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Review

Heme-Based Gas Sensors in Nature and Their Chemical and Biotechnological Applications

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Abstract: Sensing is an essential feature of life, where many systems have been developed. Diatomic molecules such as O_2, NO and CO exhibit an important role in life, which requires specialized sensors. Among the sensors discovered, heme-based gas sensors compose the largest group with at least eight different families. This large variety of proteins also exhibits many distinct ways of sensing diatomic molecules and promote a response for biological adaptation. Here, we briefly describe a story of two impressive systems of heme-based oxygen sensors, FixL from *Rhizobium* and DevS(DosS)/DosT from *Mycobacterium tuberculosis*. Beyond this, we also examined many applications that have emerged. These heme-based gas sensors have been manipulated to function as chemical and biochemical analytical systems to detect small molecules (O_2, CO, NO, CN^-), fluorophores for imaging and bioanalysis, regulation of processes in synthetic biology and preparation of biocatalysts among others. These exciting features show the robustness of this field and multiple opportunities ahead besides the advances in the fundamental understanding of their molecular functioning.

Keywords: heme-based gas sensors; DevS/DosT/DevR; FixL; bioanalytical tool; biotechnological applications

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1. Introduction

Life is a complex web of connections, where no single organism lives in complete isolation. Chemical ecology is an obvious and essential feature that emerges from this, where a multitude of molecules are produced, released, absorbed and transformed all the time. Some of them can be very important while others are extremely harmful, which sometimes requires very sophisticated alert systems. The ability to sense and respond to some molecules is a way to adapt to environmental changes, a key feature of life. Among the various molecules required to be sensed, there are very simple and small ones such as diatomic molecules that can play critical roles in life. Molecules such as H_2, N_2, O_2, NO and CO have many biological uses, and sensing systems have been found for most of them, regulating their processes [1–4].

Nature has developed exciting proteins capable of selectively binding and recognizing diatomic gases, distinguishing one single atom of oxygen from nitrogen or carbon in the triad of gases: O_2, NO and CO [2,5]. Their chemical properties as tiny gaseous molecules make it harder for proteins to bind them [1]. Indeed, most of the protein sensors for these gaseous molecules employ metal centers that are capable of making a direct bond with them. Actually, there are many highly stable metal-based compounds containing one or more CO or NO as ligands, including in metalloprotein sites (see hydrogenases, nitrogenases [6]). The use of metal ions, particularly of iron in proteins, allows sensors to interact more efficiently with O_2, NO or CO. Indeed, their binding strength can vary widely (from picomolar to micromolar), opening opportunities to probe a large range of concentrations. The most common nature of these iron-based sensing units is mononuclear iron, binuclear iron, iron-sulfur cluster or heme sites [1]. These four distinct types of iron-based sites have been

found in many systems such as NO sensor NorR from *Escherichia coli* (mononuclear iron site) [7], O_2 sensor *Vc*Bhr-DGC from *Vibrio cholerae* (binuclear iron site, hemerythrin-like) [8], O_2 sensor FNR from *Escherichia coli* (iron–sulfur cluster site [Fe_4S_4]) [9] and O_2 sensor FixL from *Rhizobium* (heme site) [10] (Figure 1). Regarding their structural organization, these metal-based sensing units have been found within a certain region of the protein, in a different domain or as a completely independent protein (Figure 1). This organizations can be exemplified by three metalloprotein gas sensors:

1. Single domain: Whib3, an NO/O_2 sensor from *Mycobacterium tuberculosis*, exhibits an iron–sulfur cluster site (sensing unit) and a DNA-binding region (responsive unit) within the same protein region (Figure 1) [9].
2. Different domains: FixL, an O_2 sensor from *Rhizobia* bacteria, contains a heme site (sensing unit) and a histidine kinase region (responsive unit) in different domains (Figure 1) [3].
3. Independent proteins: *Pa*NosP, an NO sensor from *Pseudomonas aeruginosa*, presents a heme-containing protein (sensing unit) that functions by regulating another independent histidine kinase protein (responsive unit) (Figure 1) [11].

