynthesis-related genes mediated by AppA and its partner repressor PpsR. Taken together, the findings of this previous study suggest that the AppA−PpsR regulatory system functions as an oxygen-dependent transcriptional rheostat via the heme iron complex. However, if heme discoordination occurred in solution in vitro, the heme absorption peak should move to longer or shorter wavelengths, but in this study, the heme discoordination manifested as a decrease in heme absorption intensity. Thus, it is possible that precipitation or degradation of AppA heme, or some other phenomenon, occurred in response to the increase in $O_2$ concentration. Further studies will be required to elucidate heme iron behavior in AppA in response to changes in $O_2$ concentration.



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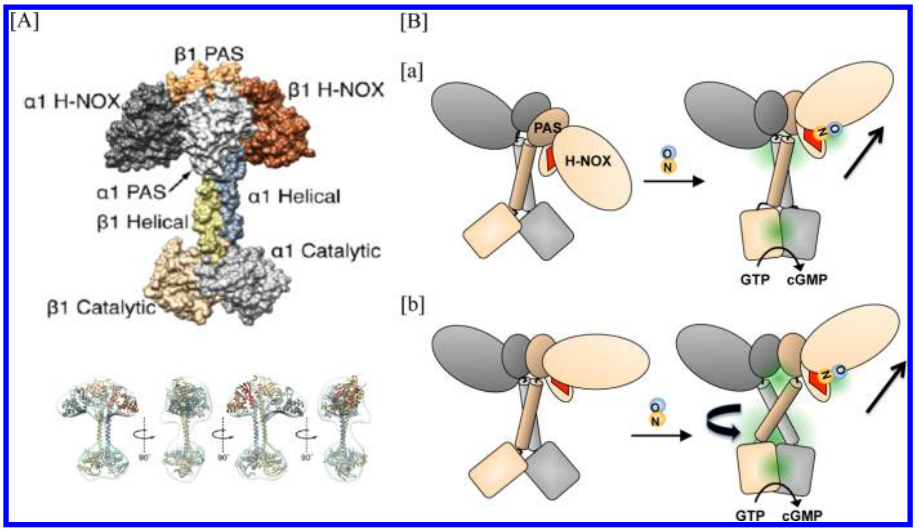


**Figure 9.** Proposed mechanisms of NO-induced activation of sGC based on both HDX-MA[250] and single-particle EM studies.[251] [A] The higher-order domain architecture is based on a recent single-particle EM study. Homology models of the individual domains could be fit into representative EM maps exhibiting two conformational extremes: the extended conformation [A] and a bent conformation (not shown). [B] NO binding releases and opens the heme-associated helix of the H-NOX domain while condensing and closing the active site pocket of the catalytic domain. Dominant points of conformational articulation are highlighted in green. The allosteric pathway bridging the sensor and output domains may involve two different mechanisms. [a] Large conformational changes at the junction between PAS and helical domains might indicate interdomain pivoting that relieves inhibitory contacts between H-NOX and catalytic domains. [b] Alternatively, the PAS-helical junction might mediate remote allosteric effects via long-range conformational changes propagated through the helical domains. Adapted from Campbell et al.[251] Reproduced with permission from ref 251. Copyright 2014 National Academy of Sciences, U.S.A. [A]. Adapted from Underbakke et al.[250] Adapted with permission from ref 250. Copyright 2014 Elsevier Inc. [B].

## 3. HEME-BASED NO SENSORS

NO is an important gas molecule involved in a wide range of signal transduction; it also contributes to cytotoxicity associated with numerous and ubiquitous important physiological and pathological functions in mammalian vascular, nervous, and immune systems.[11,12,14,15,18,22,204,205] In mammals, NO is synthesized from L-arginine by nitric oxide synthase (NOS).[11,15,20,21,204] Environmental nitrate ($NO_3^-$) and nitrite ($NO_2^-$), present in plants and food, for example, can be reduced and converted into NO, and thus represent a potentially important alternative source of NO.[25−27,206−210]

NO interacts with superoxide or molecular $O_2$ and is converted to peroxynitrite, which exerts numerous pathological functions, including tyrosine nitration.[28,29,205,211] NO itself interacts directly with cysteine thiolates on the surface of proteins, forming Cys−SNO, which plays an important role in critical functions associated with intermolecular signal transduction.[30−32,205,212−217] NO also interacts with metal centers, including nonheme iron complexes such as nitric oxide reductase, which is important for the biological denitrification process.[33,218−224] It also interacts with SoxR, NorR, iron regulatory protein, and FNR (fumarate and nitrate reduction regulator), which are involved in numerous important physiological functions through modulation of gene expression.[33,218,220,223−226] NO (or HNO) interacts with heme iron complexes, thereby regulating reactions associated with heme proteins, such as myoglobin, hemoglobin, cytochrome, and cytochrome oxidase.[10,58,227−231]

The importance of NO in the functions of heme-based NO sensors warrants particular emphasis. One of the best-known and most important heme-based NO sensors in eukaryotic physiology and pathology is sGC.[9,11,36,204,232−241] Bacterial heme-based NO sensors are also an emerging topic, providing interesting parallels with sGC from the standpoint of structure−function relationships, as detailed below in the section on H-NOX.

### 3.1. The Eukaryotic NO Sensor sGC

cGMP, a second messenger molecule that is critical for regulating diverse, important physiological functions, including smooth muscle relaxation, platelet inhibition, cell growth, and differentiation, is synthesized by the heme-based NO sensor, sGC (Table 2).[9,11,36,204,232−241] sGC senses picomolar levels of NO (~20 pmol). NO binding to the heme Fe(II) complex in the N-terminal sensing domain triggers guanylate cyclase function in the C-terminal domain, enhancing catalytic activity by ~200-fold. Despite the physiological importance of eukaryotic sGC, the structure of the full-length enzyme, and even that of the isolated heme-bound NO-sensing domain, has not been determined. This is in contrast to bacterial heme-based NO sensors (N-NOXs), which in many cases have been structurally resolved.[242−246] Accordingly, it has been suggested that structure and function relationships among bacterial heme-based NO sensors can provide valuable insight into the mechanisms of action of related eukaryotic proteins.[9,235,243,244,247−250]

**3.1.1. Protein Structure.** Full-length sGC is a heterodimer consisting of two homologous $\alpha$1 and $\beta$1 subunits, each composed of four domains (Figure 9).[235,249−251] The $\beta$1 subunit of sGC is composed of an N-terminal heme Fe-bound, NO-sensing H-NOX (heme-NO and oxygen-binding) domain, central PAS and helix domains, and a C-terminal guanylate cyclase domain. The N-terminal domain of the $\alpha$1 subunit does not contain a heme iron complex. The PAS and helical/coiled-coil domains are responsible for heterodimer formation and intramolecular, intersubunit, and interdomain signal transmission.[247,252] The C-terminal of both subunits constitute the cyclase active site, which is formed at the subunit interface.[253]


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The structure of the full-length, heme-bound heterodimeric form of sGC remains undetermined.[235] However, structures of sGC domain truncations, H-NOX, and homologues have been reported and modeled using diverse approaches, including single-particle electron microscopy (EM)[251] and hydrogen/deuterium exchange mass spectrometry (HDX-MS).[250] Single-particle EM has revealed the following structural features: (1) sGC is separated into two main globular densities and exhibits continuous flexibility; (2) the dimeric catalytic domain corresponds to a smaller, heart-shaped density; (3) the helical coiled-coil domain forms an elongated stalk that provides two points of flexible movement and rotation; (4) PAS and H-NOX domains cluster together in a larger, four-lobed density; and (5) the conformational flexibility of sGC is retained upon NO binding.[251] An HDX-MS analysis of the full-length sGC heterodimer further revealed that NO binding affects several discrete regions. Specifically, NO binding to the H-NOX domain perturbs a signaling surface implicated in PAS domain interactions; it also elicits striking conformational changes in the junction between the PAS and helical domains that propagate as perturbations throughout adjoining helices. Ultimately, NO binding stimulates the catalytic domain by contracting the active site pocket (Figure 9).[250]

Results of a FRET study using endogenous tryptophan residues as donors and the substrate analogue 2′-Mant-3′-dGTP as an acceptor suggested that NO binding to the heme Fe(II) complex drives Trp22 in the $\alpha$B helix of the $\beta_1$ heme binding domain and Trp466 in the second short helix of the $\alpha_1$ coiled-coil domain closer to the catalytic domain. It was proposed that the respective domains act like "a pair of tongs", forcing the catalytic domain into the NO-activated conformation.[254]

**3.1.2. Coordination Structures of the Heme Fe(II) Complex.** In general, binding of external ligands such as $O_2$, NO, CO, cyanide, or imidazole to the heme iron complex of a heme protein forms a 6-coordinated, low-spin complex.[1,6] Catalytic activation of sGC by NO binding to the heme iron complex is unique in that it forms an unusual 5-coordinated heme Fe(II)−NO complex. Importantly, formation of the 5-coordinated NO−heme Fe(II) complex in the $\beta$1 subunit of sGC is necessary for catalytic activation (Figure 9).[9,58,59,234,242,255] Rupture of the heme−histidine bond and formation of the 5-coordinated NO-heme Fe(II) complex in sGC have been rationalized on the basis of the crystal structure of the H-NOX protein from *Shewanella oneidensis*,[242] as described later. Time-resolved spectroscopy was used to evaluate the dynamics of heme coordination after NO release and binding.[256] After photodissociation of NO, all heme transitions occurred in the range of 1 ps to 0.2 s, notably the breaking and reformation of the bond between the heme iron and proximal His, considered major events for the activation/deactivation processes. Thus, the structural allosteric transition likely occurred in the time range 1−50 $\mu$s.

