Review Article

Hemoglobin: A Nitric-Oxide Dioxygenase

Paul R. Gardner

Miami Valley Biotech, 1001 E. 2nd Street, Suite 2445, Dayton, OH 45402, USA

Correspondence should be addressed to Paul R. Gardner; paul.gardner@mvbiotech.com

Received 11 September 2012; Accepted 4 October 2012

Academic Editors: Y. Furukawa and H. Iwano

Copyright © 2012 Paul R. Gardner. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Members of the hemoglobin superfamily efficiently catalyze nitric-oxide dioxygenation, and when paired with native electron donors, function as NO dioxygenases (NODs). Indeed, the NOD function has emerged as a more common and ancient function than the well-known role in O₂ transport-storage. Novel hemoglobins possessing a NOD function continue to be discovered in diverse life forms. Unique hemoglobin structures evolved, in part, for catalysis with different electron donors. The mechanism of NOD catalysis by representative single domain hemoglobins and multidomain flavohemoglobin occurs through a multistep mechanism involving O₂ migration to the heme pocket, O₂ binding-reduction, NO migration, radical-radical coupling, O-atom rearrangement, nitrate release, and heme iron re-reduction. Unraveling the physiological functions of multiple NODs with varying expression in organisms and the complexity of NO as both a poison and signaling molecule remain grand challenges for the NO field. NOD knockout organisms and cells expressing recombinant NODs are helping to advance our understanding of NO actions in microbial infection, plant senescence, cancer, mitochondrial function, iron metabolism, and tissue O₂ homeostasis. NOD inhibitors are being pursued for therapeutic applications as antibiotics and antitumor agents. Transgenic NOD-expressing plants, fish, algae, and microbes are being developed for agriculture, aquaculture, and industry.

1. Background and Introduction

Nitric-oxide dioxygenases (NODs) are enzymes that efficiently convert NO and O₂ to nitrate (1). Most, if not all, NODs are hemoglobins (Hbs), and most, if not all, Hbs have the capacity to function as NODs. Hb-NODs appear to be widely distributed in nature. In fact, the NOD function appears more common and ancient than the classic O₂ transport-storage function, or any other function, within the Hb superfamily [1]. Nevertheless, textbook familiarity with the O₂ transport-storage function continues to blind investigators to the enzymatic functions of various members of the Hb superfamily. For example, the genome of the nematode Caenorhabditis elegans encodes >33 candidate Hbs [2, 3], many of which are thought to store or transport O₂. Multiple globins are also normally expressed in non-erythroid vertebrate cells and tissues [4, 5], and globin expression is deranged in cancer cells [6–9]. When asking the question “what are all these oxygen-binding heme proteins doing?” [10], investigators are now obliged to thoroughly consider enzyme functions, and in particular a NOD function

\[
\text{NO} + O_2 + e^- \rightarrow NO_3^-
\] (1)

NODs are one of the most recent additions to the growing family of free radical and peroxide scavenging enzymes that includes the family of peroxidases [11] and peroxiredoxins (alkyl hydroperoxide reductases) [12], catalases [13], superoxide dismutases (SODs) [14, 15], superoxide reductases [16], and NO reductases (NORs) [17]. There is also preliminary evidence for a NO dismutase in certain methanotrophs [18].

Seminal concepts within the family of peroxide and free radical scavenging enzymes can be traced to Oskar Loew’s pioneering description of “catalase”, a heme-containing enzyme with the "power of catalyzing hydrogen peroxide" [13, 19]. In 1900, Loew wrote “There seems to exist no plant and no animal which is without that enzyme [catalase]”. He also presciently wrote “One of the functions of
this enzyme appears to be to prevent any accumulation of hydrogen peroxide which might be formed as a by-product in the series of energetic oxidations that characterize the cellular respiration process. Hydrogen peroxide is a poison for the living protoplasm, hence the activity of catalase is of vital importance.” Loew also made the early and important distinction between the substrate (H$_2$O$_2$) and enzyme in the catalytic reaction at a time when enzymes were only vaguely understood. While peroxidations had been described as early as 1855, the enzymatic nature and biological function of peroxidases would only be investigated much later [11, 20, 21]. The idea of dangerous toxic free radicals formed as by-products of an aerobic metabolism blossomed following the discovery of superoxide dismutase(s) and the demonstrations of superoxide radical actions in biological systems by Irwin Fridovich and his students [14, 15, 22]. Incalculable efforts have been expended to achieve our current understanding of the poisonous and damaging reactions, sources of the radicals and oxidants, as well as the full repertoire of detoxifying enzymes and their roles in physiology and pathophysiology. In addition, novel roles for radicals and oxidants in cell signaling and scavengers in signal modulation were appreciated by the end of the 20th century [23], but are still not fully comprehended.

Indeed, paradigms established by the pioneers of antioxidative and free radical-scavenging enzymes guided the discovery of NOD and continue to instruct investigations. Experiments have also been facilitated by a wealth of knowledge, as well as questions waiting for solution, in the areas of Hb structure, distribution, expression, functions, and evolutionary origins [24, 25]. On the other hand, the discovery of NO metabolizing enzymes followed rapidly on the heels of discoveries of the biological production and functions of NO, thus leaving much to discern and discover in a rapidly evolving field. Substantial effort is now being aimed at understanding the biological functions and enzymatic mechanisms of various NODs and the development of NOD-based technologies including research tools, recombinant plants and other organisms, and therapeutic drugs including antibiotics. Novel Hbs continue to be examined for a possible NOD function. In addition, novel non-Hb type NODs and NOD mimetics are under investigation [26].

2. Purpose and Scope

While several reactions of NO with erythrocyte Hb and muscle myoglobin (Mb) including the tight-binding of NO to the ferrous heme, the NO (Stickstoffoxyd, Salt peterstoffs gas)-mediated oxidation of the oxy-heme, and the formation of NO from nitrite have been under investigation for many decades [27–33], our investigations published in 1998 [34, 35] provided our first glimpse of the ~2 billion-year-old evolutionary link between NO and Hb vis-à-vis the NOD function of E. coli flavoHb. The observations offered a distinct and common enzymatic function for the primitive low-abundance Hbs first formally formulated and investigated by David Keilin around 1945 [36, 37], expanded and clarified by Austen Riggs [38, 39] and others [40–42], and pursued early on in the labs of Cyril Appleby [43, 44], Britton Chance [45], Hans Schlegel [46], Dale Webster [47], Jonathan and Beatrice Wittenberg [24], Bärbel Friedrich [48, 49], Robert Poole [50, 51], Austen Riggs [39, 52], Michiko Nakano [53], Robert Poyton [54], Daniel Goldberg [55–57], Keiji Shikama [58–61], Malcolm Potts [62], Robert Hill [63, 64] and many others clearing a wide path to the common NOD function. However, while appearing attractive to some, the well-known tight-binding of NO to ferrous Hbs [K$_D$ = ~ 10 pM] made the NOD enzyme hypothesis appear rather dubious in the eyes of experts. Despite this and other challenges, data supporting a primal NOD mechanism-function for diverse members of the ancient Hb superfamily has expanded appreciatively in the last 14 years. While the facts that lay obscure or dormant in the pre-1998 literature, and the findings of the first ~7 years have been dutifully and thoroughly reviewed in our 2005 and 2006 publications [65, 66] and elsewhere, my intent here is not only to describe important advances in the area of Hb/NOD research, but also to pose critical questions and discuss new concepts that will help move investigations forward and into new areas. This, of course, has demanded critiques of what I consider lingering misconceptions that continue to plague the field, stymie progress, and lead investigators off the path. It is hoped that the reader finds this paper rigorous, not trite, too pedantic, tame, or polemical. The reader is also directed to more than a dozen recent reviews presenting in-depth perspectives on various aspects of the topic [1, 67–87]. I have attempted to highlight these reviews in pertinent sections of my paper. A distillation and synthesis of the available evidence is crucial since some investigators continue to ponder upon a “common but still poorly defined function of globins” [88]. Here, I thoroughly scrutinize the merits of a common NOD function in the light of the results of more than a decade of research.

3. Evolution and Distribution of Hbs

Several recent phylogenomic studies and reviews have catalogued the broad distribution and lineages of Hbs of various subtypes in the three life kingdoms [1, 89–94]. The amazing array of globins expressed even within individual life forms [3, 42, 74, 95] including humans [96, 97] raise myriad questions that will occupy investigators for many years. Most strikingly, the genomes of most organisms encode multiple Hbs with unknown function. Moreover, relatively few Hbs have been scrutinized for a NOD function. Which subtypes function as NODs? If a life form cannot express a Hb, how does the organism metabolize NO? Why do organisms express multiple Hbs and various Hb sub-types? What primary structure features reliably predict a NOD function and allow annotation of Hb genes? What is the significance of these structural features to function? What are all the possible functions of Hb? And the biggest question—How did Hb function evolve?

Answers to these many questions have been slow to emerge, but there are fundamental concepts guiding our understanding of Hb functions and evolution. Clearly, numerous proteins possess multiple reactivities and functions [98, 99]; a property that has been colorfully referred to as “moonlighting” [100] or “catalytic promiscuity” [101].
Indeed, those reactivities serve as a framework for protein [globin] evolution. As astutely pointed out by Shikama and coworkers [61, 102], “Whatever the possible roles of such primitive or ancient globins may be [or might have been], the reversible binding of molecular oxygen to iron[III] must be the primary event to manifest their physiological functions in vivo.” The fairly unique electronic structure of the ferric heme-superoxo bond in Hbs [103] suggests an even more unique chemical-biological niche for the globins. Thus, while a focus on the evolution of globin structural differences suggests great functional variation [24, 25, 104, 105], a focus on the potentially limited reactivity of the conserved ferric heme-superoxo electronic structure suggests a common function.

As Max Perutz eloquently stated “Evolution is a brilliant chemist” [106]. Proteins evolve by eliminating old traits and creating new traits from a single protein scaffold. In the case of Hbs, the old traits of flavoHb-NODs include high O₂ affinities, high autoxidation rates, and internal electron transfer rates that are suited for rapid NO dioxygenation catalysis. New traits include the low O₂ affinities, low autoxidation rates, external electron donors, lower NO dioxygenation rate constants [65], and a single globin domain that is adapted for O₂ storage-transport [106]. What is more difficult to fathom and demonstrate is a major O₂-independent function for Hb that predates the ~2 billion year old, contemporary, and common NOD function [1]. Albeit, NO reductase [69, 107] and electron transfer [108] functions have been proposed for flavoHb and neuroglobin (Ngb), respectively. The discoveries of an estimated 3.8 billion-year-old primordial prodiglobin in a “strictly” anaerobic methanogen Methanosarcina acetivorans [109, 110] and the single domain “thermoglobin” in Aquifex aeolicus (AE000678) [52, 111] provide relic experimental systems to further explore the functional origins of the globin family.

4. FlavoHbs, Hbs, and Mbs Function as NO-Metabolizing Enzymes

Are Hbs, Mbs, trHbs, and flavoHbs simple NO scavengers or are they highly evolved enzymes? For the two domain flavoHbs, the answer has always been clear. FlavoHbs catalyze NO dioxygenation efficiently, rapidly, and with high fidelity [65, 66]. FlavoHbs belong to the oxidoreductase family utilizing NAD(P)H to incorporate two O-atoms from O₂ into the substrate NO to form nitrate (2). FlavoHbs catalyzing the reaction are formally called NODs and have been designated EC 1.14.12.17 by the Enzyme Commission of the International Union of Biochemists and Molecular Biologists. Over 4,000 flavoHb protein entries are now labeled primarily as NODs at the US National Institutes of Health web site (http://www.ncbi.nlm.nih.gov/pubmed/). Many more flavoHbs are annotated for a possible NOD function. Since my last listing of flavoHb-NODs in 2005 [65], several flavoHbs notably expressed by human or plant pathogens have been isolated and tested directly for a NOD function. Aspergillus oryzae, A. nidulans, and A. fumigatus express 2 flavoHbs showing a NOD function [112–114], Erwinia chrysanthemi [115], Giardia intestinalis [116], G. lambliia [117], and Mycobacterium tuberculosis [118, 119] flavoHbs have been also investigated. The E. chrysanthemi and Giardia flavoHbs show appreciable NOD activity in vitro.

\[2\text{NO} + 2\text{O}_2 + \text{NAD(P)}H \rightarrow 2\text{NO}_3^- + \text{NAD(P)}^+ + \text{H}^+ \] (2)

For many single domain globins (SDGs), an annotation as a NOD has always been less clear. It would appear that all single domain oxy-Hbs catalyze the NO dioxygenation reaction with reported in vitro bimolecular rate constants ranging from $10^6$ to $>10^9$ s⁻¹ M⁻¹ [65, 66, 120–125]. The Arabidopsis thaliana GLB1 and GLB2 (trHbs) convert NO to nitrate in vivo as evidenced by increased NO emissions and decreased tissue nitrate concentrations in globin-deficient mutants [126, 127]. But few SDGs have been shown to couple with a native redox partner for efficient enzymatic NO consumption [128–132]. Moreover, when demonstrations of weak or nonspecific redox coupling have been observed to support turnover in vitro, or in heterologous organisms, there has been an understandable reluctance to declare that sufficient evidence for a NOD function [133]. Clearly, full proof of a NOD function requires the demonstration of catalytic O₂-dependent NO metabolism within the native organism. Furthermore, a specific electron donor needs to be identified for demonstrations of efficient catalysis in vitro. To date, only the mammalian Cygb-NOD has been investigated both in cells [134, 135] and reconstituted with a native electron donor (ascorbate or cytochrome b₅) in vitro [135, 136]. In addition, many SDGs have shown evidence of a protective enzymatic NOD function including muscle Mb [137], Synechococcus truncated Hb [138], Synechocystis cyanoglobin [133], and the dual function nitrate reductase-fused trHb in raphidophytes [139]. However, one cannot declare a NOD function a priori. A NOD function requires a demonstration of enzymatic turnover. Protective effects of SDGs could also be attributed to nonenzymatic, rapid, and competing NO oxidation (dioxygenation) and nitrosylation reactions of Hbs similar to those of erythrocyte Hb and muscle Mb [31, 32, 140]. This is a small distinction, but a potentially important one.

5. Myriad NO Functions: Bioenergetic Intermediate, N Source, Toxin, and Signal Molecule

To understand the physiological functions of NODs (Hbs), knowledge of the burgeoning field of NO biology is required. NO is fairly ubiquitous and serves important roles in bioenergetic transformations, immunity, and signaling in diverse life forms. Quantitative knowledge of the sources of NO, target reactions of NO, and localized steady-state NO levels is also required to discern the competing and overlapping roles of NO. Moreover, within specific cells and tissues, these factors vary in complex ways [198, 199]. Nevertheless, instructive generalizations can be made.