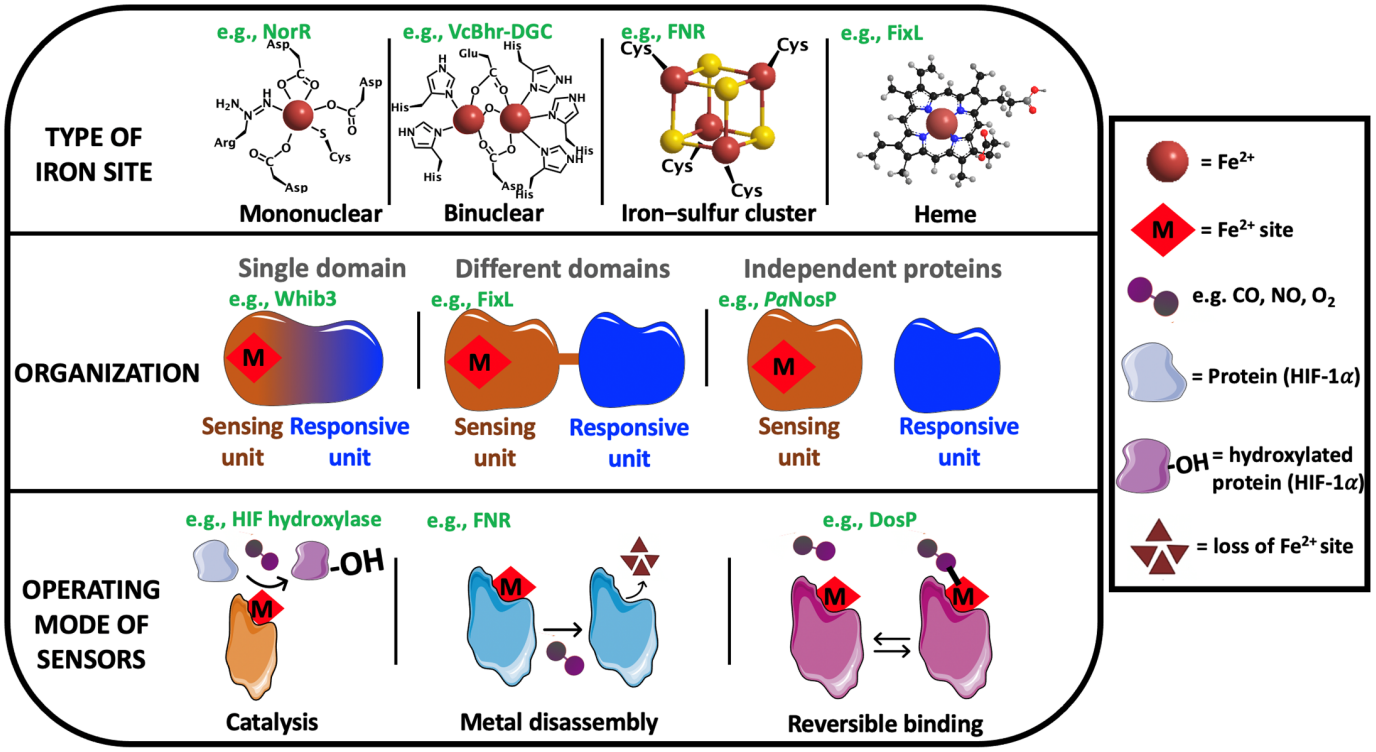

Figure 1. Basic building blocks of metal-based sensor proteins for O_2, NO and CO and molecular functioning. Firstly, four types of iron sites are briefly illustrated with examples of gas sensors: mononuclear iron site of the NO sensor NorR from *E. coli*, binuclear iron site of the O_2 sensor Bhr-DGC from *Vibrio cholerae*, iron–sulfur cluster of the O_2 sensor FNR from *E. coli*, and heme iron site of the O_2 sensor FixL from *Rhizobium*. Then, it shows the overall organization of metal-based gas sensors with three distinct structural features along with examples such as Whib3 from *Mycobacterium tuberculosis* as a single protein sensor of NO/O_2, FixL as a multi-domain O_2 sensor and *Pa*NosP from *Pseudomonas aeruginosa* with independent protein units functioning as an NO sensor and the responsive unit. The different general modes of operation of metal-based gas sensors are illustrated, where the first case involves catalysis using O_2 as shown by the O_2 sensor HIF hydroxylase. The next mode employs iron–sulfur cluster site disassembly represented by the O_2 sensor FNR, while the last case involves reversible binding to the gaseous signaling molecule as illustrated by the O_2 sensor DosP.

There are also many types of metallosensing units based on how they respond to the biological signal, as described elsewhere [1]. However, most of them function as a catalytic unit, a metal disassembling unit or a reversible binding unit [1] (Figure 1). The O_2 sensor HIF hydroxylase from *Homo sapiens* functions by catalyzing O_2-based hydroxylation of HIF-1α (a transcription factor), leading to its degradation, turning off a genetic hypoxia response [12,13]. Iron–sulfur cluster unit found in the O_2 sensor FNR from *E. coli* function by reacting with O_2 disassembly of the iron site [9,14]. However, many types of metalloprotein gas sensors function upon reversible and direct binding of the gaseous signal to the metal site such as O_2 sensor FixL that employs a heme cofactor for reversible binding [3]. Besides all of that, a validated sensing unit upon binding to these gaseous molecules must report such process to a responsive unit (also known as a functional output structure), leading to biological adaptation.

The responsive unit is another essential part of these gas sensors, being involved in the conversion of the gas binding process into a biochemical signal/information. Similarly to the sensing unit, it can also be found as part of a certain region of the protein, in one different domain or in another independent protein (Figure 1). There are many functional output units used by nature: those with enzymatic activity (e.g., histidine kinase [10,15,16], nucleotide cyclase [17–19], phosphodiesterase [18,19]), capable of interacting with a protein or DNA and others [20], meaning that the binding of O_2, NO and CO can regulate the production or degradation of molecules (e.g., c-di-GMP by DosC/DosP impacting RNA [21,22]) or the interaction with a DNA sequence enabling gene regulation (e.g., CooA [23]) or modification of a protein for degradation (e.g., HIF hydroxylase [24]), among other things [1,2,20]. These intricate arrangements have allowed nature to come up with many outstanding biological responses, regulating multiple processes as important as blood pressure, biological clock, nitrogen fixation, dormancy, bacterial biofilm, RNA degradation, virulence, among others. Unfortunately, a detailed structural mechanism of the signal transduction processes is still mainly lacking, but some important insights have emerged, as mentioned briefly here and in detail elsewhere [1,2,20,25–29].

The largest family of metalloprotein gas sensors is based on heme-containing proteins [4]. These heme-based gas sensor proteins have been commonly found containing multi-domains (e.g., FixL, DosP), but there are also standalone proteins as well (e.g., *Pa*NosP, Figure 1) [30]. Soluble guanylate cyclase (sGC) was the first discovered case of a heme-based gas sensor protein, which functions as a dedicated nitric oxide (NO) sensor [17,31]. This protein contains a heme domain, one intervening domain and an enzymatic domain that converts GTP into the second messenger cGMP and pyrophosphate (PP_i). This enzymatic activity is strongly enhanced upon NO binding to the heme domain, leading to major production of cGMP [17]. This latter molecule is a secondary messenger involved in the regulation of many physiological processes [32]. Actually, sGC is involved in many important processes in humans and other organisms, including vasodilation, platelet aggregation, memory, etc. [33,34].

In 1991, Gilles-Gonzalez discovered another heme-based gas sensor called FixL [10]. This protein was shown to be an O_2 sensor. FixL is an important upstream oxygen sensor for *Rhizobia* bacteria, which allows symbiosis to occur with leguminous plants [35]. That important discovery was soon followed by others, supporting the existence of a superfamily of heme-based gas sensors. Currently, there are at least eight major families of these sensors based on the heme fold domain, which includes the PAS, HNOB (or HNOX), globin, CooA (CRP), SCHIC, GAF, LBD (Holi) and FIST domains (Figure 2) [36,37]. This latter family is the most recent one discovered in 2017 [11], whose sensors are called NosP (nitric oxide-sensing protein). The most common diatomic gas type sensed by each family is shown in Figure 2, along with some well-known protein sensors and their biological functions.