CO binding results in formation of a 6-coordinated CO−heme (II) complex that only slightly enhances catalysis, whereas $O_2$ does not bind to the heme Fe(II) complex, probably due to strict steric constraints and/or the hydrophilic environment of the heme distal side, which would repel $O_2$ binding. A weak Fe(II)−proximal His bond has also been suggested to explain the low $O_2$ binding to sGC.[257] In addition, the redox potential value is very high (+187 mV); thus the heme Fe(II) complex is very stable and is not prone to be oxidized to the heme Fe(III) complex.[257]

Whereas high-affinity binding of NO to the sGC heme Fe(II) complex stimulates catalytic activity, oxidation of the sGC heme to the heme Fe(III) complex desensitizes the enzyme to NO because of the low affinity of NO for the Fe(III) complex. The heme-oxidized state of sGC has emerged as a potential therapeutic target in the treatment of cardiovascular disease. Reductive nitrosylation of the heme Fe(III) complex in sGC proceeds through 2 equiv of NO. However, the heme Fe(II)−NO complex generated by interaction between heme Fe(III) and NO is not the active form. In the presence of NO, a rat sGC doubly mutated at $\beta_1$Cys78 and $\beta_1$Cys122 was shown to exhibit catalytic activity comparable to that of the heme Fe(II) complex form of wild-type rat sGC. From these results, it was inferred that sGC undergoes a reductive nitrosylation that is coupled to *S*-nitrosylation of a cysteine residues in sGC, such that heme-assisted $\beta_1$Cys78 and $\beta_1$Cys122 nitrosothiol formation causes NO desensitization of sGC containing a heme Fe(III) complex.[258] It has also been suggested that both $\beta_1$Cys243 and $\beta_1$Cys122 of rat sGC are involved in desensitization.[259] For human sGC, it was suggested that interface residues are important in promoting an optimal alignment of the catalytic center and that heterodimerization is required, but not sufficient, for activity.[248]

**3.1.3. Catalytic Enhancement of sGC by Chemical Simulators and Activators.** In addition to NO donors, nitrate and nitrous, numerous chemicals, including YC-1 and BAY compounds (developed by Bayer), are known to regulate sGC catalysis even in the absence of NO or the heme iron complex.[232,233] These chemicals have potential for use as drugs to treat conditions involving cGMP-related physiology and pathology.

The first compounds developed and advanced to modulate sGC activity were highly specific and potent sGC simulators.[232,233] These simulators exert their effects in a heme-dependent, but NO-independent, manner. YC-1 and BAY 41-2272, and their advanced derivatives, have multiple hetero-aromatic rings whose structures are suitable for achieving high potency. A RR spectral study suggested that YC-1 binding substantially activates the heme Fe(II)−CO complex of sGC by causing a protein conformational change in the linker region; additional contributions to activation are provided by allosterically regulated changes in heme geometry and the formation of a 5-coodinated Fe(II)−CO complex.[260,261] This study also suggested that YC-1 and BAY 41-2272 activate sGC through a different mechanism in the presence of excess NO.[260,261] On the basis of linked equilibrium binding measurements, surface plasmon resonance, and domain truncations in *Manduca sexta* and bovine sGC, it was suggested that YC-1 binds near or directly to the H-NOX domain of the $\beta$ subunit, and this binding overcomes allosteric inhibition by the $\alpha$ subunit.[262] Catalytic and structural studies of sGC and *Nostoc* sp. H-NOX with bound BAY 58-2667 or BAY60-2770 suggested that these compounds displace the heme and act as heme mimetics through both carboxylate-mediated interactions with the conserved YxSxR motif and hydrophobic interactions, as described below (H-NOX section).[263−265]

sGC activators operate on heme-free sGC, which can be formed under oxidative stress conditions that have been causally linked to cardiovascular diseases.[233] In this latter study, the authors used a high-throughput screening method to identify an sGC activator with unprecedented potency. This compound, containing two dicarboxylic acids, acts in a completely different manner from other sGC stimulators,


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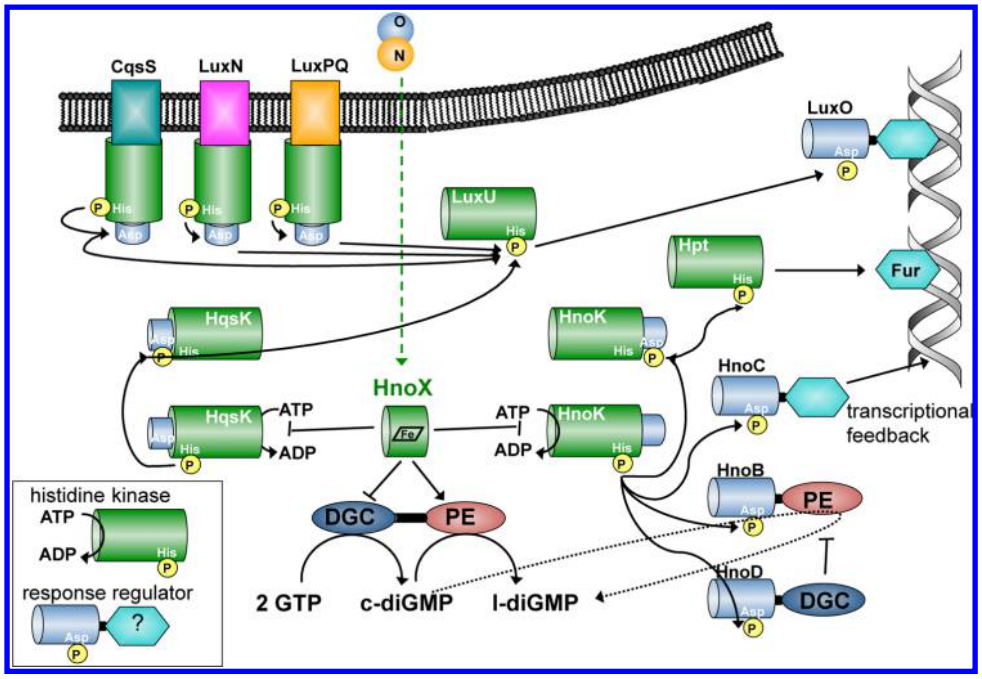


**Figure 10.** NO-induced regulation involving interactions with stand-alone H-NOX and other functional proteins. H-NOX is a stand-alone, isolated heme-bound protein whose structural changes are caused by NO binding to the heme Fe(II) complex, leading to interactions with other functional proteins and up- or down-regulation of important physiological functions (e.g., DGC, PDE, and histidine kinase in two-component systems) in bacteria. Thus, the NO sensor in this concept is different from other heme-based gas sensors in which the heme-bound gas-sensing site is located at an N-terminal site within the sensor protein that also contains a functional domain at a C-terminal site. Adapted from Plate and Marletta.[56] Note that DNR, a heme-based NO-sensing transcriptional regulator,[282−284] YybT, a heme-based NO sensor PDE,[285,286] and E75, heme-based NO-sensing nuclear receptor with the *c*-type heme,[227] are fused type sensors similar to the heme-based $O_2$ sensors, but not stand-alone type.

stimulating sGC in both an NO- and a heme-independent fashion. Detailed molecular studies that might enable exploitation of this unique activation mechanism have not yet been done.

### 3.2. Crystal Structures of the Heme-Bound, NO-Sensing H-NOX Domain of Bacterial NO Sensors: Stand-Alone Type

Crystal structures of bacterial H-NOX NO-sensing domains provide a convenient and effective tool for exploring the molecular mechanism of NO-stimulated catalytic enhancement of sGC. Importantly, many bacterial H-NOXs are stand-alone heme-based NO sensors. Accordingly, an isolated NO-sensing domain alone is capable of exerting its function by association with or dissociation from another isolated, functional protein without being covalently attached to the N-terminus of the full-length protein, as is the case for heme-based $O_2$ and CO sensors.[56]

**3.2.1. *Tt*H-NOX.** Bacterial *Tt*H-NOX domains from *Thermoanaerobacter tengcongensis* consist of an N-terminal helical subdomain and a C-terminal subdomain (Table 2).[56,246] The heme cofactor is deeply buried between the two subdomains, and the central iron is coordinated axially to a conserved histidine residue on $\alpha$-helix F (Figure 6[8]). Furthermore, the propionate groups of the heme form salt bridges with an absolutely conserved YxSxR motif, which is also required for stable heme binding to the protein and activation in sGC. Notably, the structure of the protoporphyrin IX group is distorted from planarity to an extent unprecedented for protein-bound heme groups. A comparison of the structure of two different crystal forms of *Tt*H-NOX suggests a mechanism whereby alteration in the degree of distortion of the heme group is coupled to changes on the molecular surface of the H-NOX domain and potentially to changes in intermolecular interactions.[246]

The isolated heme-bound N-terminal domain of a putative NO sensor from *T. tengcongensis*, TT-$SONO_{HD}$ (heme domain of sensor of NO from *T. tengcongensis*), was independently determined by another group (Figure 6[9]).[245] These studies showed that the tertiary topology of TT-$SONO_{HD}$ is characterized by a proximal domain with an $\alpha\beta\beta\alpha\beta\beta$ motif and an $\alpha$-helical domain. The heme is sandwiched between the two domains, and its iron atom is coordinated by His102 in the proximal helix. A modeling study suggested that a tyrosine residue is present within hydrogen-bonding distance of the terminal $O_2$ atom in the heme Fe(II)−$O_2$ complex. The protein structure of a SONO orthologue from *Clostridium botulinum* (CB-$SONO_{HD}$) was inferred from spectral studies of putative distal mutants. These studies suggested that Tyr139 in the heme distal side interacts with NO in the 5-coordinated heme Fe(II)−NO complex, thereby modulating the bond strength of the proximal Fe−His linkage and subsequently breaking the Fe−His bond.