In microbes, algae, and plants, NO serves as an important role as a bioenergetic intermediate and nitrogen source.
The process of anaerobic or microaerobic denitrification, particularly in soil microorganisms, generates NO as an obligate intermediate in the energy-yielding reductive dissimilation of NO$_3^-$ to N$_2$ [17, 200]. Nitrate reductases generate nitrite, and nitrite reductases generate NO for reduction by proton-motive force generating membrane-bound NO reductases and N$_2$O reductases. FlavoHb (NOD) can recycle the denitrification intermediate NO to NO$_3^-$ and is required for optimal microaerobic denitrification, and presumably energy production, by fungi [200] and bacteria [48]. NO is also an important intermediate in the energy-yielding pathway of microaerobic ammonia oxidation or nitrification [201, 202]. Alternatively, NO can be generated through non-specific reduction of nitrite by nitrate reductases found abundantly in plants, algae, and microbes [203–205]. Neighboring soil microorganisms and plants can assimilate N through the Hb-catalyzed dioxygenation of diffusible NO to NO$_3^-$. In addition, NO$_3^-$ generated via catalytic NO dioxygenation can provide an electron sink for fermentative energy production by plants [206], bacteria, and fungi [52, 200].

NO is also an important intermediate in the Earth's nitrogen cycle [207, 208]. Anthropogenic sources, such as N-fertilization, NO$_x$ pollution, and waste generation, may contribute to excess formation of NO and the greenhouse gas N$_2$O [207]. Rhizobial NODs serve an important role in converting NO to NO$_3^-$ in plants and in doing so avert N$_2$O formation by rhizobia and soil microbe NO reductases [87]. Indeed, _Pseudomonas stutzeri_ engineered for flavoHb overproduction emit less N$_2$O during microaerobic denitrification [209]. Photoilluminated leaf chloroplast nitrite reductase is also an important source of atmospheric N$_2$O presumably via the reduction of assimilated NO$_3^-$ to NO [210], and NO to NO$^-$ followed by the combination of 2 NO$^-$ to form N$_2$O. In this case, a chloroplast NOD (trHb) may serve as an important geochemical function by decreasing NO levels and N$_2$O formation.

_NO acts as a toxin and signal molecule throughout the biosphere._ In addition to the numerous sources of NO described above, NO is produced by nitric-oxide synthases (NOSs) and various nitrite-reducing activities in many different organisms including humans [211]. Removal of NO by catalytic NO dioxygenation can thus serve to prevent NO toxicity or attenuate NO signaling. Understanding NO toxicity and signaling in the biosphere requires an understanding of the many reactions of NO as well as its concentrations. I have listed only a few of the important biochemical reactions of NO and the relevant biological functions or consequences in Table 1. The representative list of reactions clarifies why NO is an outstanding natural antibiotic and antitumor agent and why NO metabolism by NODs or NORs provide a direct path for cellular resistance. The toxicology of NO has been discussed in greater detail in an excellent recent review by Toledo Jr. and Augusto [189]. _Not too surprisingly, many of the toxic reactions of NO have been exploited by Nature for NO signaling functions._ These bifunctional toxic reactions include ferrous heme nitrosylation (e.g., soluble guanylate cyclase activation), iron-sulfur center disruption (NsrR and IRE-BP) [193], mononuclear iron-binding (e.g., prolyl hydroxylase, NorR and ACO), and the NO/bound O$_2^-$ reaction (e.g., DevS, DosT and FixL).

When evaluating the competitive reactions of NO in complex systems, it is very valuable to know steady-state concentrations of reactants and bimolecular rate constants. These values allow us to make some simple and powerful calculations. For example, we can determine the rate of NO removal and the maximum flux of NO going to toxic peroxynitrite formation in a cell containing O$_2^-$ and oxy-Hb. If the steady-state [O$_2^-$] $= 10$ pM, [NO] $= 0.1$ mM, and [oxy-Hb] $= 0$, then the rate of NO removal and peroxynitrite formation = $k_2$ [NO] [O$_2^-$] = 7 nM NO s$^{-1}$, where $k_2$ = 6.9 $\times$ 10$^7$ s$^{-1}$ M$^{-1}$. If oxy-Hb is also present at 1 $\mu$M, and its bimolecular rate constant ($k_3$) for reaction with NO $= 3 \times 10^7$ s$^{-1}$ M$^{-1}$, then the rate of NO removal $= k_2$ [NO][O$_2^-$] + $k_3$ [NO][oxy-Hb] = 30,007 nM NO s$^{-1}$. We can also see from the relationship that at any steady-state [NO] the ratio of the NO flux to O$_2^-$ and oxy-Hb equals $k_2$[O$_2^-$]/$k_3$[oxy-Hb], or in this case 0.0023. Thus, under these physiologically relevant concentrations, only 0.23% of the NO will escape detoxification by oxy-Hb and form toxic peroxynitrite. Similar approximations can be made for other competing reactants.

### 6. FlavoHbs and Hbs Detoxify NO

A large number of flavoHbs and Hbs have been shown to detoxify NO, and many of these have been listed and described in my 2005 review [65] and in the recent Forrester and Foster review [67]. The list of organisms that are presumed to detoxify NO using flavoHbs and Hbs has expanded profoundly with the increase in genome sequencing and _in silico_ analysis. Not too surprisingly, far fewer experimental demonstrations of organisms utilizing flavoHbs or Hbs for their protection against NO have been reported. Recent experiments support NOD functions for flavoHbs or Hbs expressed by _Bacillus subtilis_ [212, 213], _Staphylococcus aureus_ [214, 215], _Aspergillus oryzae_ [112, 216], _A. nidulans_ [113], _A. fumigatus_ [114], _Yersinia pestis_ [217], _Vibrio fischeri_ [218], _Pseudoalteromonas haloplanktis_ [219], _Campylobacter jejuni_ [220, 221], the Japanese shrub _Abhuis firma_ [182], _Sinorhizobium meliloti_ [183], _Botyrtis cinerea_ [222], and _Synechococcus_ [138]. Expression of _Mycobacterium leprae_ GlbO [223] and _Synechocystis_ SynHb [133] alleviates NO toxicity in _E. coli_ supporting a NOD function. And expression of cotton non-symbiotic Hb in Arabidopsis seedlings conferred resistance to NO [224]. Furthermore, novel or special interest Hbs including the mammalian Cygb [134–136], and the algal raphidophyte _Heterosigma akashiwo_ nitrate reductase fused with a trHb (NRZ-2/2HbN) have been characterized as protective NO-metabolizing and NO-detoxifying enzymes [139]. Interestingly, the _Mycobacterium tuberculosis_ flavoHb (Rv0385) reportedly showed little NOD activity _in vitro_ [119] and provided negligible nitrosative stress protection to Hmp-deficient _E. coli_ [118]. Similar functional uncertainty exists for the NO-inducible truncated Ctb from _C. jejuni_ [225–227]. With the advent of rapid genome sequencing, _annotation of various flavoHbs and Hbs as NODs_ (EC 1.14.12.17) _in genome databases has become prodigious_.

---
| Target Reaction | Sensitivity (Est.) | Consequence | Reference |
|-----------------|-------------------|-------------|-----------|
| Aconitase (mitochondrial) | >50 nM | Citric acid cycle inhibition | [141–147] |
| IRE-BP (cytosolic aconitase) | Nanomolar | Iron homeostasis | [143, 146, 148–150] |
| 6-phosphogluconate dehydratase | >50 nM | Entner-Doudoroff pathway inhibition | [151] |
| Dihydroxy acid dehydratase | Nanomolar | Branched chain amino acid deficiency | [152–154] |
| Iron-sulfur enzymes (e.g., dehydratases above) | Nanomolar | Formation of toxic iron-dinitrosyl complexes | [146, 155–158] |
| Cytochrome oxidase (and other terminal oxidases) | Nanomolar | Respiratory inhibition | [141, 142, 159–166] |
| Catalase | Nanomolar | H2O2 damage | [167–169] |
| Prolyl hydroxylase family | Nanomolar | Hif-1α stabilization and hypoxic response, collagen cross-linking | [170, 171] |
| Cytochrome P450 family | Nanomolar | Metabolism of hormones, lipid second messengers, and so forth, Heme release and damage. | [172–174] |
| Ribonucleotide reductase (diiron) | Nanomolar-micromolar | Inhibition of DNA synthesis | [175–178] |
| Heme oxygenase family | Nanomolar | Inhibition of toxic heme breakdown | [179, 180] |
| Photosystem II | ? | Inhibition of photosynthesis | [181] |
| Nitrogenase | Nanomolar-micromolar | Inhibition of N2 fixation | [182–187] |
| Hydrogenase | Nanomolar | Inhibition of N2 fixation | [188] |
| O2 | Micromolar | NO2 damage | [189] |
| O2− | Nanomolar | Peroxynitrite damage | [189, 190] |
| Guanylate cyclase | 0.1–10 nanomolar | cGMP kinase activation and smooth muscle relaxation | [191, 192] |
| Transcription regulators (NorR, NsrR, DevS, etc.) | Nanomolar | NO defense gene expression | [80, 193–196] |
| ACO (1-aminoacyl cyclopropane-1-carboxylic acid oxidase) | Nanomolar | Ethylene production and signaling in plants | [197] |

What is needed are more reliable criteria for annotating a NOD function.

7. NOD Functions for FlavoHbs and Hbs in Microbial Pathogenesis

NO is produced as a natural antibiotic and antitumor agent in the innate immune response of animals and plants. Microbes and tumor cells have the capacity to resist NO toxicity [228]. Microarray analyses of mRNA [152, 183, 213, 214, 220, 229–238] and in silico reconstruction of transcription networks [152, 233, 239–241] in microbes and mammalian cells have revealed myriad adaptive changes potentially protecting against NO toxicity during microbial pathogenesis and inflammation. NODs, NORs [17, 151, 242], Fe-S cluster [243], and DNA repair enzymes [243], heme biosynthetic pathways [113], NO-resistant metabolic pathways (e.g., glucose metabolism and respiratory oxidases) [159–161, 240, 241], nitrite metabolism [213], and components of iron uptake systems form important and common elements of the nitrosative stress defense. Remarkably, in plants [141], Vibrio Fischeri [162], and the fungal pathogen Candida albicans [238], NO poisoning of respiration is apparently averted by the induction of NO-resistant alternative oxidase. Mycobacteria survive the hazardous environment of macrophages with GlbN (trHbN), GlbO (trHbO), and heat shock protein GroEL2 induction with GlbO providing greater protection than GlbN within macrophages [244]. Some aerobic organisms do not utilize a flavoHb-NOD, but apparently utilize sole NORs for NO metabolism [232], but these are the exception rather than the rule.

The accumulated evidence supports the hypothesis that NO metabolism and detoxification by NODs and/or NORs form the first, and most critical, line of defense against NO toxicity in microbes. However, results reviewed in the pre-2005 literature revealed only a modest advantage of the inducible flavoHbs and Hbs for microbes in the chosen infection models [65]. Tail vein injections of NOD-deficient Candida albicans showed limited effects on mouse survival [95, 238, 245], and those effects appeared NOS-independent [238]. Inhalation of Cryptococcus neoformans in mice showed similar modest extensions of mouse survival times with a flavoHb deficiency [246]. The effects of HmpX (flavoHb) deletions in Erwinia chrysanthemi infections of Saintpaulia ionantha (African violet) plants were more impressive, but interpretations were subject to potential effects of deletions of neighboring pectate lyase virulence genes [115, 247]. Nevertheless, Boccara et al. convincingly argued an important
role for HmpX in the pathogenesis of *E. chrysanthemi*. Not only does HmpX (NOD) protect against NO toxicity, it also impacts the NO-regulated hypersensitive response required for plant immunity [115, 224, 248]. Clearly, more and better models of microbial infection are required to discover the full involvement of flavoHbs and Hbs in microbial pathogenesis, *albeit the prospects appear limitless*. For example, mucosal infections by *C. albicans* are far more common and applicable than blood stream infections. Most experimental designs also ignore the effects of the metabolic state of microbes upon subsequent infectivity [249]. For example, naïve and also ignore the effects of the metabolic state of microbes in protecting alfalfa bacteroid N2 fixation [125, 259]. The rapid reactions of NO with oxy and deoxy Hb and Mb also led to the early view that these reactions would impair their O2 transport-storage functions [31, 270]. However, the role of Hbs in modulating spatial and temporal NO signaling is subtle and slowly becoming apparent. In tissues, Hb, Mb, Ngb and Cygb may act as catalytic NO sinks that together with NOS dynamically and spatially determine steady-state NO levels, soluble guanylate cyclase (sGC) activation, and myriad signaling actions [198, 199, 255, 262, 271, 272] including regulating cerebral blood flow, synaptic efficiency and neurotransmitter release [273].

**9. Beyond NO Detoxification: Signaling**

The ability of Hb to consume NO in the mammalian vasculature [262, 263] and Mb to decompose NO in muscle [137, 255, 264] has been an important issue since the conception of the role of NO in controlling blood flow. Indeed, the abundance of red blood cell Hb and myocyte Mb prompted the consideration of mechanisms that would be able to preserve NO in the vasculature or muscle tissues [265] in the face of certain destruction. These mechanisms include NO diffusion barriers [266–268], Hb/Mb-mediated NO sequestration and release [140] and, more recently, Mb or Hb-mediated NO formation from NO-derived nitrite [269]. The ability of Hb to consume NO in the mammalian vasculature [262, 263] and Mb to decompose NO in muscle [137, 255, 264] has been an important issue since the conception of the role of NO in controlling blood flow. Indeed, the abundance of red blood cell Hb and myocyte Mb prompted the consideration of mechanisms that would be able to preserve NO in the vasculature or muscle tissues [265] in the face of certain destruction. These mechanisms include NO diffusion barriers [266–268], Hb/Mb-mediated NO sequestration and release [140] and, more recently, Mb or Hb-mediated NO formation from NO-derived nitrite [269].

Griffiths and Garthwaite [271] formulated a “clamp” model for a better understanding of the consequences of NO consumption on NO steady-state levels and NO signaling functions in mammalian tissues. In this model, the sink (i.e., NOD) translates different rates of NO formation with a tissue volume into proportional steady-state NO concentrations and clamp [NO]. The NOD thus serves to “amplitude-code” NO signals. The NO inactivation rate also governs the rates of rise and fall of NO concentrations as NO sources switch on and off. In target cells, activation of sGC then causes cGMP to accumulate rapidly to levels that are graded with the prevailing NO concentration. A high NO inactivation rate endows the NO signal with temporal meaning. In addition, NO sinks add a spatial dimension for NO signals and provide additional mechanisms for regulating NO signaling such as via cellular O2 concentrations [142, 274, 275]. Furthermore, physiological inactivation mechanisms for signaling molecules generally have properties that are tuned to those of the receptors. In other words, a NOD needs to modulate NO steady-state levels at the 0.1–10 nM concentration levels that modulate sGC activity in vitro [191, 192]. Using quantitative real-time recording and modeling of neuronal NO signals via...
sGC activation in phosphodiesterase-deficient cells, Wood et al. [276] recently extended the estimates of steady-state [NO] in neuronal tissues to 0.25–3 nM with rates of NO generation estimated at 0.036 to 0.360 μM NO s⁻¹.