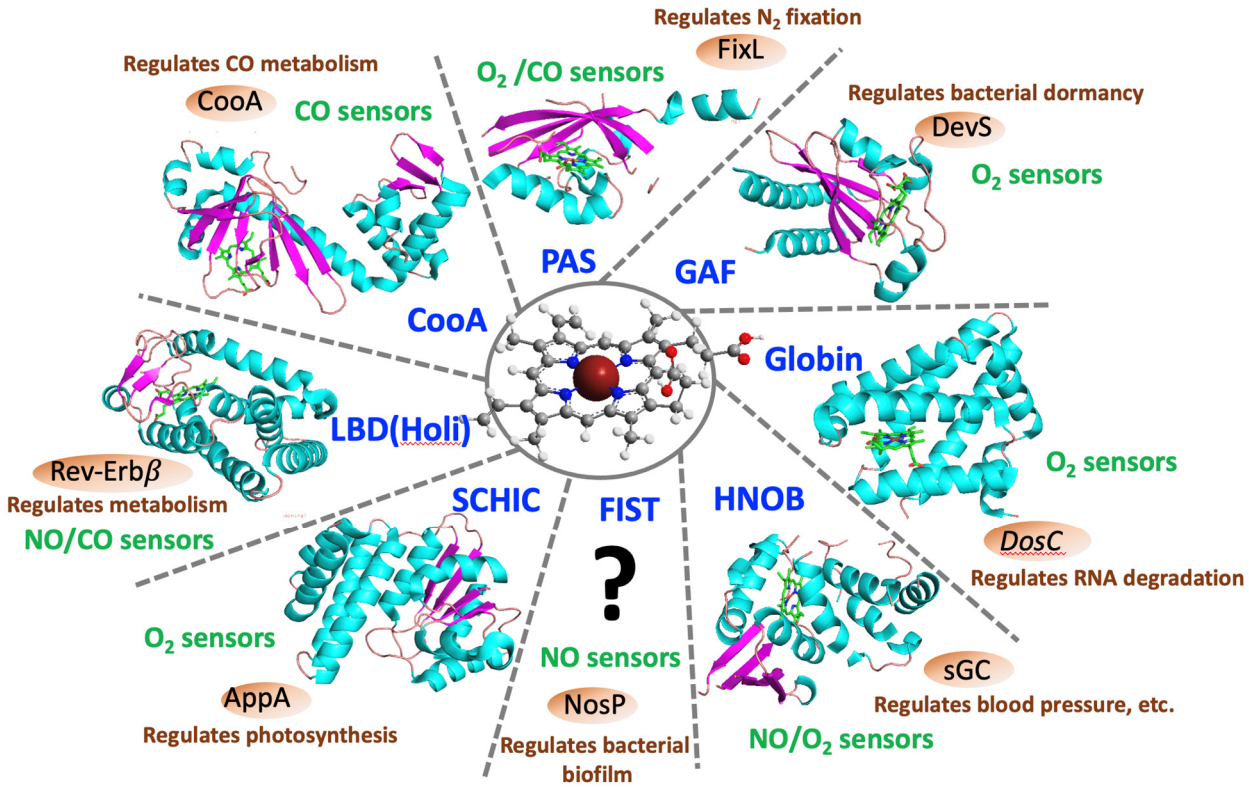

Figure 2. Heme-based gas sensor families, structural heme fold (in blue; PAS domain (pdb 1DP9), GAF domain (pdb 4YNR), globin domain (pdb 6OTD), HNOB domain (pdb 2O09), FIST domain (3D structure not solved yet), SCHIC domain (pdb 4HEH), LDB(Holi) domain (pdb 3CQV), CooA domain (pdb 2FMY)), common functionality as a gas sensor (green) and some examples of proteins of each family (brown oval) and biological function (brown).

2. FixL—A Short Story

Nitrogen fixation promoted by some bacteria is a very important process that allows proper access of a nitrogen source to plants during a symbiotic process [35,38]. This process is usually complex, and nitrogenases are also oxygen-sensitive, requiring many physiological changes to happen within the plant nodule and *Rhizobia* [38]. The plant is responsible for building an oxygen barrier in the nodule along with a large production of leghemoglobin, creating extremely low levels of free oxygen (nanomolar) [39]. In this way, it makes it possible for a nitrogenase to work. The level of oxygen within the nodule is closely monitored by FixL, which under virtual anaerobiosis becomes enzymatically active [35]. Thus, FixL can phosphorylate FixJ, another protein that functions as a transcription factor regulating genes involved in microaerobic respiration and the nitrogenase apparatus [35]. This upstream oxygen sensor along with NifA assure that a cascade of events takes place, leading to nitrogen fixation [35,38].

FixL is a multidomain sensor protein that contains a heme b group in a PAS fold domain and a histidine kinase domain in its C-terminal region (Figure 3A). Sometimes, this sensor exhibits an extra non-heme PAS domain or transmembrane segments at the N-terminal region [5]. Despite the lack of an X-ray structure for this full-length protein, there are many structural and functional studies on this sensor that have elucidated important molecular details [40–54]. One interesting feature is the ability of this protein to recognize O_2 and selectively trigger a response to the histidine kinase domain. O_2 as well as CO and NO binds to the iron heme, where O_2 has the weakest affinity of all, but at the same time it is the only one that switches off the protein enzymatic activity [15,45]. This is associated to its geometry upon binding and specific interactions with distal residues [41,53,55]. This subtle binding of O_2 causes conformational changes within the heme domain, altering the heme distortion (induced planarity) and leading to specific interactions with arginine and heme propionate groups within the PAS heme domain [41,47,53]. These changes have been associated to the signal transduction process that propagates to the enzymatic domain. Recently, SAXS studies suggested signal transduction occurs with a more subtle movement of the domains [40], not necessarily by switching contacts. A movement of the coiled-coil linker region that connects the heme PAS and histidine kinase seems to be vital for signal transduction, affecting the proximity of the ATP binding site and the histidine phosphorylatable residue [40].

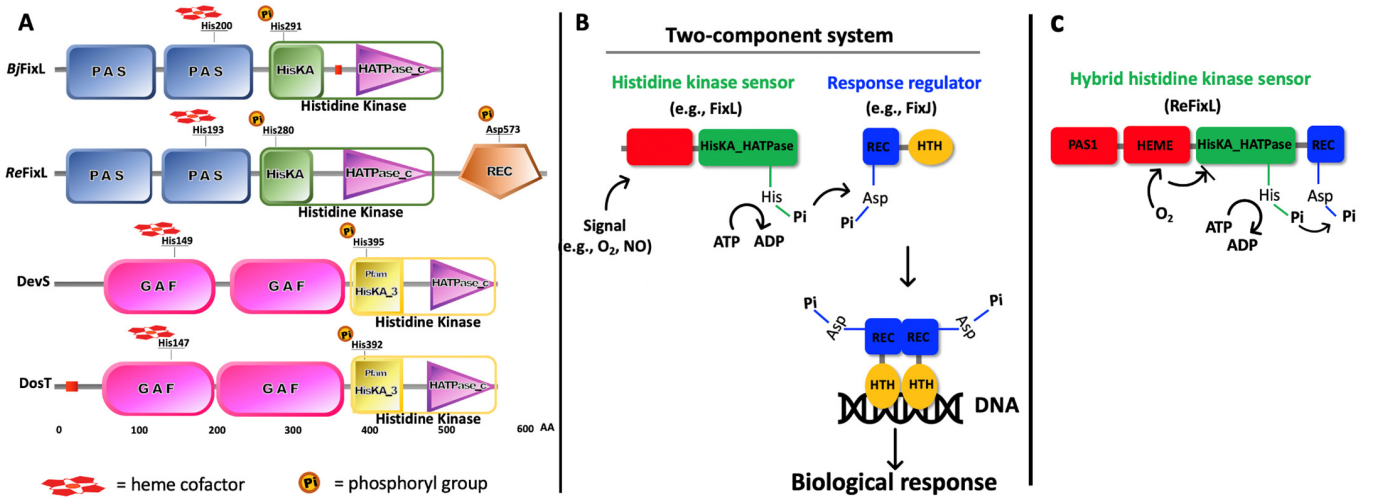

Figure 3. (**A**) Domain organization of FixL and DevS/DosT (heme as a red small structure, phosphorylation indicated by Pi; heme proximal ligand and phosphorylatable residues are shown) composed of the PAS and GAF domains along with the histidine kinase domain constituted of the HisKA and HATase_c subdomains. Hybrid FixL (*Re*FixL) presents an additional REC domain (receiver domain) usually found at the N-terminal region of the response regulator proteins. (**B**) Cartoon of the functioning of common two-component systems, where a histidine kinase sensor upon the appropriated signal becomes active, autophosphorylating a histidine residue; then, a phosphoryl group is transferred to the response regulator protein. This later becomes active upon phosphorylation and usually functions as a transcriptional factor triggering a biological response. (**C**) Autophosphorylation of the hybrid *Re*FixL, where O_2 blocks histidine kinase and phosphotransferase activities. His = histidine residue, Asp = aspartate residue, AA = amino acids.