**3.2.2. *Ns*H-NOX.** Crystal structures of ligand-free, NO-bound, and CO-bound H-NOX domains of *Ns*H-NOX, a cyanobacterial homologue of sGC from *Nostoc punctiforme*, were used as a platform for considering structure and functional relationships of eukaryotic sGC (Figure 6[10]).[243] A heme pivot-bend mechanism was proposed to explain the difference between the robust activation of sGC induced by NO and the slight activation induced by CO. NO and CO bind to the distal face of the heme and cause the heme to pivot to varying degrees away from Phe74 (Trp74 in *Ns*H-NOX). The larger pivot for CO is likely a result of two features: the limited space adjacent to Phe74 to accommodate the carbon atom, which has


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a larger radius than the nitrogen in NO, and the preference of CO to bind perpendicular to the heme. The involvement of residue 145 in NO-related catalytic regulation was also noted. It was further proposed that the second step in the NO activation mechanism involves heme bending and N-subdomain movement. The crystal structure of the activator BAY 60-2770 bound to *Ns*H-NOX revealed that BAY 60-2770 displaces the heme and acts as a heme mimetic through carboxylate-mediated interactions with the conserved YxSxR motif as well as hydrophobic interactions.[263] The hydrophobic phenyl moiety in the BAY 60-2770-bound protein has less flexibility than the BAY 58-2667-bound protein. The more rigid activator, BAY 60-2770, causes the protein region near residue 111, which is suggested to be important for the activation mechanism, to be more ordered as compared to that observed in the BAY 58-2667-bound protein.[263,265]

**3.2.3. *So*H-NOX.** High-resolution crystal structures of the H-NOX protein from *Shewanella oneidensis* in the unligated, intermediate 6-coordinated and activated 5-coordinated NO-bound states[242] revealed several structural features of the heme pocket of the unligated protein that are necessary for maintaining distortion of the heme from planarity. NO-induced scission of the Fe−His bond elicits a profound conformational change in the protein as the result of structural rearrangements in the heme pocket that permit the heme to relax toward planarity, yielding the signaling-competent, NO-bound conformation.[242] These results are consistent with suggestions based on NMR spectroscopy[266] and crystal structures of a His102Gly mutant[267] that artificial "cleaving" of the Fe−His bond induces a conformational change involving rotation of the distal subdomain relative to the proximal subdomain, centered about the heme cofactor. Paralleling this conformational change, severing the Fe−His bond was found to result in relaxation of the heme toward planarity.

These results are also consistent with a previously reported NO-induced activation model.[242,266−268] In this model, in the basal unligated Fe(II) state, the heme is distorted from planarity due to steric interactions with key conserved residues in the heme pocket. NO binding and subsequent scission of the Fe−His bond relieves the steric strain imparted on the heme, allowing it to relax toward planarity and, through intimate interactions of the protein and heme, to induce a rotation of the distal subdomain with respect to the proximal subdomain. This conformational change is thus hypothesized to communicate NO binding to downstream signaling partners.[242,266−269]

### 3.3. Functions of Bacterial NO Sensors

It has been well established that bacterial NO sensors that are separated from the functional protein they regulate are fundamentally distinct from other heme-based gas sensors in which the N-terminal heme-bound gas-sensing domain is covalently attached to the C-terminal functional domain (Table 2).[56,67]

A number of recent studies have provided details of the physiological functions of bacterial NO sensors that had previously been studied mainly from a structural and biophysical perspective (Figure 10).[56] On the basis of extensive structural and spectral studies focusing on the heme surrounding the NO-binding site, it was suggested that the heme distal side of bacterial NO sensors adopts a characteristic structure. Specifically, these studies showed that the high ligand specificity of bacterial NO sensors, which strongly favors NO over $O_2$ binding, prevents $O_2$ binding to the heme Fe(II) complex. This ligand specificity is dependent on a number of features of the protein, including the hydrophobic heme pocket, hydrogen-bonding networks containing a tyrosine residue as the NO binding site, heme distortion, protein flexibility, and a characteristic ligand access channel.[56,245,270] The crystal structure of the heme Fe(II)−$O_2$ complex of *Tt*H-NOX shows a hydrogen-bonding network in the distal pocket of the heme, consisting of Tyr140, Trp9, and Asn74. The tyrosine residue forms a direct hydrogen bond with $O_2$ bound to the heme Fe(II) complex[56,246] (Figure 6 [8]). The hydrogen-bonding network is absent in the distal heme pocket of the NO-selective *Ns*H-NOX protein and is instead replaced by nonhydrogen-bonding, hydrophobic residues[56,243] (Figure 6 [10]). Amino acid sequence alignment shows that the tyrosine and parts of the hydrogen-bonding network are conserved in the $O_2$-binding H-NOX subfamily, whereas hydrophobic residues occupy the same position in the NO-selective H-NOX proteins, including sGC.[56,243] Mutation of the Tyr to Phe in *Tt*H-NOX weakens the $O_2$-binding affinity.[271] Introduction of a Tyr into the distal heme pocket of the NO-selective H-NOX of *Legionella pneumophila* and a sGC truncation ($\beta$1[1-385]) allows the formation of a weak $O_2$ complex.[271] Incorporation of a Tyr into full-length sGC heme distal pocket alters NO binding dissociation kinetics, $O_2$ binding, and specific activity in the presence of NO,[270] confirming that the Tyr residue is a key determinant for $O_2$ binding and for NO exclusion.[56,271] However, introduction of a Tyr into full-length sGC by another group did not confer $O_2$ binding,[56,264] suggesting that the mechanism of oxygen exclusion by sGC not only involves the lack of hydrogen bonding in the distal heme pocket, but also depends on structural elements from other domains of sGC. Interestingly, this same tyrosine residue is important for GCSs containing a heme-bound globin or GAF fold,[50] as described above.

**3.3.1. *So*H-NOX: Stand-Alone Type.** HnoX (or *So*H-NOX), a stand-alone NO sensor from *S. oneidensis*, regulates the separate histidine kinase HnoK as part of a multicomponent signal transduction system (Figure 10). NO-binding to the heme Fe(II) complex in the HnoX protein inhibits autophosphorylation at the histidine residue of HnoK (see Figure 4).[67,272,273] Signal transduction by multicomponent systems plays important roles in numerous physiological functions in bacteria, as described above (see the section on FixL).[67] The phosphoryl group on the phosphorylated histidine of HnoK is transferred to an aspartate residue in multiple response regulators, switching on their output functions. The response regulators of *So*H-NOX include HnoC, which controls transcription, HnoB, which is a PDE toward c-di-GMP, and HnoD, which regulates the function of HnoB. NO-controlled repression of HnoB PDE activity leads to an increase in c-di-GMP levels, which results in a switch to a lower-motility pattern and "stickier" bacteria.[272,273]

**3.3.2. *Sw*H-NOX: Stand-Alone Type.** *Sw*H-NOX, from the biofilm-dwelling bacterium *S. woodyi*, mediates NO-induced biofilm dispersal (Figures 7 and 10).[274,275] *Sw*H-NOX is cocistronic with a gene encoding a dual-functioning DGC/PDE enzyme, designated HaCE (H-NOX-associated cyclic-di-GMP processing enzyme). In the absence of NO, *Sw*HaCE stimulates DGC activity, while PDE activity remains at basal levels. In the presence of NO-bound *Sw*H-NOX, however, *Sw*HaCE PDE activity is up-regulated, while cyclase activity remains at basal levels.


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The H-NOX-associated protein Lpg1057 (*Lp* HaCE) from *Legionella pneumophila* exhibits in vitro DGC activity that is inhibited by NO-bound H-NOX.[276]

**3.3.3. H-NOX from *Pseudoalteromonas atlantica*: Stand-Alone Type.** A stand-alone H-NOX from *P. atlantica* regulates the histidine kinase activity of a separate HahK protein as part of a two-component signal transduction system (Figures 4 and 10).[277] The autophosphorylation activity at the histidine residue in the HahK protein is inhibited by NO binding to the heme Fe(II) complex in the isolated H-NOX protein. A phosphoryl transfer reaction links HahK to a cognate response regulator, a protein annotated as a c-di-GMP-degrading enzyme termed HarR (H-NOX-associated response regulator). The resulting decrease in c-di-GMP levels modulates biofilm formation. Thus, biofilm formation is regulated, in part, by NO through the HahK−HarR two-component signal transduction system.

**3.3.4. H-NOX from *Vibrio fischeri*: Stand-Alone Type.** Many genes are involved specifically in hemin uptake as a source of iron, linking NO-H-NOX signaling to repression of hemin acquisition and utilization.[56,278] The NO-bound, stand-alone H-NOX in *V. fischeri* down-regulates expression of hemin-specific ferric uptake regulator (Fur)-associated genes through a two-component signaling pathway containing an isolated histidine kinase protein (HnoK) and a response regulator attached to its own HnoK protein (Figures 4 and 10).[278]

**3.3.5. H-NOX in *Vibrio harveyi*: Stand-Alone Type.** Bacteria use small molecules to signal their presence and gauge their surroundings, or to assess the density and identity of nearby organisms and formulate a response.[279−281] This process, called quorum sensing, regulates bioluminescence, biofilm formation, and virulence, and plays a role in communication and competition between different bacterial species. *V. harveyi* has four quorum-sensing pathways. In one, an NO-bound, stand-alone H-NOX regulates autophosphorylation of a separate kinase named HqsK that transfers a phosphoryl group to a common phospho-relay protein, LuxU, which ultimately regulates bacterial bioluminescence (Figures 4 and 10). Thus, phosphorylated LuxU transfers a phosphoryl group to LuxO, a transcription factor that, when phosphorylated, indirectly represses the master regulator of quorum sensing, LuxR, through activation of quorum regulatory RNAs (Qrrs).[56,279,280]

**3.3.6. DNR: Fused Type.** Heme-containing DNR (dissimilative nitrate-respiration regulator) of *Pseudomonas aeruginosa* is an NO sensor involved in regulating transcription of genes associated with nitrate respiration (denitrification).[282,283] DNR contains an N-terminal H-NOX domain and a C-terminal functional domain within a single protein molecule (fused type) (Figure 10), distinguishing it from the prototypal bacterial NO sensor described in the preceding sections, in which the H-NOX protein exists as an isolated form (stand-alone type). DNR belongs to the CRP-FNR (cAMP receptor protein/fumarate and nitrate reductase regulators) superfamily, whose members sense a wide variety of stimuli, such as $O_2$, NO, and redox state due to their ability to bind various cofactors. Generally speaking, CRP-FNRs are homodimers in which each monomer is composed of (1) an N-terminal sensing domain containing a fold typical of the cAMP-binding domain of CRP; (2) a dimerization-mediating $\alpha$-helix recruited to form the dimer interface; and (3) a C-terminal DNA-binding domain containing a helix-turn-helix motif. The heme iron complex in DNR is presumably located at the dimerization helix containing the axial ligands, His139 and His187. NO binding to the heme iron complex forms a 5-coordinated NO−heme Fe(II) complex and triggers its DNA binding. Protein flexibility and dynamics are key structural features essential for explaining the evolutionary success and adaptability of CRP-FNRs, and may represent a common strategy employed by heme-based redox sensors, features fundamentally different from those of canonical heme proteins. Furthermore, fluorescence anisotropy studies have shown that high-affinity, sequence-specific DNA binding of DNR occurs only when the heme is nitrosylated, consistent with the proposed function of DNR as an NO sensor and transcriptional activator. This role is further supported by the NO "trapping" properties revealed by ultrafast spectroscopy, which are similar to those of other heme-based NO-sensor proteins.[284] Further studies will be required to elucidate the structure and function relationships of DNR.