Chen and Popel [277, 278] have mathematically modeled steady-state NO production rates in vascular and perivascular tissues and estimated similar values ranging from 0.017 to 1.5 μM NO s⁻¹ that are dependent upon concentrations of the NOS isoforms and tissue O₂ concentrations. Endothelial NOS (NOS3) expressed at lower concentrations (0.045 μM) produces lower NO fluxes whereas neuronal NOS (NOS1) is expressed at higher levels (0.3–0.9 μM) and produces larger NO fluxes. In these studies, estimates of NO steady-state concentrations in the perivascular tissue ranged from 0.3 to 51 nM and were O₂ dependent. These values for [NO] are more than an order of magnitude lower than previous estimates. Importantly, the resulting steady-state NO concentrations are well within the range required for sGC activation (0.1–10 nM) [191, 192, 272]. The authors of these investigations made a number of simplifying assumptions including a rate of tissue NO consumption that was linearly proportional to NO and O₂ concentrations as expressed by the bimolecular rate equation, rate = kᵣ [NO][O₂][cells], where kᵣ = 5.4 × 10⁻¹⁰ μM⁻¹ s⁻¹ [cells/mL]⁻¹ and [cells] = 10⁸ cells mL⁻¹ [275]. However, the liver parenchymal cell activity used for the estimation of "kᵣ" may be low since mammalian cells express NO consumption activity levels ranging from 2 to 20 nmol NO min⁻¹ 10⁷ cells⁻¹. Using a cell concentration estimate of 10⁸ cells mL⁻¹, NO metabolic rates of 0.17 to 1.7 μM NO s⁻¹ corresponding to kᵣ values of 5.1 × 10⁻¹⁰ to 5.1 × 10⁻⁹ μM⁻¹ s⁻¹ [cells/mL]⁻¹ are calculated. A larger NO consumption activity and kᵣ value lowers the steady-state NO concentration estimates for the vascular and perivascular tissues. Moreover, contrary to the model, the activity shows normal Michaelis-Menten enzyme kinetics with Kₘ(NO) and Kₘ(O₂) values of 0.2 μM and 17 μM, respectively [142], thus further complicating estimates of steady-state NO levels.

Modeling of NO levels in tissues can provide quantitative and qualitative insights into the roles of potential NO consumption pathways. For example, for superoxide radical to act as a bimolecular pathway for NO removal at rates of 0.17 to 1.7 μM NO s⁻¹, steady-state O₂⁻ levels of 140 pM to 1.4 μM would be required. These concentrations are well beyond the ~8 pM O₂⁻ estimated within O₂⁻-generating mitochondria [279] and far greater than the level expected in the cytosol. On the other hand, for a Hb-NOD, like Cygb-NOD with a maximal turnover rate of ~1.2 s⁻¹ [135], to act as the sole catalyst, a tissue Cygb concentration of 0.14 to 1.4 μM would be required. These concentrations are within range of the globin concentrations typically seen in nonerythroid cells [4, 280–282] and plants [74, 283]. Moreover, an O₂-dependent NO consumption activity provides a feedback mechanism for controlling O₂ delivery to hypoxic tissues via decreased NO consumption, [NO] elevation, sGC activation, smooth muscle relaxation, and increased capillary blood flow [142, 255, 262]. Halligan et al. [134] and Liu et al. [136] recently provided evidence and arguments supporting a role for Cygb-NOD in controlling NO levels and vasorelaxation. Similar roles for Mb expressed in smooth muscle [4, 137] and oxidase-generated O₂⁻ [284] have been previously suggested. Various Hb-NODs are excellent candidates for the long sought dynamic sensor-regulator controlling tissue O₂ delivery and pO₂. The plots in Figure 1 illustrate the potential for Cygb, or other globins, to act as O₂-dependent modulators of NO steady-state levels within the physiologically relevant parameters. Caution is warranted since this simple model assumes that the globin is the major catalyst. Cells may express multiple NO dioxygenation catalysts [135]. Cytosolic globins have the potential to influence NO signaling and cell phenotypes through NO scavenging; however, enzymatic NO scavenging by cytosolic mammalian globins has not been demonstrated in most cells to date (e.g., see [285]). Cygb is the exception [134, 135]. In mammals, α and/or β-chains of the red blood cell HbA₁ have been

**Figure 1:** Modeling steady-state [NO] as a function of O₂ concentration with Cygb-NOD catalysis at a constant NO flux. The steady-state [NO] decreases with a higher Cygb-NOD turnover at higher [O₂] (solid line). At low [O₂], an elevated steady-state [NO] decreases the O₂: NO ratio (dashed line), and at O₂: NO ratios <500, NO inhibits Cygb-NOD [135]. A higher [Cygb], or lower NO synthesis rate, would be required for Cygb-NOD to maintain an O₂: NO ratio of >500:1 at a lower [O₂]. Calculations were for 1 μM Cygb, kᵣ[NO] = 3 × 10⁷ M⁻¹ s⁻¹ M⁻¹, and a NO synthesis rate of 1.7 μM NO s⁻¹. Calculations were simplified by applying the experimentally measured Kₘ(NO) = 20 μM to derive a kᵣ apparent = kᵣ[NO]/Kₘ(NO) + [O₂] for the various O₂ concentrations. The graded NO inhibition of Cygb-NOD is reflected in the apparent Kₘ(NO), and the effect of O₂ on NO synthesis rates are ignored.
detected in many different cells including hepatocytes [286], neurons and glial cells [287–291], macrophages [285], alveolar type II epithelial lung cells [282, 292], and mesangial kidney cells [293] suggesting additional Hb functions. Hb is enriched in pyramidal hippocampus and parietal grey matter neurons of Alzheimer's patients [294]. Other studies have demonstrated the expression of HbAα- and β-chain mRNA in non-erythroid cells. Visceral metastases of breast carcinoma express elevated Hb β-chain [295]. Cygb and Ngb are expressed in a variety of animal cells and tissues including neurons [296]. In cancer, tumor growth is suppressed by expressing E. coli flavoHb-NOD in brain gliomas [297], overexpressing Cygb in head and neck squamous cell carcinomas [298, 299] or, paradoxically, by decreasing Mb in breast cancer cells [7]. Globins, acting as NODs, may either decrease the NO signal eliciting a metastatic cell phenotype or increase NO detoxification and the resistance of tumor cell targets to endogenously generated NO (see Table 1). Removal of NO would decrease NO-elicited hypoxic tissue vascularization, mitochondrial biogenesis [300, 301], and the Hif-1α‑orchestrated gene array expression important for bolstering hypoxic metabolism and the metastatic phenotype. The prolyl hydroxylase controlling Hif-1α stability and function is sensitive to inhibition by NO [170]. NO induces and stabilizes Hif-1α and bolsters the hypoxic adaptation of cells [302]. Moreover, Hif-1α upregulates Mb and Ngb expression [7, 9], thus providing a potential feedback loop for [NO] homeostasis. In this model, increased NO removal would decrease prolyl hydroxylase inhibition, destabilize Hif-1α, and attenuate globin expression and NO removal.

Globin-regulated NO signaling may also be important for the normal functioning of neurons. Expression of HbAα in dopaminergic neurons is linked with pathways involved in O2 homeostasis, oxidative stress, iron metabolism, NO synthesis, and oxidative phosphorylation [287]. Hif-1α expression was decreased in HbAα expressing neurons and targets of Hif-1α were altered [287]. Ngb deficiency in mouse brain exacerbates the Hif-1α‑regulated response to hypoxia [303] further suggesting effects of the neuronal Ngb on [NO] and NO on Hif-1α stability.

There is also evidence for (flavo)Hbs modulating NO signaling pathways in fungi and bacteria. FlavOHB expression affects Dictyostelium discoideum development [304], and Aspergillus nidulans sexual development and mycotoxin production [305]. NO scavenging by flavoHb attenuates the expression of the nitrosative stress response (e.g., norVW) [151], affects the swarming behavior of E. coli [306], and maintains squid-Vibrio fischeri [218] and Medicago truncatula-Sinorhizobium melloti [260] symbioses.

R. Hill recently published an up-to-date and thorough review discussing the many known and potential roles for Hbs in modulating NO signaling processes in plants [82]. For example, the Arabidopsis Hb, GLB1, modulates salicylate, ethylene, and jasmonic acid responses to Pseudomonas syringae and Botrytis cinerea infections [307]. Lowering GLB1 levels and increasing NO levels elevates the defense against pathogens via multiple hormones. GLB1 expression also decreases ethylene-induced upward leaf movements in response to root hypoxia (hyponasty) [126].

10. Regulation of Globin Expression and NOD Function

A variety of mechanisms exist for controlling the expression of flavoHbs and Hbs in different organisms. Numerous organisms are now known to regulate globin expression in response to NO and hypoxia [65, 60] via specific NO and O2-responsive transcription factors as part of NO and hypoxic stress responses [67, 80]. Other inducing signals emanate from the inhibition of the electron transport chain [54], iron deficiency, oxidative stress, osmotic stress, cold stress, or hormones. A complex interplay of NO, O2, temperature [237, 308], iron availability, nitrate, nitrite, and oxidative stress can control Hb-NOD expression in various organisms. Moreover, it is impossible to deduce a NO detoxification function of a protein/gene solely from inducing signals since many NO-inducible proteins/genes are accessory [183] and not all NO detoxifying enzyme (NOD) genes are responsive to NO [95, 128, 142, 309, 310]. Nevertheless, given the common capacity of Hbs to function as NODs, the induction of a Hb by NO should be considered strong evidence for a NO metabolic function. It is also important to remember that NO can be produced from media nitrate and/or nitrite and that high levels of apparently constitutive (flavo)Hb expression [95, 138, 310] may be due to endogenous NO formation.

An impressive literature has amassed describing NO-responsive regulation of globin expression in various life forms since the writing of my 2005 review [65]. At that time, a role for the NO-sensitive regulator NorR in the context of the transcriptional regulation of norVW in E. coli [151, 194, 311] and norBC inRalstoniaeutrapha[312]wasknown. The mechanism of NO-sensing by NorR was subsequently elucidated by D’Autraux et al. [313]. Roles for the ferric uptake regulator (Fur), methionine repressor (MetR), superoxide response regulator (SorRS), fumarate nitrate reductase regulator (Fnr) in E. coli, orSalmonellahmp(flavoHb)transcription had also been investigated and reported [69, 314–316], but those regulators did not satisfactorily explain NO induction [229, 317–319]. The advent of genomics and systematic and comparative operon analyses [239] greatly accelerated the identification NO-sensing regulators and the characterization of globin regulation in various bacteria. Stephen Spiro recently reviewed the literature on NO sensor regulators in the larger context of gas sensors in bacterial nitrogen metabolism [80]. Efforts to identify NO-sensing regulators in representative yeast and fungi have progressed more slowly. Upregulation of globin expression in plants, algae, and animals in response to NO and hypoxia have also been described and is consistent with a NOD function. Here, I briefly review the NO and hypoxia sensing regulators of globin expression in the context of the putative NOD function.

10.1. Bacteria. NO-regulated NORs and NODs cooperate in the NO defense in bacteria. However, within any single organism, NOD and NOR appear, for the most part, to be under the control of different NO sensor regulators. On the other hand, homologous regulators control either NOR or NOD in different organisms. For example, the
**10.2. Yeast/Fungi.** Progress has also been made in understanding NO regulation of flavohb in fungi and yeast. In *Saccharomyces cerevisiae*, the Fz1p regulator is required for NO induction of *YHB* transcription [236]. In *Candida albicans*, CTA4 a zinc-finger protein is required for induction of *YHB1* transcription [245], and Cw1p acts as a repressor of a nitrosative stress regulon that includes *YHB1* [241].

NO upregulates flavohb (NOD) expression by *Aspergillus oryzae* [216], the plant pathogen *Botrytis cinerea* [222] and other fungi [65] presumably via similar mechanisms. The full details of NO sensing and transcription activation remain to be elucidated. One of the more interesting discoveries has been that the *Fusarium graminearum* virus-DK21 downregulates flavohb mRNA and protein expression by the pathogenic plant fungus *Fusarium graminearum* along with virulence [327]. A role for the flavohb in virulence is suggested.

**10.3. Photosynthetic Organisms.** Numerous examples of NO and hypoxia upregulating Hbs in plants have been described and reviewed [74, 81, 82, 328]. The fused nitrate reductase-trHb (NR2-2/2HbN) is induced by NO in the microalgal species *Heterosigma akashiwo* [139]. *Arabidopsis* class I nsHb is induced during growth with nitrate [329]. Two nonsymbiotic trHb genes are strongly induced by nitrate, nitrite, and NO in cultured rice cells [330]. Cotton and wheat nsHbs are induced by NO [224] or NO donors [331]. Only one of the five Hb genes, LjHb1 encoding a nsHb, is induced by NO and hypoxia in *Lotus japonicus* [308, 332]. Another nsHb (LjHb2) is induced by sucrose, absicic acid, and osmotic stress. Clearly, not every Hb with a capacity for a NOD function is induced by NO or hypoxia. For example, the cyanobacterium *Synechococcus sp.* GbHm provides resistance to NO, but is apparently not inducible by NO, nitrate, or nitrite [138].

**10.4. Protists.** The protective *Giardia lamblia* flavohb-NOD is induced by nitrite, nitrosoglutathione, and NO donors [116].

**10.5. Animals/Humans.** Numerous animal Hbs are reportedly induced by hypoxia, but apparently none by NO. The Hb β-chain is induced in macrophages by interferon and lipopolysaccharide [285] which also elicit NO synthesis. However, the induced Hb β-chain failed to increase NO consumption by macrophages [285]. Hbα chains are expressed in rat ischaemic rat neurons [333] and are upregulated in response to hemorrhage [291], hemin, erythropoietin [289], and oxygen-glucose deprivation [333]. The chains are not always coordinately regulated; oxygen-glucose deprivation increases rat neuronal α-chain mRNA 1.9-fold, but decreases Hb β-chain mRNA 3-fold [333]. Hbα is also reportedly present in hippocampal and parietal grey matter neurons of Alzheimer's patients [294], a hypoxic and inflammatory condition that may induce globin expression. Hb α- and β-chains are also expressed in alveolar type II lung epithelial cells in response to hypoxia [282, 292] and Hif-2α or Hif-1α control transcription [334]. Ngb is induced by hypoxia [335] and is under control of the master transcriptional regulator of the hypoxic response, Hif-1α [336–338]. Hypoxia also induces Mb in nonmuscle cells including liver, gill, and brain [5]. Numerous other studies have shown elevated expression of mRNAs for Hb α- and β-chains, Mb, Cygb, and Ngb in non-erythroid cells and especially hypoxic tumors [7, 9]. What remains unclear is whether these animal globins have a significant capacity for NO dioxygenation and modulation of NO functions and how this relates to pathophysiology. For example, Ngb-deficiency in mice leads to changes in Hif-1α-regulated pathways in response to hypoxia [303]. Is this due to deficient NO metabolism and NO stabilization of Hif-1α via prolyl hydroxylase inhibition? Is NO, like O2, a signal that normally controls Hif-1α?
While knowledge of the many factors and mechanisms controlling transcription or translation of globins in various life forms is revealing and often supportive of a NOD function, a quantitative knowledge of the NO and O₂ concentration dependencies of the responses is critical for understanding Hb function in NO homeostasis control, detoxification, and/or signal modulation. The overriding question is: What level of NO is normal and how does cell maintain and respond to changes in the concentration of NO via globin synthesis regulation?

11. Hb Structure(s) and the NOD Mechanism

The molecular, atomic, and electronic details of the NO dioxygenation reaction form the heart of our understanding of Hb structure-function and evolution. Proposals of a common, intrinsic, and ancient NOD function require a thorough understanding of the reaction and the chemical properties of Hbs or flavoHbs that make them either good or poor catalysts. There are several recognized basic requirements for a NOD function that Hbs must possess. These include

1. a high O₂ affinity,
2. a mechanism for decreasing NO binding to heme,
3. a superoxide radical-like character of the bound O₂,
4. a protected pocket for the peroxynitrite intermediate,
5. an O-atom isomerization mechanism,
6. a mechanism for nitrate egress,
7. a mechanism for univalent reduction.