The bacteria have commonly used a particular type of sensing systems to perceive and respond to environmental changes called two-component systems, which are usually formed by one histidine kinase sensor and one response regulator protein (Figure 3B) [56]. FixL and FixJ are a typical two-component system responsible for sensing O_2, where FixL is a histidine kinase sensor and FixJ is a response regulator. How these multi-domains communicate and coordinate intra- and interprotein phosphorylation are still exciting questions. The presence of additional "nonfunctional" domains in some FixL proteins raised further question on their structural roles. FixL from *Bradyrhizobium japonicum* (*Bj*FixL) is such a case, where one additional non-heme PAS domain is found at the N-terminal region followed by a heme-PAS domain and a histidine kinase (Figure 3A). The actual role of this additional PAS domain is not clear, but it may be quite important as shown in another study using a hybrid FixL sensor from *Rhizobium etli* (*Re*FixL) [57]. This latter cytosolic sensor exhibits a domain organization quite similar to *Bj*FixL (Figure 3A), but it also has an extra domain at the C-terminal region. This extra terminal domain (REC) is analogous to the receiver domain of FixJ, which contains a phosphorylatable aspartate residue (Figure 3A). Thus, this protein features as a combination of *Bj*FixL and part of *Bj*FixJ, being a great opportunity for questioning the function of multi-domains in the FixL–FixJ system. In such a hybrid FixL sensor, there are two phosphorylatable residues, one histidine residue centered at the kinase domain and one aspartate at the C-terminal receiver domain (Figure 3C). Then, the hybrid protein can promote histidine autophosphorylation followed by an intramolecular phosphotransfer to aspartate in the receiver domain, all within the same protein.

This hybrid *Re*FixL sensor brought up an opportunity to explore this cascade of phosphorylation processes and their regulation [57]. Interestingly, this sensor showed the lowest reported oxygen affinity for a heme-based gas sensor ever (K_d = 738 μM) [57], despite *Bj*FixL also having a reasonably low affinity, too (K_d = 140 μM) [58]. Despite that, *Re*FixL exhibited very tight oxygen regulation even in the air, where only 26% of the protein is bound to oxygen. The deletion of the first PAS domain (ΔPAS1 *Re*FixL) provoked major changes not only in the heme domain, but also in the histidine kinase activity. While the oxygen affinity increased about eightfold, the histidine kinase activity was completely turned off with minor measurable ligand regulation [57]. This lack of activity could be due to a damaged inactive histidine kinase domain; however, ΔPAS1 *Re*FixL was still capable of being phosphorylated by another fully functional ReFixL. This result supported the histidine kinase domain was still functional, but ΔPAS1 ReFixL was likely shifted toward an inactive equilibrium state. The PAS1 domain seems to allow an equilibrium between active and inactive states to be achieved, whose removal disrupted this conformational change, locking the protein in an inactive state. In another study, we also noticed the plasticity of their domains and differences in the overall stability when measured in the active versus inactive state [48]. These studies illustrated how sensitive the heme domain is and how major properties can be altered by interaction with other domains, not only by mutations within the heme domain itself. Indeed, this particular feature has been noticed by many other studies comparing heme properties of isolated heme domains with full-length proteins [1], reinforcing sensing domains are much more flexible as they should be to promote signal transduction.

Another exciting feature discovered in FixL is an O_2 memory effect. This phenomenon is a hysteresis response caused upon O_2 binding, which quickly inactivates histidine kinase, but FixL reactivation takes much longer once O_2 dissociates [59]. There was an initial expectation that one O_2 molecule bound to FixL is capable of turning off its histidine kinase activity. If this behavior was right, a linear dose response would be obtained for O_2 saturation of FixL versus histidine kinase activity. However, experimental data showed the dose response for *Bj*FixL was remarkably nonlinear, but it matched nicely the in vivo O_2 dose response regulation data. In other words, this result means that a few molecules of O_2 are able to inactivate many molecules of FixL due to faster O_2 rebinding in comparison to slower kinetics of histidine kinase activation. This process fits well in a hysteresis model

supporting in vitro and in vivo data. This exciting phenomenon can indeed explain how extremely low-affinity sensors work [57,59]. Actually, the nonlinear response of inhibitors (as drugs) has alerted medicinal chemists of an important aspect of association/dissociation kinetics beyond thermodynamic affinity (K_d) [60]. The memory effect may explain how oxygen can regulate *Re*FixL so tightly despite its extremely low affinity. While this process may explain FixL behavior, it was not able to explain the nonlinear dose response for other oxygen sensors (e.g., DosP [19]), where other mechanisms may take place.

Structural changes of the heme domain of FixL were observed as reasonably fast (few microseconds) events associated with planarity/distortion of the heme and its interactions with nearby residues [61–63]. Recently, an attempt to investigate the time scale of the signal transduction process was carried out with FixL from *Sinorhizobium meliloti* (*Sm*FixL) using Raman spectroscopy [64]. Despite the limitations of this study, the authors suggested conformational changes provoked in the heme domain by oxygen binding would take at least microseconds to alter the histidine kinase domain. However, it is still unknown if any further fine adjustment is required to achieve full inactivation or activation.

FixL or analogs have been found in other microorganisms, such as *Caulobacter crescentus* [65], *Brucella abortus* [66], *Chlamydomonas reinhardtii* [67] and *Burkholderia cepacia* [68,69]. This latter bacterium is part of a class of pathogens causing concerns in cystic fibrosis patients. There is growing evidence that this bacterium employs the oxygen sensor FixL to control biofilm formation, motility, intracellular invasion/persistence and virulence, opening exciting opportunities to explore this system in therapy. Beyond that, this system has also been explored in synthetic biology as mentioned later (see section "Diverse Potential Applications of Heme-Based Gas Sensors: Systems Applied in Cell Biology").

3. DevS and DosT—A Short History

The millenary disease, tuberculosis, is still a major global health issue, causing deaths of over 1 million people around the world [70]. The low number of drugs, emergence of multiple drug-resistant strains and the large reservoir of dormant bacteria within humans have caused a challenge to control and eradicate this disease [71,72]. Almost 20 years ago, a two(three)-component system (DevS–DevR, and later DosT) was identified in *M. tuberculosis* (Mtb) [73–75], which was associated to its virulence. Genetic studies showed this system was oxygen-responsive and associated to the initiation of the dormancy program in the bacterium [74,76]. The dormancy of Mtb is related to its persistence in humans, the lengthy medical treatment and difficulty to eradicate this disease in the world [77]. Importantly, this sensory system of DevS–DosT–DevR was shown as a suitable drug target, where two independent laboratories have been developing new compounds as novel anti-tuberculosis drugs (see section "Diverse Potential Applications of Heme-Based Gas Sensors: Pharmacological Use").