**3.3.7. YybT: Fused Type.** YybT family proteins (COG3887) are proteins of generally unknown function that are widely distributed among the firmicutes, including the human pathogens *Staphylococcus aureus* and *Listeria monocytogenes*.[285,286] YybT family proteins contain an N-terminal domain that shares minimum sequence homology with the PAS domains. *Bs*YybT and *Gt*YybT, two representative YybT family proteins from *B. subtilis* and *Geobacillus thermodenitrificans*, respectively, are found to bind *b*-type heme with 1:1 stoichiometry. YybT is composed of an N-terminal heme-bound NO-sensing domain and a C-terminal PDE domain (fused type), but the domain arrangement is different from that of prototypical bacterial NO sensors in which the NO sensor protein, H-NOX, exists as a separate protein that interacts with a separate functional protein to exert its regulatory effects. The coordination of NO to the heme iron complex creates a 5-coordinated heme Fe(II)−NO complex that stimulates PDE activity toward c-di-GMP, suggesting that YybT family proteins function as stress-signaling proteins involved in monitoring cellular heme or NO levels (Figures 7 and 10).[285] The heme-free form, apoYybT, has significantly higher catalytic activity than that of the Fe(II)−NO-bound form, suggesting that binding of the heme iron complex to the enzyme serves to suppress catalysis, similar to the heme-based oxygen sensors *Ec*DOS and YddV, described above. An NMR study revealed that YybT possesses a PAS domain containing a potential ligand-binding pocket, reinforcing the view that the PAS domain of this enzyme functions as a regulatory domain in modulating cellular cyclic di-AMP concentrations in response to NO.[286]

### 3.4. Insect E75: Fused Type

Extensive biochemical and physicochemical studies, including ligand binding kinetics analyses, have been conducted on E75 family nuclear receptors from the insects *Oncopeltus fasciatus* and *Blattella germanica*.[227] These studies have shown that E75 proteins contain a heme iron complex in their ligand-binding domain. Their homologous nuclear receptors, Rev-erb$\alpha$ and Rev-erb$\beta$, have been implicated as heme-responsive heme sensors, in which association of the heme iron complex with the ligand-binding domain, or dissociation from it, regulates transcriptional activity. However, the E75 protein from *O. fasciatus* was shown to exhibit covalent heme binding via heme methyl group, strongly suggesting that this protein is an NO sensor or diatomic gas sensor. It is not clear if the E75 protein acts as a redox sensor, although redox sensor proteins


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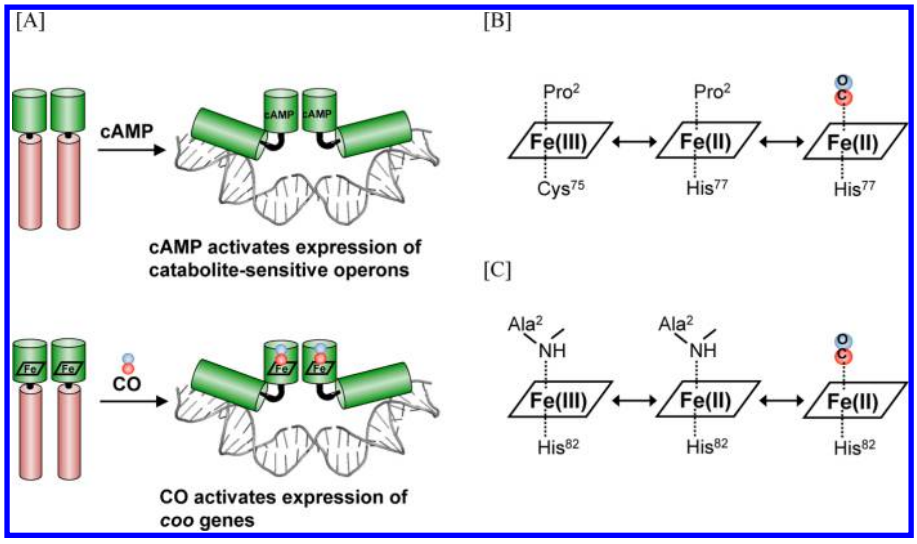


**Figure 11.** Heme coordination and protein structural changes that occur upon CO binding to the heme Fe(II) complex in *Rr*CooA ([A], lower)[310,312] as compared to the protein structural changes caused by binding of cAMP to the catabolite gene activator protein (CAP) family ([A], upper). CO binding to the heme Fe(II) complex in the dimeric *Rr*CooA protein induces profound structural changes that enable the C-terminal domain to interact with DNA in the promoter region of the *coo* gene. This CO-induced activation of *Rr*CooA is similar to cAMP-induced activation of CAP. Note that the proposed changes in coordination structure induced by redox changes and CO binding for *Rr*CooA [B] are different from those of *Ch*CooA [C].[34,332,333]

containing a covalent *c*-type heme iron complex (e.g., DcrA, GSU0582, GSU0935, and MA4561) have been characterized, as described below (see heme redox sensor with *c*-type heme).

### 3.5. Human Cystathionine β-Synthase: Fused Type

Mammalian cystathionine β-synthase (CBS) is a heme-bound enzyme that catalyzes the condensation of serine and homocysteine to produce cystathionine; it also generates $H_2S$ by catalyzing the β-replacement of cysteine with homocysteine.[145−147,287−291] Catalysis is regulated by heme redox and CO binding to the heme Fe(II) complex, as described below in the CO-sensor section. However, human CBS is inhibited by NO, which binds more rapidly and tightly to the heme Fe(II) complex than CO.[292] Thus, it is possible that human CBS is an NO sensor.

## 4. HEME-BASED CO SENSORS

Atmospheric CO is generated by numerous natural and industrial processes, such as volcanic activity and incomplete combustion (biotic and abiotic) of organic matter.[13,293] The concentration of CO is held below toxic levels by microorganisms that utilize it. In a number of aerobic and anaerobic bacteria, and also in certain archaea, CO can serve as a source of energy and/or cell carbon.[13,293]

CO is a key metabolite and signaling molecule for many species of CO-utilizing bacteria and archaea.[13,293−295] Numerous prokaryotes express distinct CO-regulated systems that catalyze catabolically productive aerobic and anaerobic CO oxidation. Aerobic organisms employ a high-affinity, oxygen-tolerant, molybdo-flavoprotein complex to effect CO oxidation. This system is encoded by *cox* genes and has been shown to be transcriptionally regulated.[13,294]

The pathways to anaerobic CO oxidation mechanisms that employ variants of the enzyme carbon monoxide dehydrogenase (CODH), the oxygen-sensitive Ni-containing protein complex that enables anaerobic CO utilization, encoded by *coo* genes, are distinct from CO oxidation in aerobes mediated by *cox* genes.[13,293−295] Anaerobic CO metabolism is usually controlled by the transcriptional regulator, CooA.[52,294−296] CooA is a heme-based CO sensor whose transcription of CO-associated metabolism in bacteria is triggered by CO binding to its heme Fe(II) complex. An additional prokaryotic CO-dependent transcriptional regulator is RcoM (regulator of CO metabolism), a heme-bound CO sensor that represents a one-component CO-sensing transcriptional factor (Table 3).[52,294]

CO gas is generated by heme oxygenase in pathogenic bacteria.[161−163,297−301] Pathogenic bacteria degrade the heme iron complex, which is biologically extracted from hemoglobin in the host cells. This degradation of the heme iron complex by heme oxygenase generates free iron, biliverdin, and CO as the enzymatic products. The main purpose of pathobacterial heme degradation is to use the heme-released iron as a nutrient. However, the various CO utilizing systems indicated above may oxidize the CO generated by heme oxygenase. Notable in this context, however, are the heme oxygenases MhuD from *M. tuberculosis* and IsdG/IsdI from *S. aureus*, which degrade the heme iron complex without forming CO; instead, they generate aldehyde-bound tetrapyrrole (mycobilin) and formaldehyde.[299]

CO is also an important signaling molecule in eukaryotes (Table 3). The physiological and pathological effects of CO in mammals, acting in part as an antioxidant, have been well documented.[12,13,16−19,302−305] In eukaryotes, CO is synthesized by heme oxygenase with the aid of NADPH cytochrome P450 reductase.[161−163,300−307] Unlike $O_2$ and NO, which are easily converted into reactive oxygen and nitrogen species, CO is a stable molecule that is difficult for eukaryotes to convert into other species. CO also interacts only with metal complexes, such as heme iron complexes or nonheme iron or molybdenum iron complexes,[308,309] and not with intracellular compounds or amino acid residues on the surface of proteins. This chemical behavior of CO is different from that of $O_2$, which interacts with amino acid residues and metal ion complexes directly and as reactive oxygen species (such as hydroxyl radical, superoxide anion, and $H_2O_2$). It also differs from that of NO, which interacts with amino acids on the protein surface directly and in its derived forms, such as peroxynitrite. Thus, probably most


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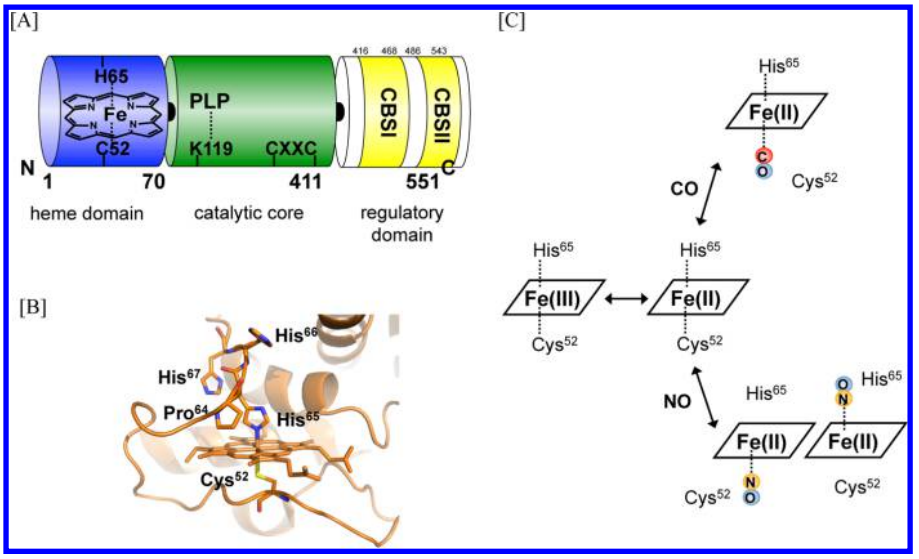


**Figure 12.** Domain organization [A] and close-up view of the heme Fe(III) complex surroundings [B] and the changes in coordination structure of the heme iron complex caused by CO and NO binding [C] in human CBS.[287,289]

(or all) eukaryotic physiological reactions associated with CO are caused by binding of CO with a heme iron complex or other metal cation complex.