Here, I will focus on new knowledge, key concepts, and current questions about Hb chemistry that are directly related to the NO dioxygenation reaction, NOD catalysis, and a NOD function. New investigators of Hb-NODs should consult the seminal literature [34, 49, 52, 339–341] and prior reviews describing (flavo)Hb structure-function-evolution [1, 65–67, 71, 72, 102, 342] for background and additional information. Mowat et al. provide an excellent recent overview of the flavoHb-NOD structure-function [72].

11.1. High O₂ Affinity.

\[
\text{HbFe}^{2+} + \text{O}_2 \rightleftharpoons \text{HbFe}^{2+}(\text{O}_2) \quad (3)
\]

By definition, all Hbs bind O₂ reversibly (3). However, Hb affinities for O₂ vary greatly with equilibrium dissociation constants (K_D) values ranging from low nM to greater than 20 μM O₂. On- and off-rates for O₂ determine O₂ affinities, and these are influenced by structure as described in an excellent recent review by Marti et al. [343]. A high O₂ affinity is critical for limiting NO inhibition during catalytic NO dioxygenation. Mutations increasing the O₂ off rate of flavoHb increase NO inhibition [341]. O₂ must be able to outcompete NO for binding the ferrous heme for a Hb to function as a NOD. Hbs with lower O₂ affinities (e.g., Cygb) function as effective NODs but only at a low [NO] relative to [O₂] [135].

11.2. Mechanisms for Decreasing NO Binding to Heme. Competition between NO and O₂ for binding the ferrous heme, as predicted from transient kinetic measurements, should prohibit a NOD function for most (flavo)Hbs [65, 344]. For many Hbs, the K_D(NO) value determined by laser photolysis and stopped-flow is ~10 pM. Yet, steady-state assays of NOD activity reveal much weaker inhibition by NO [52, 135, 341, 344] thus suggesting the existence of mechanisms for decreasing NO binding during catalysis (4)

\[
\text{HbFe}^{2+} + \text{NO} \rightleftharpoons \text{HbFe}^{2+}(\text{NO}) \quad (4)
\]

The most attractive explanation for this phenomenon is the allosteric modulation of an NO tunnel or gate by O₂ binding. In the M. tuberculosis trHbN, O₂ and NO access the ferrous heme through two different gated tunnels [345]. O₂ binding opens a long tunnel for NO and increases NO access [346–348]. Tunnel switching occurs on the picosecond to nanosecond time scale and is compatible with a role in NOD catalysis [349]. A novel role for PheE15 in gating O₂ and NO migration through channels has been suggested in which the distal H-bonding TyrB10 and GlnE11 act as the triggers [348, 350, 351]. Similar tunnels or gates may control NO access in other Hbs. Bis-histidyl ligation and structural plasticity may serve a similar role in controlling ligand access in the NOD function of plant nHb [84, 352].

Other explanations for diminished NO inhibition include NO reduction by the flavoHbs [341], yet, rates of NO reduction by some flavoHbs appear too low [52]. Interestingly, Liu et al. were able to mathematically model the Cygb-NOD activity with the available transient kinetic rate constants for NO and O₂ binding and NO dioxygenation [136] suggesting the absence of mechanisms for decreasing NO binding in some Hbs.

11.3. Superoxide Radical-Like Character of the Bound O₂. The ferrous heme in Hb transfers an electron to O₂ to form a stable Fe⁵⁺(O₂⁻) complex [353, 354]

\[
\text{HbFe}^{5+}(\text{O}_2) \rightleftharpoons \text{HbFe}^{5+}(\text{O}_2^-) \quad (5)
\]

The ferric iron forms unique bonding interactions with the bound O₂⁻ important for NOD function. The heme-Fe³⁺ (3d⁶) unpaired electron interacts with the unpaired π* O₂⁻ electron through strong antiferromagnetic coupling in a unique end-on orientation [103]. In the NO dioxygenation reaction, the unpaired NO π* electron associated with the N-atom couples with the unpaired π* O₂⁻ electron. One electron is formally transferred from NO to the half-filled π* (O₂⁻) orbital (reduction of superoxo), but not to the metal; thus the strong π O₂ bond is broken and the Fe-O bond is strengthened. The reaction is analogous to the diffusion-limited reaction of NO and O₂⁻ in solution [355] except that the strong anti-ferromagnetic coupling of an end-on O₂⁻ has the capacity to preferentially localize the unpaired π* electron density to the O-atom proximal to the iron and to direct the NO reaction (vide infra).

The anti-ferromagnetic coupling is eliminated with the return of the electron from the bound O₂⁻ to Fe³⁺ permitting...
the ferric-nitrato species. Oxy-globin model compounds, at high oxy-Hb concentrations and is slow for abundant Hbs and Mbs functioning in O2 transport-storage [356–358]. In contrast, the less abundant neuronal Ngb shows high autooxidation rates in vitro [359]. Curiously, this has led some investigators to discount a NOD function [360]. However, high autooxidation rates can be an artefact of in vitro conditions and should not be the sole basis for conclusions about function. For example, autooxidation of the Candida norvigenensis flavoHb-NOD oxy-complex is very slow, but increases when the reductase domain cofactor FAD is absent or when the reductase domain is separated from the Hb domain [61, 102].

11.4. Protected Pocket for the Peroxynitrite Intermediate. A transient ferric-peroxynitrite intermediate likely forms in the reaction of NO with globin Fe3+(O2−) [65, 66] (6)

\[ \text{HbFe}^{3+}(\text{O}_2^{-}) + \text{NO} \rightarrow \text{HbFe}^{3+}(\text{ONO2}^{-}) \] (6)

The half-life of the fleeting peroxynitrite intermediate is expected to be in the microsecond range due to facile iron-catalyzed isomerization to ferric-nitrate, whereas the ferric-nitrate species has a measured half-life of milliseconds [361, 362]. The putative HbFe3+(ONO2−) intermediate detected in reactions of oxy-Mb, -Ngb and -GhO in the millisecond time range [122, 123, 363–365] must now be assigned to the ferric-nitrate species. Oxy-globin model compounds, Fe(Por)(NH3)(O2), also react with NO at 80–100 K forming only the low-spin ferric-nitrate complexes, thus implying that peroxynitrite intermediates, if formed, also undergo very facile isomerization [366]. In contrast, NO reacts with oxy-coboglobin model compounds, Co(Por)(NH3)(O2), forming a detectable peroxynitrite intermediate and nitrate species at low temperature [367]. More recently, Navati and Friedman [365] reported preliminary evidence for Fe3+(ONO2−) formation during the reaction of NO with oxy-Mb and oxy-Hb in a special “dry” glassy matrix.

The ferric-peroxynitrite would form in the distal pocket of Hb-NODs which is well isolated from nucleophiles and solvent. Hb pockets are typically lined with a large number of hydrophobic residues with histidine, glutamate, and/or tyrosine residues forming hydrogen bonds to the O2− ligand. A protected pocket may explain how Hb functions as a high-fidelity catalyst of NO dioxygenation whereas the indoleamine dioxygenase-like oxy Turbo Mb cannot [66].

11.5. O-Atom Isomerization or Rearrangement Mechanism.

\[ \text{HbFe}^{3+}(\text{ONO2}^{-}) \rightarrow \text{HbFe}^{3+}(\text{NO3}^{-}) \] (7)

Two mechanisms for formation of nitrate from the reaction of NO and HbFe3+(O2−) (7) have been previously discussed [66]. Both provide a pathway for O−O bond breaking and O-atom isomerization or rearrangement in the peroxynitrite intermediate. The first that we envisioned was an iron-catalyzed mechanism in which ferric iron, acting as a Lewis acid, facilitates O-atom rearrangement [34]. We wrote “The heme-Fe3+ may facilitate an oxygen bond rearrangement by participating in a iron-mediated oxygen bond shift analogous to the proton-mediated shift suggested for the nonenzymic mechanism for HOONO decomposition to NO2−.” The mechanism was never fully rationalized, developed, or argued in the literature, but it was assumed that the mechanism would bear similarity to the mechanism of peroxynitrite isomerization accelerated by acidic pH (pH < 2) [368, 369]. The second, a ferryl mechanism involving peroxynitrite, O−O bond homolysis, and ferryl O-atom transfer was strongly supported by theory, overwhelmingly favored, and thoroughly argued and investigated [65, 66, 342, 364]. However, both the doubt cast by recent [121, 362] and earlier [368] investigations and the paucity of strong experimental support for the ferryl mechanism have demanded new insights and the scrutiny of alternative O-atom rearrangement mechanisms.

In 1954, Anbar and Taube [370] suggested a concerted internal O-atom rearrangement mechanism for the isomerization of peroxynitrous acid to nitrate to explain O-atom retention, and a similar mechanism, as already mentioned, was suggested as a possibility for the Hb reaction, albeit remaining poorly defined (see above) [65, 66]. Tsai et al. [371] had argued against an internal rearrangement mechanism for peroxynitrite/peroxynitrous acid in 1996 “because contracting of the O−O−N bond angle produces a strong repulsion between the terminal peroxide oxygen and the two oxygens bound to nitrogen.” Discussions with Henry Taube and recent descriptions of the unique Fe3+O2− bonding in Hbs [103] provided new insights for a novel concerted internal O-atom rearrangement mechanism (see below). The new mechanism casts doubt on the relevance and validity of mechanistic inferences drawn from experiments demonstrating peroxynitrite isomerization catalyzed by ferric Mb [372–375] or metalloporphyrins [26, 66, 376]. Models of heme-peroxynitrite adducts envision the negatively charged terminal O-atom bonded to iron [376, 377].

The two possible O-atom rearrangement mechanisms are illustrated in Figure 2. In the new reaction scheme (1), NO attacks and bonds the O-atom proximal to the iron atom breaking the strong π O2 bond and strengthening the Fe−O bond, the terminal O-atom attacks the nitrogen, and the O−O peroxide bond heterolytically breaks to form Fe3+[NO3−]. As envisioned, another electron pair donor (e.g., NO, HCN or F−) should be able to intercept the terminal O-atom to form NO2− and an oxygenated product (e.g., NO2−, CNO− or IO−). The role of the ferric iron is purely that of a Lewis acid, as we first imagined [34], and the Hb pocket shields reactive intermediates. Ferric iron forms an ionic or coordinate bond with ONOO−, withdraws electrons from the terminal peroxide O-atom, and increases terminal O-atom reactivity with N. In contrast, in the ferryl mechanism (2), NO attacks the O-atom distal to the iron atom to form Fe3+NO2−(ONOO−), the O−O bond weakens and homolytically breaks to form the “caged” Fe3+ = O(O−) and NO2 pair which rapidly combine to form Fe3+[NO3−].
The support for a ferryl mechanism was previously reviewed [65, 66, 364] and recently critiqued [121, 362, 375, 378]. From atomistic simulations of the truncated group I HbN, Mishra and Meuwly [121] concluded that the O–O bond scission energy barrier was too high and homolysis too slow thus suggesting an internal rearrangement mechanism. Su and Groves [378] have concluded that NO2 is an unavoidable product of the ferryl mechanism, yet, there is no evidence for NO2 formation or ferryl O-atom scrambling with water during (flavo)Hb-catalyzed NO dioxygenation [66]. One would expect NO2-mediated nitration damage to the B10 tyrosine residue in the distal pocket of flavoHb-NOD and a loss of NOD activity similar to the nitration of more distant tyrosines observed in Mb and Hb [66, 373, 374, 379–383]. The release of reactive toxic intermediates during NO oxidation by oxy-Hb is the antithesis of a NOD function, and the evidence for that is meager [33]. What then are the arguments and evidence suggesting a concerted Lewis acid mechanism (Figure 2, (1))? First, the reaction mechanism demands the application of density functional theory and further scrutiny. Past theoretical investigations of peroxynitrous acid isomerization [384] have not considered the effects of hydrogen ion interactions with peroxynitrous acid O-atoms. Nor has O-atom retention during peroxynitrous acid isomerization [370, 385] been investigated as a function of pH. Clearly, hydrogen ions increase the rate of the reaction [368, 369] well beyond the peroxynitrous acid pKₐ of 6.5–7 [386], but this phenomenon has never been clarified. A proton, or Lewis acid, interacting with the peroxide O-atom proximal to N would decrease the strong repulsion between the terminal peroxide oxygen and the two oxygens bound to nitrogen. In an analogous reaction, hydrogen ions catalyze O-atom transfer from H₂O₂ to I⁻ and other two-electron donors [387–390]. The only apparent catalytic mechanism is through O-atom protonation, which withdraws electrons from the proximal O-atom, increases O or OH⁻ reactivity with I⁻ and which causes peroxide bond homolysis forming water and IOO⁻ or IOH [389]. A Lewis acid (e.g., Fe³⁺) would be expected to be able to catalyze a similar O-atom transfer. There are also reasons why a mechanism involving NO attack of the O-atom proximal to iron in Hbs is attractive. The proximal O-atom is more accessible to NO. Moreover, it is the most probable location for the anti-ferromagnetically coupled unpaired π*Fe³⁺(O₂⁻) electron [103] that couples with the unpaired NO electron. Hughes and Nicklin had suggested a heterolytic mechanism for peroxynitrous acid isomerization in 1968 [368], but the potential role of hydronium or hydrogen ions in catalyzing the reaction was not considered.

11.6. Mechanisms for Nitrate Egress. Martí et al. simulated nitrate release from Mycobacterium tuberculosis trHbN [391]. The molecular dynamic simulations suggest that formation of the ferric-nitrito species causes a structural distortion of the pocket cavity walls forming pores for water entry. Water hydration weakens the bond between the heme iron atom and nitrate exits in ~5 ns via a unique pathway differing from O₂ and NO tunnels. A role for Thr22 in assisting nitrate egress was proposed. The role of plant nsHb pocket plasticity in nitrate removal has also been discussed [84]. The Mycobacterium leprae GbO ferric-nitrito intermediate showed a 10–100-fold longer half-life than other globins [122] suggesting a slow nitrate egress mechanism.