In 2005, DevS (also known as DosS) was shown to be a GAF heme-containing protein [78] (Figure 3B). Later, DevS and its ortholog, DosT, were demonstrated to function as O_2 sensors [79,80], supporting previous genetic studies [74,76]. In 2007, another laboratory suggested DevS is a redox sensor while DosT is a direct oxygen sensor [81]. These apparently conflicting conclusions were addressed by other studies as discussed elsewhere [36]. In summary, there is strong and compelling evidence supporting that DevS and DosT are indeed direct oxygen sensors, including DevS having a relatively high electrochemical potential (E_m = −10 mV vs. NHE) [82]. But why nature would employ two oxygen sensors for that process? These proteins may function by sensing and responding to distinct levels of hypoxia, which would be in agreement with their oxygen affinity [79], as indicated by genetic studies [83]. DosT with a lower oxygen affinity (K_d = 26 μM) may perceive the initial drop in oxygen concentration, allowing initial bacterial adaptation. If this condition does not persist, it may be an easier return to normal growth; however, if a further decrease in the oxygen level takes place, then DevS becomes active, leading to major physiological changes into a dormant state. Despite their differences, the accrued biochemical knowledge on FixL–FixJ helped us to better understand this system [1,3,4].

Raman spectroscopy, site-direct mutagenesis and single crystal X-ray studies provided important hints on the role of key residues involved in sensing and signal transduction [84–86]. The distal ligand in the heme domain is a tyrosine residue (Y171 in DevS and Y169 in DosT), which makes a hydrogen bond with O_2, supposedly assisting its binding. It is interesting to remark that such assistance was not validated for DevS, where the Y171F mutant showed an even higher affinity to oxygen [85], illustrating the complexity of these interactions. Unlike FixL, these proteins showed minor changes within the heme pocket in the active and inactive states, besides a modification in the hydrogen bonding network from the heme to the surface of the domain [86]. This effect might report O_2 binding to the surface of the domain and could propagate this information into other domains as a signal transduction process. Other studies were carried out to identify the role of other residues within the heme, where arginine 204 seemed to have a critical role [87]. This residue is expected to be in a polar patch at the surface of the heme domain in an interface with the other GAF domain. Notably, the R204A mutant promoted a complete disruption in the histidine kinase activity, implicating this residue in signal transduction [87]. Earlier, another study had investigated the role of the extra GAF domain, where two constructs were compared with the wild-type one containing the two GAF domains (GAF(A)–GAF(B)) and another one with only the heme GAF domain (GAF(A)) [88]. These constructs in comparison to wild-type DevS showed distinct features by Raman spectroscopy. Interestingly, the heme GAF domain upon binding to CO exhibited a Raman spectrum consistent with two conformations, while the two GAF constructs had only one, indicating that the intervening domain might mediate signal transduction initiated in the heme domain.

Recently, our laboratory showed DevS is a mixture of oligomers dimer–tetramer–octamers, and more importantly, this oligomeric distribution could be shifted by selective disturbance in the heme domain [89]. Indeed, active DevS either under anaerobic (deoxy-DevS) or CO-bound form (CO-DevS) exhibited a major fraction as octamers, while in the inactive state, either ferric (met-DevS) or oxygen-bound forms (oxy-DevS) showed mainly tetramers and dimers. Remarkably, the inhibition ratio (histidine kinase activity in the active/inactive states) matched nicely the relative amount of octameric/(dimeric–tetrameric) species, supporting the phenomenon of signal transduction promotes changes in the quaternary state of the protein. This process was also reasonably fast (a few minutes), and may occur in other heme-based gas sensors as well [90,91].

The overall mechanism of functioning of this two(three)–component system may be more complex than expected [25]. There is a series of other protein partners, protein modifiers (serine threonine kinase, acetylation) and cross-talks with other sensing systems involved, making this whole story complex, as discussed elsewhere [25]. Nevertheless, there are many opportunities ahead to unfold these details and find new applications for these systems either as drug targets or biological tools, etc.

4. Diverse Potential Applications of Heme-Based Gas Sensors

Our understanding of the molecular functioning of heme-based gas sensors and their broad diversity has also opened new opportunities, both to develop small-molecule regulators and use them as new tools and materials, as we discuss further (Figure 4).