### 4.1. CooA

*Rr*CooA from *Rhodospirillum rubrum* belongs to the catabolite activator protein (CAP) family of transcription regulators, whose members activate the expression of specific genes in response to distinct external signals.[52,310,311] *Rr*CooA is a homodimeric protein in which each monomer consists of an N-terminal effector-binding domain with a *b*-type heme and a C-terminal DNA-binding domain containing a classical helix-turn-helix motif (Figures 6[12] and 11A).[312] The two domains are connected via a long helix (the C-helix) that serves as the dimerization interface. For the heme Fe(II) complex of *Rr*CooA, His77 in both subunits provides one axial heme ligand, whereas the N-terminal nitrogen of Pro2 residues from the opposite subunit provides the other axial ligand. Binding of cAMP substantially changes the structure of homodimeric CAP proteins such that the C-terminal domain interacts with DNA.[310] CO binding to the heme Fe(II) complex activates *Rr*CooA, initiating the transcription of a series of genes encoding the CO oxidation system in *R. rubrum*.[34,52,296,313−317] On the basis of its overall structure and general comparison with CAP proteins, it is assumed that CO binding to the heme Fe(II) complex in the N-terminus of *Rr*CooA allosterically promotes adoption of similar profound structural changes in the C-terminus, enabling DNA interactions.[312]

The axial ligands for the heme Fe(III) complex of *Rr*CooA are Pro2, supplied by the other subunit in the homodimer, and Cys75 in the N-terminus.[312] Reduction of the heme Fe(III) complex to the heme Fe(II) complex causes dissociation of Cys75 from the heme Fe(II) complex, making His77 the axial ligand trans to Pro2 (Figure 11B).[318−324] Binding of CO to the heme Fe(II) complex in *Rr*CooA displaces Pro-2, which moves away from the heme, leading to substantial structural changes in the heme-bound domain that allosterically trigger C-terminal transcriptional or DNA binding activity (Figure 11).[55,325−330] Mapping of CooA-RNA polymerase interactions identified activating regions 2 and 3 in CooA.[331]

In *Ch*CooA from *Carboxydothermus hydrogenoformans*, a homologue of *Rr*CooA,[332] the axial ligands for the heme Fe(III) complex are His82 and the main chain NH of Ala2. Importantly, redox-dependent axial ligand switching does not occur for *Ch*CooA, resulting in CO binding to the heme Fe(II) complex trans to His82 (Figures 6[13] and 11C). This redox-insensitive axial ligand behavior is different from that of *Rr*CooA, in which the axial ligand Cys75 for the heme Fe(III) complex is replaced by His77 upon heme reduction to an Fe(II) complex. Thus, subtle and minute protein structural changes upon heme reduction that occur for *Ch*CooA are different from those of *Rr*CooA, as reflected in differences in these proteins' absorption and RR spectra and redox potential values. Nevertheless, the crystal structure of imidazole-bound *Ch*CooA suggests that the structural changes in *Ch*CooA caused by adding CO are similar to the profound changes in protein structure observed for *Rr*CooA.[333] Interestingly, adding NO promotes formation of a 6-coordinated heme Fe(II)−NO, yielding the DNA-binding, active form of *Ch*CooA.[334] This is in contrast to *Rr*CooA, in which adding NO causes formation of a 5-coordinated heme Fe(II)−NO complex that is inactive. However, whether *Ch*CooA is a heme-based NO sensor remains unclear.

RR spectral studies suggest that displacement of the C-helix by CO ligation is associated with DNA binding.[335,336] Additionally, it has been suggested that, once CO displaces the Pro2 ligand, the displacement of the heme into an adjacent hydrophobic cavity initiates the conformational change required for DNA binding.[337]

The low-frequency vibrational dynamics of the heme of *Ch*CooA have been investigated using vibrational coherence spectroscopy.[316] These studies suggested that distortion of the heme in the form of "doming" or "saddling" upon CO binding in *Ch*CooA is associated with the heme environmental and geometry changes that lead to activation of the DNA-binding domain. DNA binding to the *Rr*CooA heme Fe(II)−CO complex changes CO rebinding behavior, suggesting the presence of an allosteric transition in *Rr*CooA.[315]



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### 4.2. RcoM

RcoM is a single-component, CO-dependent transcriptional regulator that appears to regulate the expression of *coo* and *cox* genes involved in CO metabolism in different bacteria.[294] The RcoM proteins from *Burkholderia xenonorans*, $RcoM_{Bx}$-1 and $RcoM_{Bx}$-2, are composed of an N-terminal heme-bound PAS domain containing a 6-coordinated *b*-type heme Fe(II) complex and a C-terminal LytTR transcriptional/DNA-binding domain.[294,338] CO binding to the heme Fe(II) complex substantially enhances the transcriptional activity of $RcoM_{Bx}$.[294] $RcoM_{Bx}$-2 undergoes heme redox-dependent ligand switching in which the axial ligands in the heme Fe(III) complex, His74 and Cys94, change to His74 and Met104 in the heme Fe(II) complex and to His74 and CO in the heme Fe(II)−CO complex.[339,340] The ligand switching events caused by redox changes and/or CO binding induce profound structural changes in the heme-bound domain of the enzyme. $RcoM_{Bx}$-1 has a high CO affinity and multiple low-affinity DNA-binding states, suggesting a transcriptional "accumulating switch" that senses low but persistent CO levels.[341] Thus, the CO-dependent regulation of RcoM is significantly different from that of CooA, another CO-dependent transcriptional regulator.

### 4.3. Cystathionine β-Synthase

Mammalian CBS is a pyridoxal phosphate-dependent heme-binding enzyme that catalyzes the condensation of serine and homocysteine to yield cystathionine; in yeast and the protozoan *Trypanosoma cruzi*, the corresponding enzymes do not contain heme.[145−147,287−291] CBS also catalyzes the β-replacement of cysteine with homocysteine to generate $H_2S$ and the corresponding thiol ether.[145−147,287−291,342]

Mammalian CBS is composed of an N-terminal heme-bound domain, a central catalytic core, and a C-terminal regulatory domain, which consists of two CBS domains (Figure 12).[287,289−291,343−347] Pyridoxal phosphate is bound to Lys119 in the catalytic core, and *S*-adenosylmethionine is bound to the CBS domain, where it acts as an allosteric activator to exert intrasteric regulation.

For mammalian CBS, catalysis is regulated by heme redox and CO binding such that CBS containing a heme Fe(III) complex is active, whereas CO (or NO) binding to the heme Fe(II) complex inhibits catalysis.[287,289−291,348] In these enzymes, the role of the heme iron complex in catalysis is similar to that of heme-based oxygen sensors such as *Ec*DOS and YddV, in which the heme-free forms exhibit optimal catalytic activity and changes in the heme-redox state or $O_2$ binding regulate (switch on or off) catalysis. This suggests that supplying the heme iron complex to the enzyme serves to suppress catalysis, and changes in heme redox status or $O_2$ binding to or dissociation from the heme iron complex release this suppression.[50,116,120] Therefore, the role of the heme iron complex in catalysis of mammalian CBS appears critical for its functional role in mammalian cells. Considering that yeast and protozoan CBS enzymes have no heme, the heme-containing CBS enzyme would appear to represent an evolutionarily advanced, more sophisticated form that is particularly suited for mammalian survival in response to environmental stimuli.

CO binding to the heme Fe(II) complex of human CBS inhibits catalysis. The axial ligands for both heme Fe(III) and Fe(II) complexes of human CBS are Cys52 and His65 (Figures 6[14] and 12B]). CO binding to the heme Fe(II) complex displaces Cys52, and the axial ligands of the heme Fe(II) complex become CO and His65 (Figure 12C).[343−345,348] CO binding to the heme Fe(II) complex inhibits catalysis, probably because CO binding breaks the salt bridge between Arg266 and Cys52.[147,287,289−291,349]

The redox potential of the heme iron complex of human CBS is low (−350 mV), suggesting that the heme Fe(III) complex is extremely stable as compared to the heme Fe(II) complex. Because CO binds only to the Fe(II) complex and not to the heme Fe(III) complex, this raises questions about the feasibility of forming a heme Fe(II)−CO complex under physiological conditions. Human methionine synthase reductase, an NADPH-dependent diflavin oxidoreductase, is suggested to play a significant role as a CBS reductase, providing a possible resolution of this apparent conundrum.[147,350]

NO binding to the heme Fe(II) complex of CBS inhibits catalysis.[287,289,290] NO has been shown to bind to the heme Fe(II) complex of human CBS more rapidly and tightly than CO, with a $k_{on}$ value of $\sim 10^4$ $M^{-1}$ $s^{-1}$ and a $K_d$ value $\leq 0.23$ μM.[292] Therefore, NO may act together with CO and $H_2S$ as a gasotransmitter in CBS function to play a critical (patho)-physiological role in cell signaling.