11.7. Mechanisms for Univalent Reduction. Each NO dioxygenation reaction consumes a single electron that must be supplied by an electron donor for catalytic turnover. The ferric heme is reduced by an electron (8a) for the O₂ binding reaction (3). Importantly, Hbs require mechanisms to prevent the transfer of a second electron to the higher potential ferric superoxo complex. A second electron would generate ferric-peroxide (8b), and a third electron would generate ferryl. Reduction of the putative ferric-peroxynitritre intermediate could release peroxynitrite or generate hydroxyl radical (8c). These reactive species would be expected to cause damage to the heme and/or protein.

\[
HbFe^{3+} + e^- \rightarrow HbFe^{2+} \tag{8a}
\]

\[
HbFe^{3+} (O_2^-) + e^- + H^+ \rightarrow HbFe^{2+} (-OOH) \tag{8b}
\]

\[
HbFe^{3+} (ONOO^-) + e^- + H^+ \rightarrow \text{HbFe}^{3+} (\text{ONO}^-) + \text{OH} \tag{8c}
\]

Native electron donors are known for only a handful of Hb-NODs, but distinct patterns are emerging. The ultimate electron donors for the flavoHb-NOD [65] and dual function raphidophyte trHb-nitrate reductase [139] are flavin-containing reductases linked by a multidomain structure.
The C-terminal flavoHb reductase domain [39, 49] and C-terminal trHb-nitrate reductase domain structures belong to the FNR superfamily which includes NAD[P]H:ferredoxin oxidoreductase and NADH:cytochrome b₅ oxidoreductase. The truncated cyanoglobin (GlbN) and ferredoxin reductase genes are also linked in a bi-cistronic operon controlled by the NO sensor-regulator NsrR in Legionella pneumophila [239]. Together, the data suggest a common role for ferredoxin reductases and other FNR-like proteins as electron donors for trHbs and SDGs either linked in multi-domain structures or as separate proteins. The role of the pre-A sequence for truncated HbN-NOD activity in Mycobacteria may be to facilitate interactions with a specific ferredoxin reductase [392]. Ferredoxin:NADP⁺ oxidoreductase from E. coli was systematically tested for its capacity to support NOD activity of various Hbs in vitro [133], however, in those reactions, “NOD activity” is impossible to discern from reactions of O₂−. High concentrations of SOD are required to prevent reactions of NO with the O₂− invariably released by reductases [344]. Red blood cell Hb is reduced by cytochrome b₅, and cytochrome b₅ can support the Cygb-NOD activity in vitro [135]. Ascorbate also supports the Cygb-NOD activity at concentrations found within Cygb expressing fibroblasts and neurons [135, 136], and a putative binding site for ascorbate has been identified [135]. An accessory role for NADPH:cytochrome P450 oxidoreductase in CygbFe³⁺ reduction has also been suggested [136]. The barley nsHb can be reduced by an ascorbate-dependent monodehydroascorbate reductase [132]. A mass spectrometry approach has been utilized to identify the Ngb and Mb interactome [393]. The method may also reveal novel electron donor candidates.

Hb structures suggest two mechanisms for controlling univalent electron transfer. Bis-histidyl ligation in hexacoordinate nsHbs, Cygb, and Ngb provides structural plasticity [84] and a mechanism for controlling electron donor binding and electron transfer. For example, movements of the E-helix and ArgE₁₀ with changes in O₂ binding may modulate ascorbide binding and electron transfer in Cygb [135]. In this case, bis-histidyl ligation to the ferric iron would be expected to induce an ascorbate binding-site, and bis-histidyl ligation to Fe³⁺(O₂−) to decrease ascorbate binding. A rotating water bridge may also provide a mechanism for controlling univalent electron transfer from reduced flavins. The flavoHb and SDG structures [49, 65] and electron pathway analyses [394] suggest an important role for water molecules bridging the FAD and heme in mediating electron transfer. In the E. coli flavoHb-NOD structure [339], the bridging water molecule hydrogen-bonded and anchored by LysF₇ ammonium group has the capacity to rotate to ON and OFF orientations (Figure 3). Strengthening of a short, strong hydrogen bond between water and the heme propionate, with O₂ binding and a pKₐ shift of the heme propionate, provides a mechanism for controlling the bridging water orientation and regulating univalent electron transfer in Hb-NODs. The oxygen core repels electrons in the OFF orientation and would block electron tunneling. In support of the hypothesis, mutation of LysF₇ dysregulates univalent electron transfer, oxidatively destroys the heme, and incapacitates the NOD function [395].

The many adaptations of the Hbs for the NOD function show that Mother Nature is indeed a brilliant chemist!

**12. The FlavoHb Denitrosylase (O₂ Nitrosylase) Concept**

Following the report of NO metabolism by the NO-inducible E. coli flavoHb and kinetic investigations supporting a NOD mechanism [34, 52, 341], Hausladen et al. argued for a major revision by suggesting a denitrosylase mechanism for NO conversion to nitrate [396, 397]. NO binding to the ferrous heme, and Fe²⁺ reducing NO to Fe³⁺(NO⁻) and N₂O was reported, and evidence for a reaction of O₂ with Fe³⁺(NO⁻) to form nitrate (9) under more relevant physiological O₂.
and NO concentrations was argued. The rationale for the
denitrosylase hypothesis was largely motivated by transient
kinetic measurements showing a 1500-fold greater affinity of
the ferrous heme for NO than O$_2$ [52, 341] coupled with the
observation of flavoHb-catalyzed NO metabolic activity at
an exceptionally high $\sim 1:6$ NO:O$_2$ ratio [398]. Hausladen
et al. concluded that the NO and O$_2$ affinities necessarily
indicated an unfeasible competition of O$_2$ for the flavoHb
heme under physiologically relevant conditions. Thus, the
high NO affinity supposedly negated any possibility of a
NOD mechanism at physiological O$_2$ and NO levels. The
argument epitomized the dogmatic belief that NO binding
to any ferrous hemoglobin would prevent O$_2$ binding and
thus a catalytic NO metabolic activity that was dependent
upon O$_2$ binding. NO competition, or the lack thereof,
remains relevant in investigations and assignments of a NOD
function-mechanism for various hemoglobins (see above)
[133, 135, 136, 344], but it does not prohibit the mechanism

$$\text{HbFe}^{3+} (\text{NO}^-) + \text{O}_2 \rightarrow \text{HbFe}^{3+} + \text{NO}_3^-$$  \(9\)

Despite a dearth of either experimental or theoretical
support for the flavoHb denitrosylase mechanism after more
than 10 years, a handful of investigators persistently assign
merit to the mechanism [67, 69, 70, 215, 299] or equate
the two mechanisms [87, 400] and in so doing obscure the
true function of (flavo)Hbs and impede general progress.
Moreover, proponents of the denitrosylase mechanism con-
tinue to reject the dioxygenase mechanism and nomen-
clature, but have received only a partial scientific rebuttal
[65, 344, 397, 401]. Hence, a refutation of the denitrosylase
mechanism is demanded.

The following is a list of reasons why a denitrosylase
mechanism is theoretically unfeasible and weakly supported
by experiment. (1) NO reduction ($E^0 = −0.8 \text{V}$) [189, 402]
to the nitroxyl (NO$^-$) intermediate has a large energy barrier.
$V_{\text{max}}$ values for NO reduction by flavoHb are $>2000$–fold lower
than the $V_{\text{max}}$ values for NO dioxygenation [52]; (2) the
reaction of Fe$^{3+}$ (NO$^-$) with O$_2$ is not kinetically favored [123,
403]; (3) O$_2$ would be more rate-limiting for a denitrosylation
at low physiological O$_2$ concentrations than it would for
the NOD mechanism; (4) the mechanism requires O$_2$
 to react with the N-atom liganded to the iron which does not
 provide an obvious reaction path for O-atom rearrangement;
(5) O$_2$ binding and NO scavenging are affected by the flavoHb
distal pocket structure (TyrB10), but NO binding is not
[341]; (6) CO competitively inhibits NO metabolism with
respect to O$_2$, not NO, throughout the physiological [O$_2$]
range [52, 344]; (7) the NO metabolic activity of flavoHb is
saturable by O$_2$ [52, 341, 344] with no evidence of an activity
increase at low O$_2$ as suggested and reported [396, 398];
(8) NO inhibits the NO metabolic activity of flavoHb at
NO:O$_2$ ratios $>1:100$ and not as potently as predicted from
NO affinity measurements [52, 341, 344]; (9) flavoHb-NOD
activity is induced by NO providing an effective escape from
the otherwise problematic NO inhibition at low [O$_2$] [151,
404]; (10) NO reductases are also expressed at low O$_2$
concentrations thus substituting for lost flavoHb function due to NO
inhibition [151, 404]; (11) the denitrosylase proposal assumes
that micromolar NO concentrations are required for NO
toxicity and thus the NO detoxification function of flavoHb.
Presumably, under these high NO levels, a dioxygenase
mechanism would be impossible. But, even a “tiny” 50 nM
NO is toxic to aerobic E. coli [35] and flavoHb protects
against this toxicity [35, 151, 404]. Thus, flavoHb-NOD
can detoxify NO without significant NO inhibition even at
$5 \text{µM}$ O$_2$. Furthermore, NOD turnover under normoxia ($\sim
400 \text{NO} \cdot \text{s}^{-1} \cdot \text{heme}^{-1}$) far exceeds the activity expected from
the denitrosylase mechanism. NO reduction by flavoHb, a
step in the denitrosylase mechanism, has never exceeded
0.2 NO s$^{-1}$ heme$^{-1}$ [65, 107]. For the two mechanisms to be
comparable in rate, the NOD mechanism would need to be
inhibited by 99.95%. At 50 nM NO, the available [O$_2$]
would need to be $<5 \text{nM}$ for 99.95% NOD inhibition for a
remotely plausible and equivalent NO scavenging function.
(12) Finally, the initially troubling observation of flavoHb
metabolizing NO and NADH at an NO:O$_2$ ratio of $>1:6$
[398] can be explained by the fact that unusually high levels
of flavoHb (0.5–1 µM) were present in the reactions such that
the added NO (35 µM) was so rapidly consumed as not
ever to achieve $>5 \text{µM}$ NO! (see Figure 1(a), [398]).
Moreover, the substantial NADH oxidase activity of flavoHb
can account for NADH oxidation observed at these high
concentrations of flavoHb (see Figure 1(d), [398]).
These unfavorable conditions for NOD catalysis may also explain
the relatively high yield of nitrite formation and would
even compromise the deduced O$_2$:NO:NADH reaction
stoichiometry [398]. The concentrations of flavoHb protein
used in steady-state kinetic analyses are typically 5,000-fold
lower and involve high turnover numbers [52, 341, 344].

Unresolved issues nevertheless remain. For example, why
is the affinity of flavoHb for NO $\sim15$-fold greater when deter-
mined by the flash photolysis-rebinding method than when
estimated from steady-state kinetic analysis? NO removal by
the flavoHb NO reductase activity during catalysis is one
possibility [341], but the activity may be insufficient to
account for NO resistance. Similar questions arise for all
other globins including the mammalian Cygb. The Cygb
NO:O$_2$ affinity ratio of $\sim125,000$:1 immediately suggests a
much greater inhibition by NO than that observed at NO:O$_2$
ratios of $>1:500$ [135]. Liu et al. [136] have recently used
modeling simulations to argue that the steady-state kinetics
measured for the Cygb-NOD activity are allowed by the high
NO affinity ($K_D = 8 \text{pM}$), but the model required assump-
tions including NO affinity decreases with temperature. Less
consideration has been given to structural dynamics and
changes in NO access [346, 348] as a common mechanism
for limiting NO binding and inhibition of catalysis (see
above).

13. Why So Many Different Hb-Type NODs?

While there is certain danger of a fallacy of composition
in suggesting a NOD function for all Hbs, the literature
now shows that many different Hb structures in many
different forms of life function as NODs. This realization
raises important questions. If various Hbs function as NODs,
why the structural diversity and the multiplicity of Hbs within organisms?

Cursory answers to these questions can be readily gleaned from the anti-oxidant and radical scavenging enzyme systems. For example, in order to scavenge H$_2$O$_2$, organisms express catalase, glutathione peroxidases (8 isoforms in humans), cytochrome c peroxidase, ascorbate peroxidase, and peroxiredoxins (6 isoforms in mammals) [12]. And, for O$_2^-$ removal, multiple SOD isoforms are expressed (7 isoforms in Arabidopsis) [11, 15, 405]. These enzyme isoforms show differential localization in subcellular compartments, expression during development and stresses, electron sources, catalytic requirements, posttranslational regulation, and subtle differences in physiological functions.

The analogy implies a similar ubiquity and richness of NO- and Hb-related functions in nature. Moreover, examples are emerging to support this rubric. Aspergillus flavoHb-NODs are differentially distributed to the cytosol and mitochondria because FHb2 bears a mitochondrial N-terminal signal sequence [112, 216]. A class I truncated Hb localizes along chloroplasts thylakoid membranes in algae [406]. Other examples of Hbs with membrane localization signals or affinities include the M. tuberculosis HbO [407, 408], VHB [409, 410], the myristoylated Crab globin [411], and the myristoylated or palmitoylated fish GlbX [88]. Membrane association may also facilitate reduction by membrane-bound reductases. Saccharomyces cerevisiae flavoHb localizes to mitochondria and cytosol without an apparent signal sequence [412]. The wheat nsHb interacts with, and apparently cofunctions with, photosystem I and II [331]. Many Hbs show tissue-specific and developmental regulation of expression [82, 116, 222, 304, 336, 413]. Some Hb-NODs are induced by NO stress, and others are not [95, 116, 216, 310]. Some flavoHb-NODs prefer NADH while others prefer NADPH [52, 114, 216], and expression appears to correlate with the availability of the electron source. The Cygb-NOD utilizes ascorbate as an electron donor in vitro [135, 136] and is expressed in ascorbate-rich fibroblasts and neurons [296]. Transcript network analysis strongly suggests a role for ferredoxin: NADP$^+$ oxidoreductase as the electron donor for cyanoglobin (GlbN) in Legionella pneumophila [239], and GlbN would be required to interact specifically with the reductase. Some globins are suited for NOD catalysis at relatively high micromolar NO concentrations and fluxes (e.g., flavoHbs) [52, 341] whereas others show lower turnover rates and are more susceptible to NO inhibition (e.g., Cygb and Ngb) [135]. This is reasonable since some globins serve primary NO detoxification functions while others subserve NO signaling functions. Some Hbs may function better at low or high temperatures [111, 219] while others may function under dehydrating osmotic stresses [308]. Potential mechanisms for post-translational regulation are also emerging. Reeder et al. have reported that lipid binding to Cygb alters heme coordination and suggest that this provides a mechanism for lipids to alter or regulate Cygb function [414]. Protein phosphorylation and thiol-disulfide interchange have been suggested as mechanisms regulating Ngb function [415–417]. In addition, some globins (e.g., muscle Mb and legume Hb) may be suited to serve dual functions in O$_2$ transport-storage and NO metabolism [125, 137, 255, 262]. These many differences are achieved by many unique Hb structures while intrinsic O$_2$ binding and NO dioxygenation activity appear to be preserved in all Hbs.

### 14. Are Hemoglobins Unique O$_2$-Dependent Catalysts for NO Metabolism?

The simple rapid bimolecular reaction between NO and O$_2^-$ to generate peroxynitrite ($k_2 = 6.9 \times 10^9$ M$^{-1}$ s$^{-1}$) [189, 355], and ultimately yield ∼70% nitrate, initially suggested only minimal requirements for efficient O$_2$-dependent NO metabolism in biological systems [284]. While attractive, the very serious consequences of peroxynitrite toxicity [189, 190] were often overlooked. It is now clear that many organisms metabolize NO to nitrate via Hb-type NODs. What is not clear is whether Hb is the only protein family capable of catalyzing significant O$_2$-dependent NO decomposition in cells. Other O$_2^-$-binding heme or copper catalysts yield peroxynitrite reaction products [26, 418] or scramble the O-atoms with water [66] suggesting a unique capacity of Hbs for high-fidelity NO dioxygenation. On the other hand, an oxygenated Rhodobacter spaeroides cytochrome apparently dioxygenates NO in vitro, forms a complex with an electron-carrying b-type cytochrome and may function as a novel NOD [419].