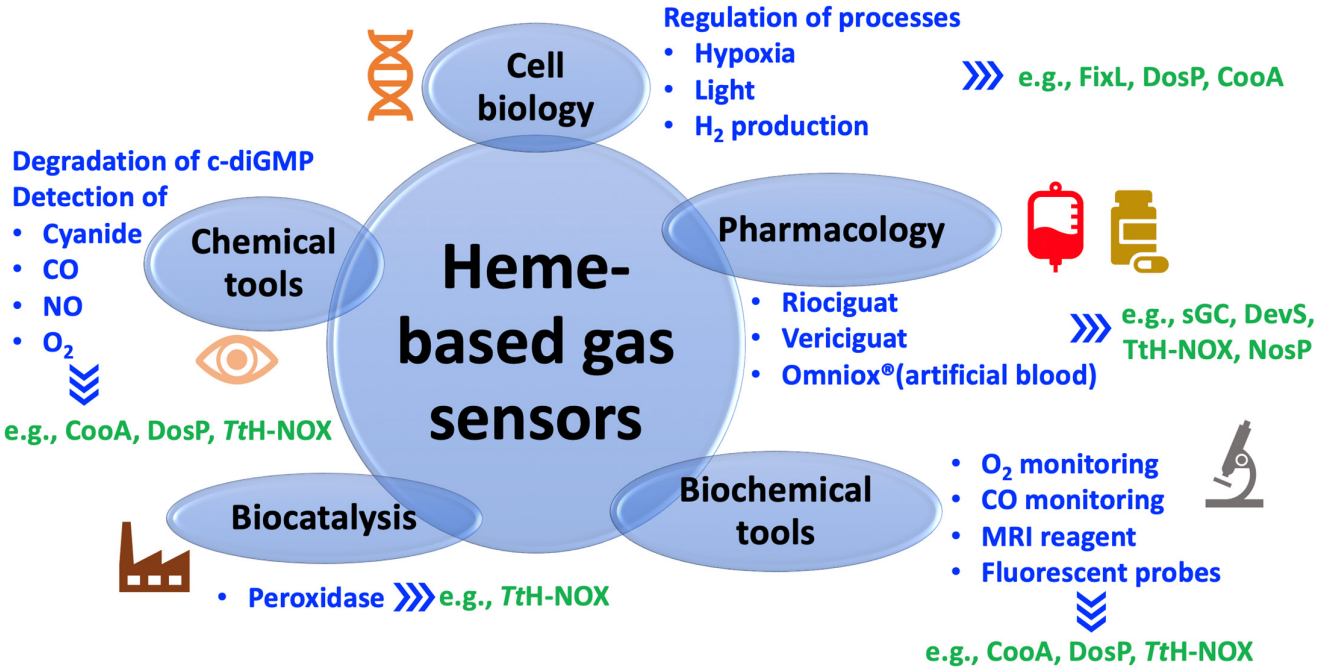

Figure 4. Chemical and biotechnological applications of heme-based gas sensors. There are five segments of investigations with some developed applications of heme-based gas sensors in cell biology (e.g., FixL was employed to achieve hypoxia and light control), pharmacology (e.g., two FDA-approved drugs are available targeting sGC, while others are under study, along with a blood substitute candidate Omniox®), biochemical tools (e.g., CooA, DosP and *Tt*H-NOX are explored for in vivo monitoring of O_2, CO and other uses such as MRI or fluorescent reagents), biocatalysis (e.g., *Tt*H-NOX has been explored as a peroxidase catalyst), chemical tools (e.g., CooA and *Tt*-HNOX have been explored for in vitro detection and measurement of cyanide, CO, NO and O_2).

4.1. Pharmacological Use

Currently, there are many heme-based gas sensors involved in key biological processes both in humans and pathogens, making them suitable drug targets for investigation [92]. Here, we described a few interesting cases, some of them still in very early stage while others with drugs approved for clinical use.

The first heme-based gas sensor discovered, sGC, has many physiological roles in humans, making it a very desirable drug target for the treatment of endothelial, cardiovascular and pulmonary disorders [93,94]. A series of nitric oxide (NO) donors was earlier employed targeting sGC, even before this sensor was known or this small molecule (NO) found to be biologically active. The binding of NO to sGC strongly stimulates its cyclase activity, converting GTP into cGMP [27]. This is one of the reasons for the development of NO donor molecules, which can be employed to lower blood pressure, hypertension crisis, angina pectoris, glaucoma, among other applications [94]. However, the lack of selectivity and acquired tolerance to NO can limit its action in continuous use. Some strategies have emerged to deal with these issues, particularly providing more selective delivery strategies either by using light, nanoparticles or other stimuli [1,94]. At the same time, other small molecules have been investigated, starting with YC-1, the first direct sGC stimulator discovered [95]. Currently, there are many organic- and inorganic-based molecules investigated as sGC stimulators or activators likely able to treat many conditions [92–94,96]. Two drugs (riociguat, 2013, and vericiguat, 2021) that function by targeting

sGC are already approved for clinical use, opening many opportunities. Other heme-based gas sensors found in humans have also been explored as potential drug targets involved in the mood and metabolic disorders (e.g., NPAS2, Rev-erbα/β) [92,97].

The disruption of sensing systems in bacteria has emerged as a promising strategy for the development of novel antibiotics with likely minor selective pressure [98]. Among these systems, there are heme-based gas sensor proteins as potential drug targets [1,92]. In certain cases, the use of NO donors can be beneficial to assist with the elimination of the bacterial infection through interaction with heme-based gas sensors [94]. One of the major obstacles to eliminate bacteria is caused by the production of biofilm that leads to antibiotic tolerance [99]. The NO sensor NosP found in *P. aeruginosa* and *V. cholerae* is involved in the regulation of biofilm production, where binding to NO promotes biofilm dispersal [100]. This has opened new opportunities for the use of NO donors, particularly in combination with antibiotics. Recently, we and others have been able to show that NO donors can function in synergy with antibiotics, promoting the disruption of biofilms and enhancement of antibiotic action [101–103].

The O_2 sensor two(three)–component system of *M. tuberculosis*, DevS/DosT–DevR, has also become an important drug target [72,92,104]. Two laboratories have made efforts to identify new molecules to inhibit this sensing system. Tyagi's laboratory pioneered this study in identifying phenylcoumarin-based compounds through an initial in silico screening (2.5 million compounds), but their hits exhibited low biological activity [105]. Then, they carried out other studies looking at small peptides targeting DevR and DevS using the phage display technology [106,107]. Unfortunately, the most active peptides identified did not show suitable biological efficiency. Recently, Abramovitch's laboratory developed a cell-based phenotypic screening assay with a reporter gene under DevR regulation [108]. In this study, they employed a library of 540,288 compounds, where six classes of compounds were identified with apparent distinct mechanisms of action. These compounds showed quite promising biological activity toward Mtb with apparently distinct mechanisms of action, including a synergistic effect with anti-tuberculosis drugs (e.g., isoniazid) [104,108,109]. One interesting case was with artemisinin, one class of drug found in the screening, that was shown to target the heme of DevS and DosT [108]. Other laboratories had shown the potential anti-tuberculosis activity of this old compound also in combination with isoniazid (and other anti-TB drugs) [110,111], but there was no clue about its potential target. These exciting studies may continue and eventually provide a leading compound targeting dormant bacteria, a major issue during tuberculosis treatment. Besides this case, other heme-based gas sensors might function as a drug target in the development of antibiotics or antiparasitic agents, deserving a close look, including in certain FixL systems (found in the pathogens *Burkholderia cepacia* and *Brucella abortus*) [66,68]), *Bpe*GReg (found in *Bordetella pertussis*, a pathogen that causes whooping cough) [112], Hpk2 (found in *Treponema denticola* that causes periodontal disease) [113], DosC/DosP (found in *E. coli*) [19,89] and HemAC-Lm (found in the parasite *Leishmania*) [114].

Beyond this, due to the large diversity of heme domains reported for heme-based gas sensors, they should be further investigated as potential antidotes for carbon monoxide poisoning, delivery of gasotransmitters (e.g., Sanguinate® [115]) or even as blood substitutes as carried out with neuroglobins [116] and modified hemoglobins (e.g., Hemopure®) [117]. There is one expired patent on the potential use of globin-based proteins including heme-based gas sensor proteins HemAT from *Halobacterium salinarum* and *Bacillus subtilis* in medicine as blood substitutes and microsensors [118]. As far as we are aware, there is only one case of a heme-based gas sensor protein being investigated as a blood substitute, which was reported for the H-NOX sensor from *Thermoanaerobacter tengcongensis* (*Tt*H-NOX, Omniox®) [119]. In this study, a modified *Tt*H-NOX heme domain exhibited promising results in preserving myocardial contractility during acute hypoxia in an ovine model of myocardial injury, whereas NO sequestration was not an issue, commonly faced when using hemoglobin-derived biotherapeutics. This heme-based gas sensor has been explored in many other applications as described further.