### 4.4. BK (Slo1) and Kv1.4 $K^+$ Channels, Nav1.5 $Na^+$ Channel, and Connexin Hemichannels

The heme Fe(III) complex binds to the $Ca^{2+}$-dependent large-conductance $K^+$ (BK) channel, also known as Slo1,[351] and the voltage-dependent $K^+$ channel Kv1.4,[352] and regulates their functions. Heme oxygenase-2 is part of the BK/Slo1 channel complex, and CO, a product of heme oxygenase, significantly regulates BK/Slo1 channel function.[353−356] CO also mediates the antiapoptotic effects of heme oxygenase-1 in medulloblastoma DAOY cells via $K^+$ channel inhibition,[357] and inhibits the function of the cardiac $Na^+$ channel, Nav1.5.[358] CO inhibits connexin hemichannel opening as well.[359]

Interestingly, it has been suggested that the heme Fe(II) complex is a CO receptor in $Ca^{2+}$-dependent $K^+$ channels.[360] Domain structure and function relationships involving CO generated from human heme oxygenase-2 and the heme iron complex and BK/Slo1 channel under both hypoxic and normoxic conditions have been proposed.[361,362] However, notable in this context, CO binds only to the heme Fe(II) complex and not to the heme Fe(III) complex. Thus, it is not clear how the heme Fe(III) complex bound to those channel proteins and reduced to the heme Fe(II) complex, the CO acceptor. The molecular mechanisms underlying heme-induced functional regulation, notably including how CO engages this function to form a heme Fe(II)−CO complex, remain unresolved.

### 4.5. NPAS2, CLOCK, Rev-erbα, Rev-erbβ, E75, Per1, and Per2

CO appears to significantly contribute to the transcription regulation associated with circadian rhythms, and the heme iron complex, in particular the heme Fe(II) complex, is clearly an acceptor of CO in this system. In fact, transcriptional regulatory factors associated with circadian rhythms, including NPAS2,[363−368] CLOCK,[369,370] Rev-erbα,[371−373] Rev-erbβ[371,374−376] (Figure 6[15]), E75,[227,375,377] Per1,[378] and Per2,[379−384] are heme-binding proteins. It has been suggested that these proteins are heme-based CO or NO sensors, because either CO or NO binds to the heme Fe(II) complex bound to those transcriptional regulatory proteins. Importantly, the transcription regulatory activity of the heme-bound NPAS2-bMAL1 dimer is inhibited by CO binding to the heme Fe(II)


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complex.[363] It has also been suggested that heme oxygenase is involved in the transcriptional regulation by NPAS2 and bMAL, and subsequent action of mouse Per2. This implies that CO contributes to the transcription mediated by heme-bound transcriptional factors.[382] It has also been suggested that these proteins are heme sensors in which Cys serves as the axial ligand for the heme Fe(III) complex; thus, association/dissociation of the heme iron complex regulates transcription associated with circadian rhythms.[7,46,362,385]

E75 from *O. fasciatus* has been suggested to be an NO or CO sensor, because both NO and CO bind to the heme Fe(II) complex in this protein. However, a heme iron complex in this protein is covalently attached to the protein with the heme methyl group; this contrasts with E75 from *Drosophila melanogaster* and *Bombyx mori*, where the heme iron complex is bound to the protein with coordination bonds acting as axial ligands to the heme iron complex.[227,375,377]

However, it is not clear how the heme Fe(III) complex, which reportedly binds to the above-mentioned transcriptional regulatory factors, is reduced to the CO-binding heme Fe(II) complex. One possibility is that NO reduces the heme iron complex; in this scenario, the heme Fe(III) complex bound to CLOCK undergoes reductive nitrosylation in the presence of NO to generate the heme Fe(II)−NO complex.[369] CO might then substitute with NO and form the heme Fe(II)−CO complex. Clearly, however, additional research will be required to establish the molecular mechanism by which the heme Fe(II)−CO complex is involved in transcription associated with circadian rhythms.

## 5. HEME REDOX SENSORS WITH *c*-Type AND Non-*c*-Type HEME IRON COMPLEXES

Various regulatory sensors continually monitor the redox state of the internal and external environments and control the processes that work to maintain redox homeostasis.[386] Apart from its gas ($O_2$, NO, and CO) sensor role, the redox state of the heme iron complex is critical for the regulation of various functions in bacteria (Table 4). Heme-based redox sensors with *c*-type (covalently attached: Figure 1E) heme have been reported.[387−389] Importantly, autophosphorylation, perhaps as part of a two-component signal transduction system, is regulated by heme redox changes in MA4561, a heme-based sensor with a *c*-type heme.[387] Among other heme-based sensors with *c*-type heme, including GSU582/GSU935,[388,390−393] DcrA has been extensively studied with respect to structural and physicochemical properties of the isolated heme domain.[389] Unfortunately, catalytic and functional regulation of DcrA by heme redox changes has not been examined. A defining characteristic of sensors with *c*-type heme is their low reduction potential values, from −85 to −250 mV. This property suggests that the heme Fe(II) complex is more stable than the heme Fe(III) complex and that redox-dependent ligand switching occurs, probably accompanied by profound structural changes in the heme-bound site. Redox-dependent ligand switching and/or protein structural changes induced by gas binding to the heme were observed for most heme-based gas sensors whose crystal structures were determined. In addition, these changes in the coordination and/or protein structures appear to be tightly linked to the signal transduction from the heme-bound domain to the functional domain.

Cytochrome *c* is not a gas-binding heme protein and does not undergo redox-dependent ligand switching, but it is involved in electron transfer reactions.[8] Thus, heme sensors with a *c*-type heme should act as redox sensors in response to environmental redox changes. Global protein structural alterations in the heme-bound site accompanied by changes in coordination structure also appear to occur upon ligand (NO, CO) binding, suggesting the additional possibility that heme-based sensors with a *c*-type heme could act as gas (NO, CO) sensors.[227,389,390] The covalently attached "non-*c*-type " heme proteins are formed under various conditions, suggesting possibilities of emerging and new functions of heme proteins.[394−396] Furthermore, a heme redox sensor with non-*c*-type heme (NtrY) has been reported for the NtrY/NtrX two-component signal transduction system.[74] Other heme proteins that mediate heme redox-dependent catalysis and putative heme redox sensors are described below.

**Table 4. Functions of Heme Redox Sensors[a]**

| | functions | refs |
|---|---|---|
| Bacterial | | |
| MA4561 | histidine kinase/autophosphorylation | 387 |
| GSU582 and GSU935 | MCP | 388, 391−393 |
| DcrA | MCP | 389 |
| NtrY | TC with NtrX | 74 |
| phenylacetaldoxime dehydratase (OxdB) | dehydration of acetaldoxime | 397 |
| Insect | | |
| E-75 | transcription regulator | 227 |

[a]DcrA, heme redox sensor from *Desulfovibrio vulgaris* Hildenborough; E-75, a putative heme redox sensor or NO sensor from *Oncopeltus fasciatus*; GSU582 and GSU935, heme redox sensors from *Geobacter sulfurreducens*; MA4561, heme redox sensor from the methanogenic archaeon *Methanosarcina acetivorans*; MCP, methyl-accepting chemotaxis protein; NtrY, histidine kinase with the heme-bound PAS domain and a part of the NtrY/NtrX two-component system from *Brucella* spp.; phenylacetaldoxime dehydratase, phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1 (OxdB) that catalyzes the dehydration reaction of *Z*-phenylacetaldoxime to produce phenylacetonitrile; TC, two-component system.

### 5.1. MA4561

MA4561 of *Methanosarcina acetivorans* is a multidomain sensory kinase composed (in N- to C-terminal order) of PAS, GAF, PAS, GAF, and histidine kinase-like ATP domains.[387] The heme iron complex is covalently attached to Cys656 via a vinyl side chain in the second GAF domain of MA4561. The reduction potential value of MA4561 is −85 mV. MA4561 proteins containing a heme Fe(III) or heme Fe(III)−DMS (dimethyl sulfide) complex autophosphorylate, whereas those containing heme Fe(II), heme Fe(II)−CO, or heme Fe(II)−DMS do not, suggesting that this kinase is a redox sensor. MA4561 appears to be a sensor kinase in a two-component signal transduction system that regulates methyl sulfide metabolism. Notably, MA4561 is the only one heme redox sensor with a *c*-type heme whose catalytic or functional activity has been clearly demonstrated.

### 5.2. GSU582 and GSU935

The crystal structures and physicochemical properties of periplasmic sensor domains from two methyl-accepting chemotaxis proteins, GSU582 and GSU935, from *Geobacter sulfurreducens* have been characterized.[388] These studies showed that the sensor domains of both proteins form a swapped dimer and exhibit a PAS-type fold. Structural analysis of these heme


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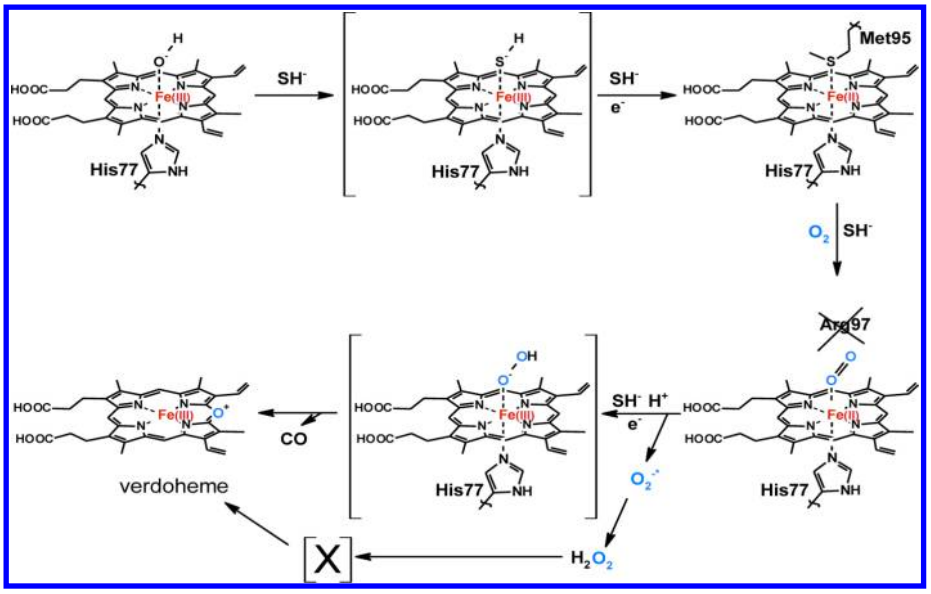


**Figure 13.** $H_2S$-induced heme modification. The mutations Arg97Ala and Arg97Ile, corresponding to the direct $O_2$-binding residues on the heme distal side, modify the porphyrin ring and cause formation of verdoheme, an oxygen-incorporated porphyrin, in the presence of excess $H_2S$.[159,160] Sulfheme, a well-known sulfur-incorporating porphyrin, is not formed under these conditions, unlike the case for hemoglobin and myoglobin.