In many cases, the catalysts of O$_2$-dependent NO metabolism in microbes, plants, brain tissue [199, 271, 274, 420, 421], lung tissue [422], liver parenchymal cells [275], various cultured cell lines [142, 423], endothelial cells [424], macrophages [425], and the aortic wall [426, 427] remain to be defined. Besides the Hbs, cytochrome c oxidase [428], succinate-cytochrome c reductase [429], dihydrolipoidamide dehydrogenase [430], and cytochrome P450s [423] join a growing list of cellular NO removal catalysts put forward for consideration.

### 15. Other Enzymatic Functions for Hemoglobins?

In addition to NO dioxygenation, several specialized enzymatic activities and functions have been proposed for various Hbs. Discerning a meaningful biological function from myriad putative in vitro activities of Hbs [65] continues to be an important challenge. In all cases, investigators must always ask whether (1) other enzymes coexist in cells that could better serve the proposed function, (2) the structure evolved for the proposed function, and (3) the reaction is beneficial for long-term survival of an organism. In evaluating proposed functions of Hbs, it is also important to consider the fundamental argument of Shikama and coworkers [61, 102] that (4) “the reversible binding of molecular oxygen to iron(II) must be the primary event to manifest their physiological functions in vivo.” Of the following recently proposed enzymatic functions, surprisingly, only the heme oxygenase activity meets the last and most basic of these criteria.
15.1. NO Reductase. Anaerobic NO reduction was first suggested to be an important biological function for the *E. coli* flavoHb by Kim et al. [107]. The reaction (10) served to explain the anaerobic induction of flavoHb by NOx [50] and the anaerobic growth protection flavoHb provided against nitrosothiols [55]

\[
2 \text{NO} + 2e^- + 2 \text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \quad (10)
\]

However, the proposed function has critical weaknesses. For example, other microbial flavoHbs show much lower [52], or negligible [48], NO reductase activity. The maximal turnover rate for NO reduction is >2000-fold lower than that measured for NOD activity, and is unstable (unpublished results). Furthermore, the flavoHb shows no NO reductase activity within anaerobic *E. coli* [404]. Moreover, there are more efficient NO reductases in organisms expressing flavoHb or other Hbs [17, 151], and flavoHbs are capable of reducing nitrosothiols directly [52], thus explaining the protection observed. Yet, uncertainty, and confusion, over a NO reductase function clearly persists in the field of reducing nitrosothiols directly [52], thus explaining the avocadoHb or other Hbs [17, 151], and avocadO Hbs are capable of reducing nitrosothiols directly [52], thus explaining the protection observed. Yet, uncertainty, and confusion, over a NO reductase function clearly persists in the field of nitrosative and oxidative stress research [431].

15.2. Peroxynitrite Isomerase. The commonly accepted NOD mechanism includes the efficient isomerization of a ferric heme-bound peroxynitrite intermediate to nitrate (7). Given the formation of toxic peroxynitrite in cells from the rapid reaction of NO and O$_2^-$, Herold and others have suggested that various Hbs and Mb may also function as scavengers of peroxynitrite [125, 138, 432]. However, large rate constants (>10$^6$ s$^{-1}$ M$^{-1}$) for peroxynitrite isomerization were only measured for distal E7 histidine mutants [372] suggesting that entry, binding or isomerization of the ONOO$^-$ anion in the heme pocket is normally hindered by E7 histidine. Exposure of Mb or Hb to exogenous peroxynitrite also nitratates and damages the protein [373, 374, 379–383]. Hence, these heme structures do not appear adapted for a peroxynitrite isomerase function. Secondly, peroxynitrite reacts with a variety of abundant biomolecules (e.g., CO$_2$ and glutathione) [189], and these biomolecules would compete with ferric Hb or Mb. Moreover, proposals of a peroxynitrite isomerase function ignore (1) the important function of SODs and NODs in preventing toxic peroxynitrite formation within cells and (2) reactions with the predominantly oxy Hb within cells [382, 383].

15.3. Nitrite Reductase. As early as 2003, Mark Gladwin and others began pursuing possible NO-generating nitrite reductase functions for the mammalian red blood cell Hb [433, 434] and muscle Mb [269, 435, 436]. The reaction (11) produces NO from nitrite under aerobic and hypoxic conditions in *vitro* and *in vivo* and is stimulated by acidic conditions that occur in tissue ischaemia

\[
\text{NO}_2^- + e^- + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O} \quad (11)
\]

A vital role for the reaction in the hypoxic vasodilation of capillaries and homeostatic control of tissue O$_2$ delivery was proposed. In addition, the reputed nitrite reductase function has been extended to other members of the Hb superfamily. For example, dithionite-reduced Ngb catalyzes NO production from millimolar nitrite under anaerobic conditions with rate constants estimated at 0.12 to 5 s$^{-1}$ M$^{-1}$ [416, 437]. Furthermore, Gladwin and co-workers have reported effects of distal histidine interactions, disulfide bond formation, and protein phosphorylation on the rate of the deoxy Ngb-nitrite reaction [415, 416]. The reaction rate constant increased to physiologically relevant values (∼50 s$^{-1}$ M$^{-1}$) with the elimination or weakening of the distal histidine-heme interaction. Li et al. reported similar low levels of nitrite reducing activity (0.14 s$^{-1}$ M$^{-1}$) for Cygb and demonstrated a significant role for the activity in soluble guanylate cyclase activation in cultured aortic smooth muscle cells with 10 μM nitrite [438]. This is remarkable since the theoretical maximum rate of NO generation under these conditions with an upper [deoxy-Cygb] estimate of 1 μM is 1.4 pM s$^{-1}$. Neighboring endothelial cells normally generate and release NO at a far higher rate of 17 to 1500 nM s$^{-1}$ [277, 278]. As pointed out by Sturms et al., “the modest (low micromolar) levels of nitrite typically found in mammalian tissues diminish the likelihood that nitrite reductase activity is a major function of these proteins” [439]. Rather, Sturms and Hargrove suggest that plant hexacoordinate Hbs may be more suited to nitrite reduction since nitrite levels are higher in plant tissues. They have demonstrated anaerobic nitrite reduction by deoxy forms of rice Hb and *Synechocystis* Hb with respective bimolecular rate constants of 166 and 130 s$^{-1}$ M$^{-1}$ [439], but these Hbs are of relatively low abundance. Tiso et al. subsequently reported comparable bimolecular rate constants of 5 and 20 s$^{-1}$ M$^{-1}$ for the *Arabidopsis thaliana* nonsymbiotic Hbs [440].

It is important to note, however, that the reaction of nitrite with globins nitrosylates ferrous heme and cysteine sulfhydryls [437] and that the reaction of nitrite with globins in the presence of H$_2$O$_2$ nitratates proteins [441, 442]. Moreover, high nitrite concentrations under mildly acidic aerobic conditions form nitrime, a damaged heme [443]. Thus, the anoxic NO$_2^-$ reduction reaction is likely a non-specific and damaging reaction of Hbs. Furthermore, the NO-generating reactions of Hbs must always be evaluated with other enzyme systems that also act as non-specific nitrite reductases [211] including the plant nitrate reductase [204] and mitochondria [444].

15.4. Hydroxylamine Reductase. Sturms et al. have suggested an additional role for plant and cyanobacterial deoxyHbs in reducing hydroxylamine (12), an intermediate in nitrite reduction, under anaerobic conditions [445]. The proposed role presumes a failure of the nitrite reductase to adequately fulfill the function

\[
\text{NH}_2\text{OH} + 2e^- + 2\text{H}^+ \rightarrow \text{NH}_3 + \text{H}_2\text{O} \quad (12)
\]

15.5. Alkylhydroperoxide Reductase. Bonamore et al. have reported an anaerobic alkyl hydroperoxide reductase activity for *E. coli* flavoHb with turnover rates of ∼1 s$^{-1}$ [71, 446]. Given the high affinity of the flavoHb distal pocket for hydrophobic lipids, the authors have suggested a function
for flavoHbs and related single domain Hbs in protecting cells from lipid-related oxidative stress. However, the benefit of flavoHb for protecting cells against peroxide stress is questionable given the exquisite sensitivity of the heme to destruction by peroxide(s) [447].

15.6. Peroxidase. Numerous peroxidative activities of Hbs have been demonstrated in vitro suggesting potential peroxidase functions. Recent suggestions and investigations of Hbs functioning as peroxidases include the *Synecochoccus* GlbN (a class 1 trHb) [138], Cygb [448–451], Ngb [450, 452], and several Arabidopsis Hbs [441]. As pointed out by Paul [453], when evaluating a peroxidase function, one needs to determine overall turnover rate, rates of elementary steps, and specificity for electron donors and compare them with those reported for genuine peroxidases. A peroxidase function must also be demonstrated within the living organism in the normal background of peroxidases and catalase. Only in the case of the peroxidative dehalogenation of phenols by the polychaete *Amphitrite ornate* Hb [454] has a peroxidase function been convincingly demonstrated.

15.7. Lactate Dehydrogenase. Gupta et al. [118] have recently reported the failure of *Mycobacterium tuberculosis* flavoHb (Rv0385) to meet the qualifications of a NOD. The protein is hexa-coordinate and shows very low NOD activity [119] and little ability to protect against nitrosative stress imposed by acidified nitrite when expressed in *E. coli* or *M. smegmatis* [118]. A function as a D-lactate specific dehydrogenase was suggested. The proposed mechanism is similar to that of the L-lactate oxidizing flavocytochrome b$_2$ [72] where lactate transfers electrons to the flavin, and the flavin transfers electrons to the heme. A rather low in vitro turnover number for D-lactate oxidation of 0.026 s$^{-1}$ is estimated from the published data [118]. The possibility of post-translational modifications influencing activity was not considered. Interestingly, the putative *M. tuberculosis* flavoHb (Rv3571) was previously suggested to function in NO detoxification [455], but the work has not been confirmed or extended.

15.8. Electron Carrier. Other electron transfer functions, absent a role for O$_2$, have been suggested for Hbs. Brittain et al. have suggested that the hexa-coordinate Ngb transfers electrons to the mitochondrial cytochrome c to prevent apoptosis [108], and additional roles in reductive stabilization of Hif-1α and Nrf2 during hypoxia have recently been argued [456]. However, Ngb-deficient mouse models do not support a role for Ngb in preventing apoptosis during hypoxia [303]. The reported changes in the Hif-1α-regulated transcription response in Ngb-deficiency [303] may be explained by impaired NO metabolism and NO inhibition of the Hif-1α destabilizing prolyl 4-hydroxylase [170, 171].

15.9. Heme Oxygenase. The flavoHb heme is readily destroyed and iron is released upon exposure to H$_2$O$_2$ [447]. FlavoHbs, and presumably other Hbs, thus have the capacity to act as heme oxygenases. Moreover, multielectron reduction of the heme-bound O$_2$ to H$_2$O$_2$ and/or ferryl by the reductase domain of a flavoHb (8b) generates the necessary intermediates for initiating the heme oxygenase-like mechanism [457]. When the distal TyrB10 and His E7 residues are mutated in the *C. jejuni* truncated Ctb, Ctb can act as a heme oxygenase [226].

15.10. Anaerobic CO Metabolism. The 3.8–4.1-billion-year-old *Methanosarcina acetivorans* protoglobin [MaPgb] binds CO tightly and is thought to function in anaerobic CO fixation possibly through CO interactions [110]. An O$_2$-dependent NOD function appears to have been discounted because the organism is “strictly” anaerobic. However, the authors ignore the fact that many strict anaerobes tolerate brief O$_2$ exposures in their natural habitats. Indeed, the genome of *Methanosarcina acetivorans* contains genes for the O$_2$ defensive enzymes Cu,Zn-SOD (sodC) and catalase/peroxidase (*katG*). Moreover, assumptions of an origin of O$_2$-generating reactions with increasing atmospheric O$_2$ concentrations (~2.45 billion years ago) ignore uncertain O$_2$ utilization rates and potential pockets of O$_2$ generation on the Archaean Earth [458].

16. DNA-Binding and Other Regulatory Functions of Hb Domains

There are numerous examples of the Hb scaffold being used by Nature as part of multi-domain DNA-binding transcription regulators and enzymes including kinases, guanylate cyclases, and phosphodiesterases [1]. These ‘globin-coupled sensors’ were outlined and discussed by Maqsudul Alam and his colleagues in their 2005 review [459]. Not surprisingly, the Hb domains in these proteins are generally thought to bind and sense O$_2$. They regulate important physiologic functions such as biofilm formation by *Salmonella* [460]. Several of the >33 *C. elegans* globins are fused within multi-domain proteins and likely possess O$_2$-sensing regulatory functions [2, 3]. Whether SDGs function in a similar capacity, but in non-covalent association with enzymes, is an important consideration when evaluating the function of Hbs. For example, oxidized ferric Ngb has been found act as a heterotrimeric Ga-protein guanine nucleotide dissociation inhibitor and has been proposed to regulate Ga-protein signaling [461, 462].

Importantly, the facile NO dioxygenation reaction may also be relevant to the biological function of some multi-domain sensor-regulators. For example, the O$_2$ binding *Azotobacter vinelandii* AvGReg, a globin–coupled sensor, reacts rapidly with NO and is thought to serve a NO detoxification [463] or NO sensing function. In the case of the *Mycobacterium tuberculosis* PAS domain sensors DevS and DosT, NO rapidly reacts with the oxy forms to generate nitrate and the ferric heme form [196]. The putative NO dioxygenation reaction triggers the dormancy program through subsequent formation of the kinase activating ferrous-N0 form. The results are intriguing and may explain the ability of NO to induce and maintain the antibiotic-resistant dormant state of the bacterium in *tuberculosis* [464, 465]. A similar NO dioxygenation reaction of the FixL regulator may explain a major part of the NO transcriptional response of *Sinorhizobium meliloti* [183].
17. Hb-NOD Technologies

Progress in understanding the NO-scavenging NOD function and mechanism of various (flavo)Hbs continues to inspire medical, agricultural, and industrial inventions and is helping to unravel the complex biology of NO in a variety of organisms.

For example, human red blood cell Hb has been modified and is being tested for use as an injectable long-lived NO and oxidant scavenger for treatment of septic shock [466]. Future designs that incorporate a catalytic Hb-NOD activity are anticipated. Alternatively, small molecule NOD mimetics [26] may find therapeutic applications once shielding of reactive intermediates in the NO dioxygenation reaction can be achieved.

Mechanistic inhibitors of NODs are being investigated for therapeutic use against microbial infections, hypertensivity, and malignant tumors [467–469]. Imidazoles bearing bulky hydrophobic groups such as the antifungal agent miconazole are particularly effective inhibitors of the flavoHbs [469]. X-ray crystal structures of miconazole and other imidazoles with *R. eutropha* flavoHb reveal key interactions with hydrophobic residues (e.g., Ile25) in the distal heme pocket [470]. These interactions suggest strategies for the rational design of therapeutic inhibitors such as the addition of hydrophobic residues (e.g., Ile25) in the distal heme pocket [470].

In agriculture, Monsanto Corp. is engineering corn, soybean, and other crop plants expressing *E. coli*, *Erwinia chrysanthemi* and yeast flavoHb-NODs for increased resistance to stresses generating NO, increased capacity for soil nitrogen (NO) sequestration in the form of nitrate, and improved growth and fruit production characteristics [472]. Using the patented technology, projected growth and fruit yields increase by ~20% and ~5%, respectively. Many others have engineered transgenic plants to express the *Vitreoscilla* Hb, or other SDGs with the capacity for catalytic NO dioxygenation [75, 129, 256]. The potential beneficial effects for growth, resistance to hypoxia, nitrosative stress, and so forth as well as potential detrimental effects have been recently reviewed [75]. In aquaculture, hypoxia resistant fish are being created through uniform expression of the *Vitreoscilla* Hb directed by the carp actin promoter [257].