4.2. Chemical and Biological Tools

DosP (earlier called *Ec*DOS) is a heme-based O_2 sensor from *E. coli*, which upon binding to oxygen exhibits a steep increase in c-di-GMP phosphodiesterase activity [120]. This dinucleotide is an important signaling molecule in bacteria with important roles in humans as well [121]. A recent study showed that DosP can be efficiently encapsulated into mesoporous silica, where its regulated phosphodiesterase activity is reasonably preserved along with stability [120]. This type of material might have interesting chemical and biological applications. The easier chemical production of linear pGpG from c-di-GMP phosphodiesterase might be one potential use of this material. In addition to that, its capacity to degrade c-di-GMP might have therapeutic features against microbes that could be explored [122].

The analytical use of heme-based gas sensors as probes for diatomic gases (O_2, NO and CO) or anions (cyanide) has been explored. The heme-based CO sensor from *Rhodospirillum rubrum*, CooA, was designed to report CO levels in vitro and in vivo [123]. This was achieved by fusing a fluorescent protein in the C-terminal domain, where a major conformational change occurs when CO binds. Notably, this sensor exhibited an increase in fluorescence upon binding to CO with a K_d of 2 μM, which was minimally disturbed by NO, O_2, H_2S, imidazole, glutathione or CN^-. This probe was also investigated in vivo by transfecting HeLa cells with a probe-expressing vector, where fluorescence images were obtained either using 5 μM CO (externally applied) or a known CO donor molecule (CORM-2, used even at 1 μM).

A mutant of the H-NOX sensor from *Thermoanaerobacter tengcongensis* (*Tt*H-NOX Y140F) was developed to function as an NO sensor. This mutant exhibited great properties such as no binding to oxygen, high stability to heat (up to 70 °C) and oxidation, but a very strong affinity to NO. Notably, this protein can be used to measure very low levels of NO even in a large background of O_2. Another modification of *Tt*H-NOX was carried out by replacing its native heme with a ruthenium(II)-mesoporphyrin IX [124]. In this case, the protein scaffold hosted a luminescent ruthenium-based cofactor that upon binding to O_2 exhibits light emission suppression, functioning as an oxygen sensor. Indeed, the authors showed they could detect up to 4.2 μM O_2, claiming this is reasonably close to commercial O_2 sensors [124].

In another study, meso-alkynylation of heme b cofactor was carried out, allowing further conjugation with many molecules, including fluorophores. The authors showed this modified porphyrin could be efficiently incorporated in the *Tt*-H-NOX protein, then labeled with an azide-based fluorophore (rhodamine) [125]. This procedure offers more flexibility where labeling is not in the protein chain, making it possible to explore fluorescence imaging as well as Raman microscopy. In a recent study, a highly fluorescent near-infrared compound, phosphorus corrole, was investigated as replacement of the native heme of the *Cs*H-NOX sensor from *Caldanaerobacter subterraneus* [126] (formerly known as *Thermoanaerobacter tengcongensis* H-NOX or *Tt*-H-NOX sensor [127]). The authors showed phosphorus corrole was able to replace its native heme cofactor, providing better properties such as long wavelength emission and high quantum yield. Corrole-based compounds exhibit some structural similarity with protoporphyrin IX, but they can be much better emitters, which in combination with a robust protein scaffold can generate promising new fluorescent systems [126]. Besides these cases, this same heme-based gas sensor was also explored in other applications, such as cyanide sensor (*Tt*H-NOX P115A mutant) [128] and MRI probes using Mn(II/III) and Gd(III) porphyrins as a substitute to the native heme [129].

4.3. Systems Applied in Cell Biology

An early study produced a sensory chimera (CskA) containing part of FixL [130], aiming mostly to understand the essential elements for building a functional sensor. These investigators combined the oxygen-sensing heme domain of *Sm*FixL from *Sinorhizobium meliloti* with the histidine kinase domain of ThkA from the hyperthermophile *Thermotoga maritima*. In that work, two out of five constructs showed oxygen affinity close to full-length *Sm*FixL, but none of them exhibited oxygen regulation of histidine kinase. Nonetheless, some constructs showed even higher autophosphorylation activity if compared to ThkA [130], indicating modulation of the enzymatic activity in the chimera. This frustrated attempt to prepare an oxygen-regulated system illustrated the key role of the linkers to allow proper interaction of the domains and likely allow regulation. Other attempts were more successful in achieving signal regulation as described below.

In 2008, Moffat's laboratory developed a chimeric protein by replacing the heme domain of the oxygen sensor FixL with the light–oxygen–voltage-sensing domain (LOV domain of YtvA from *Bacillus subtilis*) [131]. Their goal was to control reversibly the histidine kinase activity of FixL using blue light instead of oxygen. Interestingly, they prepared some protein constructs with remarkable regulation of enzymatic activity using blue light, achieving over 1000-fold inhibition. This system was also investigated in *E. coli*, where light irradiation was able to shut off 70-fold a gene reported based on this system [131]. This study opened exciting opportunities for a broad use of sensing systems. Another independent laboratory prepared a chimera of FixL with CckA, this latter being a histidine kinase sensor from *Caulobacter crescentus*, as a biological tool [132]. This system combined the sensing unit of CckA with the histidine kinase of FixL and full-length FixJ along with a fluorescent gene reporter responsive to phosphorylated FixJ. The use of this biological tool allowed them to better understand the molecular signaling mechanism associated with the asymmetric cell division of *C. crescentus* [132].

Another chimeric protein was developed using the heme PAS domain of DosP, an oxygen sensor in *E. coli*, combined with a yellow fluorescent protein (Venus YFP) [133]. This new protein, called ANA-Y (anaerobic/aerobic sensing yellow fluorescence protein), has a coiled-coil linker optimized to provide suitable fluorescence response upon oxygen binding to the heme domain. This system can report oxygen concentrations in the micromolar range, which was employed as a genetic probe in synthetic biology to monitor initial photosynthetic production of oxygen in cyanobacteria [133]. In the previous subsection (see "Diverse Potential Applications of Heme-Based Gas Sensors: Chemical and Biological Tools"), we also presented a CO probe based on a chimeric protein of CooA. This sensor regulates bacterial processes involving the consumption of CO with the generation of H_2 such as in the water–gas shift reaction ($CO + H_2O \rightarrow CO_2 + H_2$). This process is catalyzed by two enzymes, carbon monoxide dehydrogenase (CODH) and carbon monoxide-dependent hydrogenase (CO-Hyd) under regulation by CooA [134]. Aiming to enhance the production of H_2 by *Citrobacter amalonaticus* Y19, an engineered strain was prepared containing a plasmid carrying the cooA gene for overexpression [134]. This genetic alteration promoted an expressive increase in H_2 production (3.4-fold) by this bacterium, supporting this type of biotechnological strategy.

4.4. Biocatalysts

In 2018, Dr Frances Arnold was awarded a Nobel Prize in Chemistry for her contribution to the development of novel biocatalysts based on directed evolution [135]. Despite that, biocatalysts have been developed not only based on directed evolution (random mutations), but also with exchange of metal ions and incorporation of new cofactors that have broadened our horizon. Indeed, enzymatic catalysis of reactions not reported to be carried out by nature were developed by designing novel biocatalysts. Myoglobin, a non-enzymatic hemeprotein, has been widely explored and converted into an outstanding novel catalyst for many distinct biological and nonbiological reactions, as reviewed elsewhere [136]. The same has been carried out for many other metalloproteins, including other hemeproteins (e.g., cytochrome c, cytochrome P450). This may be a great opportunity to explore many distinct heme domains found in the families of heme-based gas sensors. As far as we are aware, there is only one case explored so far, as described below.