Fe(III)-bound sensor domains revealed that the heme iron complex is covalently bound to the protein. His143-Fe(III)-water coordination was observed for GSU582, whereas His144-Fe(III)-Met260 and His344-Fe(III)-water coordination were observed for monomers A and B of GSU935, respectively. The redox potentials of both proteins are very low: −156 mV for GSU582 and −251 mV for GSU935. Although the structures of the heme Fe(II) complex-bound forms were not determined, redox-dependent ligand switching of the heme iron complex accompanied by profound protein structural changes were predicted for both proteins. These molecular characteristics may be related to the signal transduction mechanism of those chemotaxis-regulating proteins. Biochemical reactions in a reconstituted system have not been reported.

### 5.3. DcrA

The chemotaxis signal-transducing protein DcrA from *Desulfovibrio vulgaris* Hildenborough contains a *c*-type heme in its periplasmic domain that senses redox changes and/or $O_2$.[389] Physicochemical studies, including absorption and RR spectroscopy, and kinetic analyses of CO association/dissociation were performed on the heme-bound domain of DcrA. These studies showed that the CO affinity of the DcrA-bound heme Fe(II) complex ($K_d \approx 138$ $\mu$M) and redox potential of the protein (−250 mV) are both extremely low. Similar to the heme-sensors GSU582 and GS0935 containing a *c*-type heme, DcrA exhibits redox-dependent ligand switching. Again, biochemical reactions in a reconstituted system have not been reported.

### 5.4. NtrY/NtrX

NtrY, a histidine kinase containing a heme-bound PAS domain, is one component of the *B. abortus* NtrY/NitrX two-component signal transduction system.[74] The $O_2$-bound Fe(II) complex is very unstable, showing rapid oxidation to the heme Fe(III) complex in the presence of $O_2$. Under low $O_2$ tension, the active NtrY form containing a heme Fe(II) complex is formed, executing autophosphorylation and phosphoryl transfer reactions to the cognate response regulator NtrX. The phosphorylated form of NtrX binds to specific promoter regions and thereby controls the expression of genes encoding nitrogen respiration enzymes.

### 5.5. OxdB

Phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1 (OxdB) catalyzes the dehydration of *Z*-phenylacetaldoxime (PAOx) to yield phenylacetonitrile. OxdB contains a heme iron complex that serves as the active center of the dehydration reaction.[397] The enzymatic activity of the heme Fe(II) complex form of OxdB is ∼1150-fold higher than that of the heme Fe(III) complex form, indicating that the heme Fe(II) complex is the active form in OxdB catalysis. The catalytic mechanism has been suggested to include the following features: (1) the aldoxime residue in the substrate, PAOx, binds directly to the heme Fe(II) complex in the heme distal side; (2) dehydration proceeds through a general acid catalysis mechanism involving the heme Fe(II) complex with the aid of His306; and (3) phenylacetonitrile and water are generated as the final products. The affinity of the substrate, PAOx, for the heme Fe(III) complex in OxdB is much lower than that for the heme Fe(II) complex. Therefore, OxdB catalysis is regulated by the heme redox state. This suggests that OxdB is a heme-redox sensor, although the heme iron complex is directly involved in catalysis, but does not act as signaling receptor/sensor.

### 5.6. Insect E75

Insect E75 from *O. fasciatus* has an N-terminal covalently bound heme.[227] This heme is not a typical *c*-type heme that binds via a thiol-vinyl link, thus strongly altering the properties of the iron moiety. Rather, the heme in insect E75 is bound covalently to the heme methyl groups,[227] making it unlikely that insect E75 is the redox sensor. On the basis of spectral studies, it was suggested that insect E75 is a heme-based NO sensor, as described above.

### 5.7. *Ec*DOS

*Ec*DOS is a heme-based oxygen sensor PDE that acts on the substrate c-di-GMP as described above in the $O_2$ sensor section.[115,117,119,120] In *Ec*DOS, the heme iron complex is bound to the PAS domain through coordination bonds with


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amino acids of the protein, as the axial ligands, and through ionic bonds between the heme propionate and an arginine residue of the protein, but no *c*-type (covalently attached) heme is formed.[51,85] The *Ec*DOS-associated PDE activity toward cAMP is regulated by the heme redox state of the enzyme[53,121−123,125] such that the heme Fe(II) complex form of *Ec*DOS is active toward c-AMP, whereas the heme Fe(III) complex form is inactive toward the same substrate. A gene knockout of *Ec*DOS in *E. coli* results in high intracellular concentrations of cAMP, suggesting that cAMP is an alternative substrate of *Ec*DOS, although the activity of *Ec*DOS toward cAMP is very low as compared to that toward c-di-GMP.[54,124,125]

## 6. HEME-BASED $H_2S$ SENSORS

No heme-based $H_2S$ sensors have been reported to date. Notably, however, catalytic activity of the heme-based oxygen-sensing *Ec*DOS PDE is substantially enhanced by the addition of $H_2S$ under aerobic conditions as already shown in the *Ec*DOS section (Figure 13).[158,160] Although this enhancement is mainly caused by changes in the coordination structure of the heme iron complex in the enzyme, it is unlikely that *Ec*DOS is a heme-based $H_2S$ sensor, because the $H_2S$ concentration needed to stimulate catalysis is in a physiologically irrelevant (∼100 $\mu$M) range. However, it is assumed that deep-sea marine organisms living near volcanic hypothermal vents use $H_2S$-associated biochemistry and physiology. Thus, it is possible that future studies will identify heme-based or non heme-based $H_2S$ sensors.

## 7. COMMON CHARACTERISTICS OF HEME-BASED GAS SENSORS

(1) Ligand binding and heme redox change induce profound structural changes in most heme-based gas sensors. Such profound protein structural changes are not observed for prototypical heme proteins, such as hemoglobin, myoglobin, cytochrome *c*, and P450.

(2) The rank order of affinity for gas binding to the heme Fe(II) complex is $O_2 \ll CO \ll NO$. A "sliding scale rule" was proposed to explain differences in affinity between these three gas molecules.[58,59,398] Accordingly, for heme-based gas sensors, structural deformation of the heme plane, the protein structure surrounding the gas binding channel, and/or the distal structure of the gas-bound heme complex in the protein significantly affects the specificity of gas binding to the heme Fe(II) complex.

(3) Heme sensors, but not heme-based gas sensors, in which association/dissociation of the heme Fe(III) complex to the protein per se regulates functions, can also be gas sensors. However, these gas molecules bind only to the heme Fe(II) complex; thus, changes in heme redox status must occur to convert them from heme sensor to gas sensor. In addition to reductases, NO might act as a reducing agent. Yet, the molecular mechanism by which a small molecule reductant or reductase might facilitate reduction of the heme Fe(III) complex to the heme Fe(II) complex remains elusive.

(4) Heme-based gas sensors can also be converted into heme sensors when the heme Fe(II) complex is changed to the heme Fe(III) complex under oxidative stress conditions through (auto)oxidation, the converse of characteristic (3). NO might act as the oxidizing agent under nitrosive/oxidative conditions.

(5) Results obtained for *Ec*DOS and YddV suggest that incorporation of a heme iron complex in heme-based gas sensors may serve to suppress catalysis, with gas binding acting to release catalytic suppression.[116,120,123] However, this is not always the case because the heme-free forms of *Ax*PEA1[131] and HemAC-*Lm*[133] have very low catalytic activities. It is possible that binding of the heme iron complex is needed to form a protein structure appropriate for optimal signal transduction or catalytic functions.

## 8. CONCERNS THAT NEED TO BE ADDRESSED

There is considerable confusion in understanding the differences between heme-responsive heme sensors and heme-based gas sensors. For heme-responsive heme sensors, the association/dissociation of the heme iron complex to/from the protein regulate(s) its functions, including catalytic reactions (such as kinase and proteolysis) or DNA binding. Indeed, the association/dissociation of the "heme Fe(III) complex" to/from the protein has been studied in several cases. In contrast, gas molecules such as $O_2$ and CO bind only to the "heme Fe(II) complex". Therefore, under reducing conditions, a heme-responsive heme sensor can be converted into a heme-based gas sensor. However, it is not clear which reductase or reductant (substance or chemical) is involved in the reduction of the heme Fe(III) complex to the heme Fe(II) complex present in some heme-based gas sensors. For example, NPAS2 was first reported to be a heme-based CO sensor.[363] Later, however, NPAS2 was found to have an additional function as a heme-responsive heme sensor, in that the association/dissociation of the heme Fe(III) complex regulates DNA binding of this protein,[366] but the reductase or reductant of the heme-bound protein has not been identified. For many sensors, this point is elusive and requires exploration in greater detail.

Similar confusion is present for heme-redox sensors, because it is not clear whether the redox sensor is an actual redox sensor or a heme-based oxygen sensor. It is not practical for many laboratories to take advantage of anaerobic conditions, making it difficult to isolate and separate the heme Fe(II) or Fe(II)−$O_2$ complex as the active species under aerobic conditions. In addition, some heme Fe(II)−$O_2$ complexes are unstable, resulting in rapid conversion to the heme Fe(III) complex except when the heme iron complex has a high redox potential. Thus, it is only possible to determine the real active heme species (heme Fe(II), Fe(II)−$O_2$, or Fe(III) complex) of the redox sensor when the reconstituted system can be used under anaerobic conditions.