*Vitreoscilla* Hb is now being frequently exploited to increase the industrial production of proteins and other molecules by bacteria and yeasts under hypoxic reactor conditions [473–481], and the primary benefit appears to be increased microaerobic respiration *vis-à-vis* the NOD function [256, 258]. Another potential application is the use of the recently discovered dual function algal raphidophyte NOD-nitrate reductase [139] in bioremediation reactors to scavenge NO produced in fossil fuel combustion and to supply algae with nitrate for increased production of biofuels [482].

More recently, the flavoHb-NOD has been touted as a molecular tool for determining the role of NO in biological processes [483]. As stressed by Forrester et al. [483], the strategy has clear advantages over pharmacological methods employing NOS inhibitors or gene knock-outs. Indeed, flavoHb and SDGs have already been used to discover and dissect the role of NO in glioma growth and cancer [297], fungal development and mycotoxin production [305], the plant hypersensitive response [115, 224, 484], symbiosis and nodule development [87, 260], *N*₂ fixation [183, 184], and plant senescence [485].

18. Summary and Outlook

In all fields, we find change and permanence in thinking [20] and a maturation of obscure phenomena to fundamental theories and advances through the scientific process, as lucidly stated by Werner Heisenberg (1901–1976).

“For an understanding of the phenomena, the first condition is the introduction of adequate concepts; only with the help of the correct concepts can we really know what has been observed. When we enter a new field, very often new concepts are needed, and these new concepts usually come up in a rather unclear and undeveloped form. Later they are modified, sometimes they are almost completely abandoned and are replaced by better concepts which then, finally are clear and well-defined.”

Only through the introduction of “correct concepts”, controlled experiments, exacting scrutiny, careful retrospection, and the revision or refutation of faulty concepts can basic truths be established from obscure phenomena. This has been aptly illustrated by our progress in understanding the biology of Hb and NO. Sir Humphry Davy’s investigations of the “respiration” of *Stickstoffoxyd* (NO/NO₂) by blood and tissues in 1800 produced the first relevant, yet unclear, phenomena including the remarkable oxidation of the red pigment of blood by the gas [30]. Since then, the red-pigmented proteins Hb/Mb have been shown to carry O₂⁻/H₂O₂ generation by flavoHb in *S. aureus* in addition to blocking the NOD function [471].

In agriculture, *Vitreoscilla* is now being frequently exploited to increase the industrial production of proteins and other molecules by bacteria and yeasts under hypoxic reactor conditions [473–481], and the primary benefit appears to be increased microaerobic respiration *vis-à-vis* the NOD function [256, 258]. Another potential application is the use of the recently discovered dual function algal raphidophyte NOD-nitrate reductase [139] in bioremediation reactors to scavenge NO produced in fossil fuel combustion and to supply algae with nitrate for increased production of biofuels [482].

More recently, the flavoHb-NOD has been touted as a molecular tool for determining the role of NO in biological processes [483]. As stressed by Forrester et al. [483], the strategy has clear advantages over pharmacological methods employing NOS inhibitors or gene knock-outs. Indeed, flavoHb and SDGs have already been used to discover and dissect the role of NO in glioma growth and cancer [297], fungal development and mycotoxin production [305], the plant hypersensitive response [115, 224, 484], symbiosis and nodule development [87, 260], *N*₂ fixation [183, 184], and plant senescence [485].
agents, and vasomodulators. FlavoHbs are being widely used as tools to assess the involvement of NO in the physiology and pathophysiology of plants, animals, fungi, and bacteria. Plants are being genetically modified for (flavo)Hb expression for increased NO resistance, nitrogen assimilation, growth, and crop yields. Hbs and flavoHbs are also being used to increase the productivity of bacteria, yeasts, and algae in biotechnological and environmental remediation applications. It is hoped that success in any of these or other applications provides tangible rewards for past and current efforts aimed at understanding the NOD function of Hbs.

Finally, I end with a tribute to the spirit of the discoverer of the “Sauerstoffsaugung” (Hbs O₂-absorbing function) and pioneer of molecular biology, Friedrich Hünfeld [486], by echoing his motto 172 years later.

Whatever is still hidden reveals ages in the light!

**Abbreviations**

NOD: Nitric-oxide dioxygenase

Hb: Hemoglobin

Mb: Myoglobin

flavoHb: Flavohemoglobin

SDG: Single domain globin

Cyg: Cytooglobin

Ngb: Neuroglobin

trHb: Truncated hemoglobin

nsHb: Non-symbiotic hemoglobin

SOD: Superoxide dismutase

NOR: Nitric-oxide reductase

NOS: Nitric-oxide synthase

sGC: Soluble guanylate cyclase

IRE-BP: Iron responsive element binding protein.

**Acknowledgments**

Many colleagues contributed to the evolution of the ideas and knowledge presented in this paper. If the author was remiss in citation, it was due to the rapid expansion of the literature and breadth of the field. The author is especially thankful to Dr. Anne Gardner for many thoughtful discussions and experimental contributions. The author thanks his students, assistants, and collaborators listed on many of the publications for their contributions. The author gratefully acknowledges discussions with the late Professor Henry Taube that led him to formulate the internal O-atom rearrangement mechanism presented herein. This work was initiated with support from the American Heart Association Scientist Development Grant (9730193N), the Cincinnati Children's Hospital Trustee Grant, and the National Institutes of Health Grant (R01 GM65090).

**References**

[1] S. N. Vinogradov and L. Moens, “Diversity of globin function: enzymatic, transport, storage, and sensing,” *The Journal of Biological Chemistry*, vol. 283, no. 14, pp. 8773–8777, 2008.

[2] D. Hoogewijs, S. de Henau, S. Dewilde et al., “The Caenorhabditis globin gene family reveals extensive nematode-specific radiation and diversification,” *BMC Evolutionary Biology*, vol. 8, no. 1, article 279, 2008.

[3] D. Hoogewijs, E. Geuens, S. Dewilde et al., “Wide diversity in structure and expression profiles among members of the *Caenorhabditis elegans* globin protein family,” *BMC Genomics*, vol. 8, article 356, 2007.

[4] Y. Qiu, L. Sutton, and A. F. Riggs, “Identification of myoglobin in human smooth muscle,” *The Journal of Biological Chemistry*, vol. 273, no. 36, pp. 23426–23432, 1998.

[5] J. Fraser, L. V. de Mello, D. Ward et al., “Hypoxia-inducible myoglobin expression in nonmuscle tissues,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 8, pp. 2977–2981, 2006.

[6] T. A. Gorr, D. Wichmann, C. Pilarsky et al., “Old proteins–new locations: myoglobin, haemoglobin, neuroglobin and cytoglobin in solid tumors and cancer cells,” *Acta Physiologica*, vol. 202, no. 3, pp. 563–581, 2010.

[7] G. Kristiansen, J. Hu, D. Wichmann et al., “Endogenous myoglobin in breast cancer is hypoxia-inducible by alternative transcription and functions to impair mitochondrial activity. A role in tumor suppression,” *The Journal of Biological Chemistry*, vol. 286, no. 50, pp. 43417–43428, 2011.

[8] G. Xinarianos, F. E. McDonald, J. M. Risk et al., “Frequent genetic and epigenetic abnormalities contribute to the deregulation of cytoglobin in non-small cell lung cancer,” *Human Molecular Genetics*, vol. 15, no. 13, pp. 2038–2044, 2006.

[9] U. Oleksiewicz, N. Daskoulidou, T. Liloglou et al., “Neuroglobin and myoglobin in non-small cell lung cancer: expression, regulation and prognosis,” *Lung Cancer*, vol. 74, no. 3, pp. 411–418, 2011.

[10] A. F. Riggs and T. A. Gorr, “A globin in every cell?” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 8, pp. 2469–2470, 2006.

[11] T. Gaspar, C. Penel, T. Thorpe, and H. Greppin, *Peroxidases 1970–1980: A Survey of Their Biochemical and Physiological Roles in Higher Plants*, Universitè de Genève, Geneva, Switzerland, 1982.

[12] S. G. Rhee, H. Z. Chae, and K. Kim, “Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling,” *Free Radical Biology and Medicine*, vol. 38, no. 12, pp. 1543–1552, 2005.

[13] O. Loew, “A new enzyme of general occurrence in organisms. A preliminary note,” *Science*, vol. 11, pp. 701–702, 1900.

[14] J. M. McCord and I. Fridovich, “Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein),” *The Journal of Biological Chemistry*, vol. 244, no. 22, pp. 6049–6055, 1969.

[15] I. Fridovich, “Superoxide radical and superoxide dismutases,” *Annual Review of Biochemistry*, vol. 64, pp. 97–112, 1995.

[16] F. Bonnot, T. Molle, S. Menage et al., “Control of the evolution of iron peroxide intermediate in superoxide reductase from *Desulfoarculus baarsii*. Involvement of lysine 48 in protonation,” *Journal of the American Chemical Society*, vol. 134, no. 11, pp. 5120–5130, 2012.

[17] W. G. Zumft, “Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type,” *Journal of Inorganic Biochemistry*, vol. 99, no. 1, pp. 194–215, 2005.

[18] K. F. Ettwig, M. K. Butler, D. Le Paslier et al., “Nitrite-driven anaerobic methane oxidation by oxygenic bacteria,” *Nature*, vol. 464, no. 7288, pp. 543–548, 2010.
[19] R. E. Oesper, “Oscar Loew,” *Journal of Chemical Education*, vol. 7, no. 2, pp. 314–315, 1930.
[20] P. Nicholls, “The oxygenase-peroxidase theory of Bach and Chodat and its modern equivalents: change and permanence in scientific thinking as shown by our understanding of the roles of water, peroxide, and oxygen in the functioning of redox enzymes,” *Biochemistry*, vol. 72, no. 10, pp. 1039–1046, 2007.
[21] A. N. Bach and R. Chodat, “Untersuchungen über die Rolle der Peroxyde in der lebenden Zelle, IV Über Peroxydase,” *Berichte der Deutschen Chemischen Gesellschaft*, vol. 35, pp. 2466–2470, 1902.
[22] I. Fridovich and P. Handler, “Detection of free radicals generated during enzymic oxidations by the initiation of sulfite oxidation,” *The Journal of Biological Chemistry*, vol. 236, pp. 1836–1840, 1961.
[23] S. J. Neill, R. Desikan, A. Clarke, R. D. Hurst, and J. T. Hancock, “Hydrogen peroxide and nitric oxide as signalling molecules in plants,” *Journal of Experimental Botany*, vol. 53, no. 372, pp. 1237–1247, 2002.
[24] J. B. Wittenberg and B. A. Wittenberg, “Mechanisms of cytoplasmic hemoglobin and myoglobin function,” *Annual Review of Biophysics and Biophysical Chemistry*, vol. 19, pp. 217–241, 1990.
[25] R. E. Weber and S. N. Vinogradov, “Nonvertebrate hemoglobins: functions and molecular adaptations,” *Physical Reviews*, vol. 81, no. 2, pp. 569–628, 2001.
[26] M. P. Schopfer, B. Mondal, D. H. Lee, A. A. N. Sarjeant, and K. D. Karlin, “Heme/O$_2$-/NO nitric oxide dioxygenase (NOD) reactivity: phenolic nitration via a putative heme-peroxynitrite intermediate,” *Journal of the American Chemical Society*, vol. 131, no. 32, pp. 11304–11305, 2009.
[27] L. Hermann, “Über die Wirkung des Stickstoffoxydges auf das Blut,” *Archiv für Anatomic, Physiologie und Wissenschaftliche Medicin*, pp. 469–481, 1865.
[28] D. Keilin and E. F. Hartree, “Reaction of nitric oxide with hemoglobin and methemoglobin,” *Nature*, vol. 139, no. 3517, article 548, 1937.
[29] H. Davy, *Researches Chemical and Philosophical, Chiefly Concerning Nitrous Oxide or Dephlogisticated Nitrous Air, and Its Respiration*, J. Johnson, London, UK, 1800.
[30] H. Davy, “Humphry Davy’s physiologische-chemische Untersuchungen über das Athmen, besonders über das Athmen von oxydartem Stickgas,” vol. 2, Menerschen Buchhandlung, Lemgo, Germany, 1814.
[31] M. P. Doyle and J. W. Hoekstra, “Oxidation of nitrogen oxides by bound dioxygen in hemoproteins,” *Journal of Inorganic Biochemistry*, vol. 14, no. 4, pp. 351–358, 1981.
[32] R. F. Eich, T. Li, D. D. Lemon et al., “Mechanism of NO-induced oxidation of myoglobin and hemoglobin,” *Biochemistry*, vol. 35, no. 22, pp. 6976–6983, 1996.
[33] R. S. Wade and C. E. Castro, “Reactions of oxyhemoglobin with NO, NO$_2$-, and NO$_3$- under argon and in air,” *Chemical Research in Toxicology*, vol. 9, no. 8, pp. 1382–1390, 1996.
[34] P. R. Gardner, A. M. Gardner, L. A. Martin, and A. L. Salzman, “Nitric oxide dioxygenase: an enzymic function for flavohemoglobin,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 18, pp. 10378–10383, 1998.
[35] P. R. Gardner, G. Costantino, and A. L. Salzman, “Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli*: Role of nitric-oxide dioxygenase in the protection of aconitase,” *The Journal of Biological Chemistry*, vol. 273, no. 41, pp. 26528–26533, 1998.
[36] D. Keilin and Y. L. Wang, “Haemoglobin in the root nodules of leguminous plants,” *Nature*, vol. 155, no. 3930, pp. 227–229, 1945.
[37] D. Keilin, “Haemoglobin in fungi: occurrence of haemoglobin in yeast and the supposed stabilization of the oxygenated cytochrome oxidase,” *Nature*, vol. 172, no. 4374, pp. 390–393, 1953.
[38] A. F. Riggs, “Aspects of the origin and evolution of nonvertebrate hemoglobins,” *Integrative and Comparative Biology*, vol. 31, no. 3, pp. 535–545, 1991.
[39] H. Zhu and A. F. Riggs, “Yeast flavohemoglobin is an ancient protein related to globins and a reductase family,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 11, pp. 5015–5019, 1992.
[40] T. Suzuki and K. Imai, “Evolution of myoglobin,” *Cellular and Molecular Life Sciences*, vol. 54, no. 9, pp. 979–1004, 1998.
[41] L. Moens, J. Vanfleteren, Y. de Peer et al., “Globins in nonvertebrate species: dispersal by horizontal gene transfer and evolution of the structure-function relationships,” *Molecular Biology and Evolution*, vol. 13, no. 2, pp. 324–333, 1996.
[42] R. C. Hardison, “A brief history of hemoglobins: plant, animal, protist, and bacteria,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 12, pp. 5675–5679, 1996.
[43] C. A. Appleby, “Electron transport systems of *Rhizobium japonicum*, II. *Rhizobium* haemoglobin, cytochromes and oxidases in free-living (cultured) cells,” *Biochimica et Biophysica Acta*, vol. 172, no. 1, pp. 88–105, 1969.
[44] C. A. Appleby, J. D. Tjepkema, and M. J. Trinick, “Hemoglobin in a nonleguminous plant, parasponia: possible genetic origin and function in nitrogen fixation,” *Science*, vol. 220, no. 4600, pp. 951–953, 1983.
[45] R. Oshino, N. Oshino, and R. Chance, “Studies on yeast hemoglobin. The properties of yeast hemoglobin and its physiological function in the cell,” *European Journal of Biochemistry*, vol. 35, no. 1, pp. 23–33, 1973.
[46] I. Probst, G. Wolf, and H. G. Schlegel, “An oxygen-binding flavohemeprotein from *Alcaligenes eutrophus*,” *Biochimica et Biophysica Acta*, vol. 576, no. 2, pp. 471–478, 1979.
[47] R. P. Dikshit, K. L. Dikshit, Y. Liu, and D. A. Webster, “The bacterial hemoglobin from *Vitreoscilla* can support the aerobic growth of *Escherichia coli* lacking terminal oxidases,” *Archives of Biochemistry and Biophysics*, vol. 293, no. 2, pp. 241–245, 1992.
[48] R. C. Hardison, B. Friedrich, and B. Friedrich, “Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*,” *The Journal of Biological Chemistry*, vol. 269, no. 10, pp. 7349–7354, 1994.
[49] U. Ermker, N. Siddiqui, R. Cramm, and B. Friedrich, “Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution,” *EMBO Journal*, vol. 14, no. 24, pp. 6067–6077, 1995.
[50] R. K. Poole, M. F. Anjum, J. Membrillo-Hernández, S. O. Kim, M. N. Hughes, and V. Stewart, “Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12,” *Journal of Bacteriology*, vol. 178, no. 18, pp. 5487–5492, 1996.
[51] M. F. Anjum, N. Ioannidis, and R. K. Poole, “Response of the NAD(P)H-oxidising flavohaemoglobin (Hmp) to prolonged oxidative stress and implications for its physiological role in..."
P. R. Gardner, A. M. Gardner, L. A. Martin et al., “Nitric-oxide dioxygenase activity and function of flavohemoglobin. Sensitivity to nitric oxide and carbon monoxide inhibition,” *The Journal of Biological Chemistry*, vol. 275, no. 41, pp. 31581–31587, 2000.