Aiming to explore and enhance potential catalytic properties of the heme-based gas sensor *Tt*-H-NOX, its heme domain was studied and subjected to site-direct mutagenesis at the distal tyrosine ligand [137]. Interestingly, this protein showed capacity to decompose H_2O_2, which was strongly enhanced in the Y140H mutant and lowered in the Y140A mutant. Their peroxidase activities were investigated using the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) substrate along with H_2O_2, where the WT protein showed moderate activity but the Y140H mutant was significantly better (70% higher) in degrading ABTS. Despite this protein showed a much lower turnover compared to other known catalytic proteins, the authors claimed there is a comparable efficiency in oxidizing substrates as in some native catalysts (e.g., but using different substrates, *Tt*H-NOX $k_{cat} = 0.06\ s^{-1}$ for ABTS versus cytochrome P450BM-3 $k_{cat} < 0.008\ s^{-1}$) [137]. This study did not provide appealing catalytical results, but it highlighted the opportunities ahead, taking advantage of the thermostability, easy production and structural robustness of that heme-based gas sensor protein.

In another study, a multidomain protein containing one heme PAS domain showed an unexpected oxidative N-demethylase activity based on the heme cofactor [138]. The PAS domain is indeed a typical signal transduction unit employed by many types of sensors, including heme-based gas sensors, which has never exhibited any reported catalytic activity. Despite this protein might not be a true heme-based gas sensor, it has exposed the heme PAS domain as a biocatalytic unit deserving further studies. This unique case highlights the broad opportunities to explore biocatalysis using many other types of heme domains from heme-based gas sensors.

As shown here, there are many opportunities to create novel tools and materials by manipulating heme domains. One strategy to explore this is to replace the native heme by another metalloporphyrin analogs, providing novel functions to the protein as described before. To achieve this, engineered *E. coli* strains or growth conditions have been explored to facilitate direct incorporation of heme analogs or production of hemeless proteins [139–142]. The engineered RP523 strain of *E. coli* was prepared, exhibiting disruption of biosynthesis of the heme (hemB and hemC) along with the heme permeability phenotype [139]. This feature allowed direct incorporation of metalloporphyrins once they were provided in the cell culture medium [139], but oxygen sensitivity and the toxicity of cofactors are a concern for its success. Another strategy was to use an *E. coli* BL21(DE3) strain containing a plasmid bearing the heme transporter ChuA allowing the uptake of heme analogs provided in the cell medium [141]. In addition to that, *E. coli* was grown in an iron-depleted medium to minimize heme biosynthesis, but up to 5% of the protein could still bear the heme. Alternatively, it was shown that the hemeproteins overexpressed in *E. coli* BL21(DE3) employing the M9 medium are mainly hemeless, where the incorporation of exogenous metalloporphyrins can be easily achieved by mixing with lysed cells [140]. These approaches are important technical advances helping the success of using heme-based proteins.

5. Final Considerations

Heme-based gas sensor proteins are a remarkable widespread superfamily of sensing systems. These moderately old sensors always surprise us with their diversity, appearance of new families and mechanisms of action. There is no doubt many more are still going to be discovered. The multiple applications of these systems are just unfolding, covering biological and chemical tools, biocatalysis and drug targets, making them a hot spot of opportunities much beyond our necessity of understanding their molecular functioning.

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Abbreviations

*Bpe*GReg	*Bordetella pertussis* globin-coupled regulator
CckA	*Caulobacter crescentus* histidine kinase protein A
c-di-GMP	Cyclic dimeric (3′,5′) guanosine monophosphate
cGMP	Cyclic guanosine monophosphate
*Cs*H-NOX	H-NOX sensor from *Caldanaerobacter subterraneus*, formerly known as *Tt*H-NOX
CskA	Chimeric sensory kinase A
CooA	CO oxidation activator protein
CORM-2	CO-releasing molecule 2
DevS	Differentially expressed in virulent strain sensor protein, the same protein as DosS
DevR	Differentially expressed in virulent strain response regulator protein, the same protein as DosR
DosC	Direct oxygen sensor cyclase protein
DosP	Direct oxygen sensor phosphodiesterase protein
DosS	Dormancy survival sensor protein
DosT	Dormancy survival sensor T protein
*Ec*DOS	*Escherichia coli* direct oxygen sensor protein, the same protein as DosP
FIST	F-box and intracellular signal transduction proteins domain
FixL	Rhizobial nitrogen fixation gene L protein
FixJ	Nitrogen fixation gene J protein
FNR	Fumarate and nitrate reduction protein
GAF	cGMP-specific phosphodiesterases, adenylyl cyclase, and FhlA proteins domain

GTP	Guanosine-5′-triphosphate
HemAC-Lm	Heme-containing adenylate cyclase from *Leishmania major*
HemAT	Heme-based aerotactic transducer sensor protein
HIF	Hypoxia-inducible factor
HNOB	Heme NO-binding domain
HNOX	Heme NO- and oxygen-binding domain
HTH	Helix-turn-helix domain
HOLI	Ligand-binding domain of hormone receptors
Hpk2	Histidine protein kinase 2 from *Treponema denticola*
YC-1	5-[1-(phenylmethyl)- 1H-indazol-3-yl]-2-furanmethanol
LBD	Ligand-binding domain of the nuclear receptor
LOV	Light–oxygen–voltage-sensing domain
MRI	Magnetic resonance imaging
Mtb	*Mycobacterium tuberculosis*
NifA	Oxygen-responsive nitrogen fixation sensor protein A
NorR	NO reductase regulatory protein
NosP	Nitric oxide-sensing protein
PAS	Per, period circadian protein, Arnt, aryl hydrocarbon receptor nuclear translocator protein, Sim, single-minded protein domain
pGpG	Linearized form of cyclic di-GMP, 5′-phosphoguanylyl- (3′ −> 5′)-guanosine
SAXS	Small-angle X-ray scattering
SCHIC	Sensor-containing heme instead of cobalamin domain
sGC	Soluble guanylate cyclase
ThkA	*Thermotoga maritima* histidine kinase A
*Tt*H-NOX	H-NOX sensor from *Thermoanaerobacter tengcongensis*
VcBhr-DGC	*Vibrio cholerae* bacterial hemerythrin diguanylate cyclase sensor protein
Whib	regulator originally associated to the gene locus involved with the conversion of white multinucleoidal aseptate aerial hyphae into chains of mature grey uninucleoidal spores
Whib3	NO and/or O_2 sensor from *Mycobacterium tuberculosis*
YFP	Yellow fluorescent protein

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