Many more careful studies of systems reconstituted from purified proteins are needed to unequivocally understand the molecular mechanisms of heme-responsive heme sensors and heme-based gas sensors. Solid and reliable conclusions require combined studies conducted from both biological and chemical points of view.

## 9. PERSPECTIVES

The molecular mechanisms of heme-based gas sensors have attracted considerable research attention from microbiologists, biophysicists, biochemists, and bioinorganic chemists. Crystal structures for some bacterial and archeae heme-based gas sensors have been solved, reflecting the relative ease of overexpressing and purifying bacterial gas-sensing proteins in *E. coli* as compared to related proteins from eukaryotic systems. However, medical professionals often ask, what are the heme-


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based gas sensors in humans or higher organisms and how do they act to regulate important physiological functions? This interest is natural and reflects a desire to solve clinical problems of unknown cause combined with a sense that some serious diseases might be associated with heme iron complexes and gaseous molecules. This is in fact a valid perspective because human sGC and CBS are really heme-based NO or CO sensors whose impairment can cause serious diseases associated with their enzymatic functions.[399] Because new heme proteins with novel features are continuously being discovered, it is expected that future studies will reveal additional important heme-based, disease-associated gas sensors. However, bacterial heme-containing sensor proteins are also important subjects of investigation in a clinical context because they represent interesting targets for the generation of new antibacterial and antimicrobial drugs with minimal side effects on eukaryotic organisms.

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### Notes

The authors declare no competing financial interest.

### Biographies

Toru Shimizu received his B.S., M.S., and Ph.D. degrees in chemistry from Tohoku University, Sendai, Japan. After several years as a postdoctoral researcher at Albert Einstein College of Medicine, New York, with Jack Peisach, and Keio University School of Medicine, Tokyo, with Yuzuru Ishimura, he returned to Tohoku University where he held the positions of Associate Professor and Professor before retiring in 2012. He is now a Professor Emeritus of Tohoku University, Professor of Shantou University Medical College, Visiting Professor of Charles University in Prague, and Visiting Researcher of AIST. He won the Academic Award from the Chemical Society of Japan in 1991. His main research interest is the structure and function relationships of heme-sensor proteins.

Donyang Huang received a B.S. in clinical medicine from China Medical University, Shenyang, China, in 1984 and a Ph.D. in biochemistry from Kagawa Medical University, Takamatsu, Japan, in 1997. While at Kagawa Medical University, he received a fellowship from the Japan Society for the Promotion Science (JSPS). After several years working as an Assistant Lecturer, Lecturer, and Professor at China Medical University, he became a faculty member of Shantou University Medical College in 2002. He is now Chairperson and Professor of Cell Biology and Genetics and Executive Associate Dean of Shantou University Medical College, China. In cooperation with Toru Shimizu, he is conducting research in the field of heme-based $O_2$ sensors.

Fan Yang received a B.S. in pharmacy from Henan University, Kaifeng, China, in 2012. She is now undertaking a Master's degree at Shantou University Medical College. Her main research interests are the $H_2S$-induced structural and catalytic changes of the heme-based $O_2$ sensor phosphodiesterase, *Ec*DOS.

Martin Stranava received a M.S. in biochemistry from Charles University in Prague. His diploma thesis was dedicated to the



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preparation, purification, and enzymatic characterization of cytochrome P450 reductase, a protein involved in xenobiotic metabolism. Currently he is a Ph.D. student in the laboratory of Markéta Martínková and, in cooperation with Toru Shimizu, is investigating the heme sensor proteins YddV and *Af*GcHK.

Martina Bartosova studied the properties of medicinal plants during her Bachelor studies. She focused on the NADP/dehydrogenase activity and contents of phenolic compounds and flavonoids in medicinal plants. During her Master's studies, she focused on the heme sensor protein *Af*GcHK and its interaction with sodium sulfide. She now works at SOTIO, a Czech biotechnological company.

Veronika Fojtíková completed a Master's Degree in Science at Charles University in Prague in 2014, specializing in clinical and toxicological analysis. During her studies, she focused on the biochemistry of regulation and influence of exogenous conditions, applied analytical chemistry, toxicokinetics, and pharmacology. The aim of her Master's studies was to determine the kinetics parameters of the reaction catalyzed by the globin coupled histidine kinase, *Af*GcHK. Currently she is a Ph.D. student in the Department of Biochemistry, Charles University in Prague. She is interested in heme-containing gas sensing proteins and their properties.

Markéta Martínková received a Ph.D. degree in 2003 from Charles University in Prague for her work on cytochromes P450 and peroxidases from an enzymology perspective. In 2004, she was awarded a postdoctoral fellowship by the Japan Society for the Promotion of Science (JSPS) to work on heme-containing sensor proteins at the laboratory of Toru Shimizu (Tohoku University, Sendai). Currently, she is an Associate Professor of Biochemistry at the Department of Biochemistry, Faculty of Science, Charles University in Prague, and a Vice-Dean of the same faculty. Her professional interests lie in the characterization of various hemoproteins, including heme-based gas sensors.

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## ABBREVIATIONS

| | |
|---|---|
| Aer2 | aerotaxis transducer |
| AppA | antirepressor of PpsR from *Rhodobacter sphaeroides* |
| BLUF | sensors of blue light using FAD |
| CAP | catabolite activator protein |
| CBS | cystathionine $\beta$-synthase |
| c-di-GMP | cyclic-di-GMP or bis(3′-5′)-cyclic dimeric guanosine monophosphate |
| CheA/CheY | two-component signal transduction system involved in bacterial aerotaxis |
| CODH | carbon monoxide dehydrogenase |
| CooA | a heme-regulated CO-sensing transcriptional factor (e.g., *Rr*CooA from *Rhodospirillum rubrum* and *Ch*CooA from *Carboxydothermus hydrogenoformans*) |
| CRP | cAMP receptor protein (a cAMP-dependent transcriptional regulator) |
| CRP-FNR | cAMP receptor protein/fumarate and nitrate reductase regulator |
| DGC | diguanylate cyclase |
| DNR | dissimilative nitrate respiration regulator |
| DosS/DosT | $O_2$ sensor histidine kinases |
| DosR | DNA-binding transcriptional regulator (effector of DosS/DosT) |



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| | |
|---|---|
| *Ec*DOS | *E. coli* direct oxygen sensor (heme-regulated $O_2$ sensor phosphodiesterase), also designated *Ec*DosP |
| *Ec*DosC | heme-based oxygen sensor diguanylate cyclase from *E. coli*, also designated YddV |
| *Ec*DosP | heme-regulated $O_2$ sensor phosphodiesterase from *E. coli*, also designated *Ec*DOS |
| EM | single-particle electron microscopy |
| EPR | electron paramagnetic resonance |
| EXAFS | extended X-ray absorption fine structure |
| FixL | two-component $O_2$-sensor histidine kinase, for example, from *R. meliloti* (*Rm*FixL) or *B. japonicum* (*Bj*FixL) |
| FixJ | cognate response regulator of FixL |
| FRET | Förster (or fluorescence) resonance energy transfer |
| FTIR | Fourier transform infrared spectroscopy |
| GAF | cGMP-specific and -stimulated phosphodiesterase, adenylate cyclase, and *E. coli* formate hydrogen lyase transcriptional activator |
| GcHK | globin-coupled histidine kinase (e.g., *Af*GcHK from *Anaeromyxobacter* sp. Fw109-5) |
| GCS | globin-coupled $O_2$ sensor |
| GReg | globin-coupled regulator ($O_2$-sensor with DGC activity; e.g., *Av*GReg from *Azotobacter vinelandii* and *Bpe*GReg from *Bordetella pertussis*) |
| HaCE | H-NOX-associated cyclic-di-GMP processing enzyme |
| HAMP | histidine kinase, adenylate cyclase, methyl accepting protein, and phosphatase |
| Hb | hemoglobin |
| HDX-MS | hydrogen/deuterium exchange mass spectrometry |
| HemAC-*Lm* | a globin-coupled $O_2$-sensor adenylate cyclase from *Leishmania major* |
| HemAT | a globin-coupled $O_2$-sensor MCP from *Halobacterium salinarum* and *Bacillus subtilis* |
| HemDGC | heme-containing DGC |
| heme Fe(III) | protoporphyrin IX-ferric complex (hemin) |
| heme Fe(II) | protoporphyrin IX-ferrous complex |
| HK | histidine kinase |
| H-NOX | heme-nitric oxide/oxygen binding domain/protein |
| $K_a$ | equilibrium association constant |
| $K_d$ | equilibrium dissociation constant |
| $k_{ox}$ | autoxidation rate constant |
| $k_{off}$ | dissociation rate constant |
| $k_{on}$ | association rate constant |
| l-di-GMP | linear-di-GMP (pGpG) |
| MALDI-TOF | matrix-assisted laser desorption/ionization-time of flight |
| Mant-NTP | (*N*-methyl)anthraniloyl-substituted nucleotide |
| *Ma*Pgb | protoglobin from *Methanosarcina acetivorans* C2A |
| Mb | myoglobin |
| MCP | methyl-accepting chemotaxis protein |
| NMR | nuclear magnetic resonance |
| NtrY/NtrX | nitrogen regulation proteins |
| PAS | Per (*Drosophila* period clock protein), Arnt (vertebrate aryl hydrocarbon receptor nuclear translocator), and Sim (*Drosophila* single-minded protein) |
| PDE | phosphodiesterase |
| RcoM | a prokaryotic regulator of CO metabolism |
| PDEA1 | phosphodiesterase A1 (e.g., *Ax*PDEA1 from *Acetobacter xylinum*) |
| RR | resonance Raman spectroscopy |
| SAXS | small-angle X-ray scattering |
| SCHIC | sensor containing heme instead of cobalamin |
| $SONO_{HD}$ | sensor of NO heme domain (e.g., TT-$SONO_{HD}$ from *T. tengcongensis* and CB-$SONO_{HD}$ from *Clostridium botulinum*) |
| SW Mb | sperm whale myoglobin |
| sGC | soluble guanylate cyclase |
| YddV | see *Ec* DosC |

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