M. J. Crawford and D. E. Goldberg, “Role for the flavohemoglobin gene in *Bacillus subtilis*,” *Journal of Bacteriology*, vol. 178, no. 13, pp. 3803–3808, 1996.

X. J. Zhao, D. Raitt, P. V. Burke, A. S. Clewell, K. E. Kwast, and R. O. Poyton, “Function and expression of flavohemoglobin in *Saccharomyces cerevisiae*. Evidence for a role in the oxidative stress response,” *The Journal of Biological Chemistry*, vol. 271, no. 41, pp. 25131–25138, 1996.

M. J. Crawford and D. E. Goldberg, “Regulation of *Saccharomyces cerevisiae* flavohemoglobin gene expression,” *The Journal of Biological Chemistry*, vol. 270, no. 12, pp. 6991–6996, 1995.

D. R. Sherman, B. Guinn, M. M. Perdok, and D. E. Goldberg, “Components of sterol biosynthesis assembled on the oxygen-avid hemoglobin of Ascaris,” *Science*, vol. 258, no. 5090, pp. 1930–1932, 1992.

H. Iwaasa, T. Takagi, and K. Shikama, “Amino acid sequence of yeast hemoglobin in a two-domain structure,” *Journal of Molecular Biology*, vol. 227, no. 3, pp. 948–954, 1992.

H. Iwaasa, T. Takagi, and K. Shikama, “Protozoan myoglobin from *Paramecium caudatum*. Its unusual amino acid sequence,” *Journal of Molecular Biology*, vol. 208, no. 2, pp. 355–358, 1989.

H. Iwaasa, T. Takagi, and K. Shikama, “Protozoan hemoglobin from *Tetrahymena pyriformis*. Isolation, characterization, and amino acid sequence,” *The Journal of Biological Chemistry*, vol. 265, no. 15, pp. 8603–8609, 1990.

G. Kobayashi, T. Nakamura, H. Ohmachi, A. Matsuoka, T. Ochiai, and K. Shikama, “Yeast flavohemoglobin from *Candida norvegensis*: its structural, spectral, and stability properties,” *The Journal of Biological Chemistry*, vol. 277, no. 45, pp. 42540–42548, 2002.

R. D. Hill, T. J. Belbin, M. V. Thorsteinsson et al., “GlbN (cyanoglobin) is a peripheral membrane protein that is restricted to certain *Nostoc* spp.,” *Journal of Bacteriology*, vol. 178, no. 22, pp. 6587–6598, 1996.

A. W. Sowa, S. M. G. Duff, P. A. Guy, and R. D. Hill, “Altering hemoglobin levels changes energy status in maize cells under hypoxia,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 17, pp. 10317–10321, 1998.

R. D. Hill, “What are hemoglobins doing in plants?” *Canadian Journal of Botany*, vol. 76, no. 5, pp. 707–712, 1998.

P. R. Gardner, “Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases,” *Journal of Inorganic Biochemistry*, vol. 99, no. 1, pp. 247–266, 2005.

P. R. Gardner, A. M. Gardner, W. T. Brashears et al., “Hemoglobins dioxygenate nitric oxide with high fidelity,” *Journal of Inorganic Biochemistry*, vol. 100, no. 4, pp. 542–550, 2006.

M. T. Forrester and M. W. Foster, “Protection from nitrosative stress: a central role for microbial flavohemoglobin,” *Free Radical Biology and Medicine*, vol. 52, no. 9, pp. 1620–1633, 2012.

L. A. Bowman, S. McLean, R. K. Poole, and J. M. Fukuto, “The diversity of microbial responses to nitric oxide and agents of nitrosative stress close cousins but not identical twins,” *Advances in Microbial Physiology*, vol. 59, pp. 135–219, 2011.

R. K. Poole, “Nitric oxide and nitrosative stress tolerance in bacteria,” *Biochemical Society Transactions*, vol. 33, no. 1, pp. 176–180, 2005.

M. Angelo, A. Hausladen, D. J. Singel, and J. S. Stamler, “Interactions of NO with hemoglobin: from microbes to man,” *Methods in Enzymology*, vol. 436, pp. 131–168, 2008.

A. Bonamore and A. Boffi, “Flavohemoglobin: structure and reactivity,” *IUBMB Life*, vol. 60, no. 1, pp. 19–28, 2008.

C. G. Mowat, B. Gazar, L. P. Campbell, and S. K. Chapman, “Flavin-containing heme enzymes,” *Archives of Biochemistry and Biophysics*, vol. 493, no. 1, pp. 37–52, 2010.

A. Tillmann, N. A. R. Gow, and A. J. P. Brown, “Nitric oxide and nitrosative stress tolerance in yeast,” *Biochemical Society Transactions*, vol. 39, no. 1, pp. 219–223, 2011.

K. H. Hebelstrup, A. U. Igamberdiev, and R. D. Hill, “Metabolic effects of hemoglobin gene expression in plants,” *Gene*, vol. 398, no. 1-2, pp. 86–93, 2007.

S. Jokipii-Lukkari, A. D. Frey, P. T. Kallio, and H. Häggman, “Intrinsinc non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants,” *Journal of Experimental Botany*, vol. 60, no. 2, pp. 409–422, 2009.

K. J. Gupta, K. H. Hebelstrup, L. A. Mur, and A. U. Igamberdiev, “Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide,” *FEBS Letters*, vol. 585, no. 24, pp. 3843–3849, 2011.

A. U. Igamberdiev, N. V. Bykova, and R. D. Hill, “Structural and functional properties of class I plant hemoglobins,” *IUBMB Life*, vol. 63, no. 3, pp. 146–152, 2011.

A. D. Frey, M. Shepherd, S. Jokipii-Lukkari, H. Häggman, and P. T. Kallio, “The single-domain globin of *Vitreoscilla*. Augmentation of aerobic metabolism for biotechnological applications,” *Advances in Microbial Physiology*, vol. 58, pp. 82–139, 2011.

P. T. Kallio, C. J. T. Bollinger, T. Koskenkorva, and A. D. Frey, “Assessment of biotechnologically relevant characteristics of heterologous hemoglobins in *E. coli*,” *Methods in Enzymology*, vol. 436, pp. 255–272, 2008.

S. Spiro, “Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors,” *Philosophical Transactions of the Royal Society B*, vol. 367, no. 1593, pp. 1213–1225, 2012.

V. Garrocho-Villegas, S. K. Gopalasubramaniam, and R. Arredondo-Peter, “Plant hemoglobins: what we know six decades after their discovery,” *Gene*, vol. 398, no. 1-2, pp. 78–85, 2007.

R. D. Hill, “Non-symbiotic haemoglobins—what’s happening beyond nitric oxide scavenging?” *AoB Plants*, vol. 2012, Article ID pls004, 13 pages, 2012.

P. Ascenzi and P. Visca, “Scavenging of reactive nitrogen species by mycobacterial truncated hemoglobins,” *Methods in Enzymology*, vol. 436, pp. 317–337, 2008.

F. Spyra, F. J. Luque, and C. Vippani, “Structural analysis in nonsymbiotic hemoglobins: what can we learn from inner cavities?” *Plant Science*, vol. 181, no. 1, pp. 8–13, 2011.
[85] U. Oleksiewicz, T. Liloglou, J. K. Field, and G. Xinarianos, “Cytooglobin: biochemical, functional and clinical perspective of the newest member of the globin family,” *Cellular and Molecular Life Sciences*, vol. 68, no. 23, pp. 3869–3883, 2011.

[86] M. A. Marti, L. Capece, A. Bidon-Chanal et al., “Nitric oxide reactivity with globins as investigated through computer simulation,” *Methods in Enzymology*, vol. 437, pp. 477–498, 2008.

[87] C. Sánchez, J. J. Cabrera, A. J. Gates, E. J. Bedmar, D. J. Richardson, and M. J. Delgado, “Nitric oxide detoxification in the rhizobia-legume symbiosis,” *Biochemical Society Transactions*, vol. 39, no. 1, pp. 184–188, 2011.

[88] M. Blank, J. Wollberg, F. Gerlach et al., “A membrane-bound vertebrate globin,” *PLoS ONE*, vol. 6, no. 9, Article ID e25292, 2011.

[89] S. N. Vinogradov, D. Hoogewijs, X. Bailly et al., “A phyletic-nomic profile of globins,” *BMC Evolutionary Biology*, vol. 6, article 31, 2006.

[90] D. Hoogewijs, S. Dewilde, A. Verstraete, L. Moens, and S. N. Vinogradov, “A phylegetic analysis of the globins in fungi,” *PLoS ONE*, vol. 7, no. 2, Article ID e31856, 2012.

[91] S. N. Vinogradov, “Tracing globin phylogeny using PSIBLAST searches based on groups of sequences,” *Methods in Enzymology*, vol. 436, pp. 571–583, 2008.

[92] S. N. Vinogradov, D. Hoogewijs, X. Bailly et al., “A model of globin evolution,” *Gene*, vol. 398, no. 1–2, pp. 132–142, 2007.

[93] S. N. Vinogradov, D. Hoogewijs, and R. Arredondo-Peter, “What are the origins and phylogeny of plant hemoglobin,” *Communicative and Integrative Biology*, vol. 4, no. 4, pp. 443–445, 2011.

[94] D. A. Vuletich and J. T. Lecomte, “A phylogenetic and structural analysis of truncated hemoglobins,” *Journal of Molecular Evolution*, vol. 62, no. 2, pp. 196–210, 2006.

[95] B. D. Ullmann, H. Myers, W. Chiranand et al., “Inducible defense mechanism against nitric oxide in *Candida albicans*,” *Eukaryotic Cell*, vol. 3, no. 3, pp. 715–723, 2004.

[96] T. Burmester, M. Haberkamp, S. Mitz et al., “Neuroglobin and cytooglobin: genes, proteins and evolution,” *IUBMB Life*, vol. 56, no. 11-12, pp. 703–707, 2004.

[97] D. Hoogewijs, B. Ebner, F. Germani et al., “Androglobin: a chimeric globin in metazoans that is preferentially expressed in Mammalian testes,” *Molecular Biology and Evolution*, vol. 29, no. 4, pp. 1105–1114, 2012.

[98] K. Kirschner and H. Bisswanger, “Multifunctional proteins,” *Annual Review of Biochemistry*, vol. 45, pp. 143–166, 1976.

[99] T. Ramasarma, “One protein–many functions,” *Current Science*, vol. 67, pp. 24–29, 1994.

[100] C. J. Jeffery, “Moonlighting proteins,” *Current Science*, vol. 45, no. 1, pp. 8–11, 1999.

[101] P. J. O’Brien and D. Herschlag, “Catalytic promiscuity and the evolution of new enzymatic activities,” *Chemistry and Biology*, vol. 6, no. 4, pp. R91–R105, 1999.

[102] K. Shikama and A. Matsuoka, “Structure-function relationships in unusual nonvertebrate globins,” *Critical Reviews in Biochemistry and Molecular Biology*, vol. 39, no. 4, pp. 217–259, 2004.

[103] L. W. Chung, X. Li, H. Hirao, and K. Morokuma, “Comparative reactivity of ferric-superoxo and ferryl-oxo species in heme and non-heme complexes,” *Journal of the American Chemical Society*, vol. 133, no. 50, pp. 20076–120079, 2011.

[104] J. T. J. Lecomte, D. A. Vuletich, and A. M. Lesk, “Structural divergence and distant relationships in proteins: evolution of the globins,” *Current Opinion in Structural Biology*, vol. 15, no. 3, pp. 290–301, 2005.

[105] J. B. Wittenberg, M. Bolognesi, B. A. Wittenberg, and M. Guertin, “Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants,” *The Journal of Biological Chemistry*, vol. 277, no. 2, pp. 871–874, 2002.

[106] M. F. Perutz, “Mechanisms regulating the reactions of human hemoglobin with oxygen and carbon monoxide,” *Annual Review of Physiology*, vol. 52, pp. 1–25, 1990.

[107] S. O. Kim, Y. Orii, D. Lloyd, M. N. Hughes, and R. K. Poole, “Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide,” *FEBS Letters*, vol. 445, no. 2-3, pp. 389–394, 1999.

[108] A. Fago, A. J. Mathews, L. Moens, S. Dewilde, and T. Brittain, “The reaction of neuroglobin with potential redox protein partners cytochrome b5 and cytochrome c,” *FEBS Letters*, vol. 580, no. 20, pp. 4884–4888, 2006.

[109] T. A. K. Freitas, S. Hou, E. M. Dioum et al., “Ancestral hemoglobins in *Archaea*, *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 17, pp. 6675–6680, 2004.

[110] S. Abbruzzetti, L. Tillman, S. Bruno et al., “Ligation tunes protein reactivity in an ancient haemoglobin: kinetic evidence for an allosteric mechanism in *Metanovacula acetivorans* protoglobin,” *PLoS ONE*, vol. 7, Article ID e36314, 2012.

[111] J. J. L. Miranda, D. H. Mailliet, J. Soman, and J. S. Olson, “Thermoglobin, oxygen-avid hemoglobin in a bacterial hyperthermophile,” *The Journal of Biological Chemistry*, vol. 280, no. 44, pp. 36754–36761, 2005.

[112] S. Zhou, S. Pushinobu, Y. Nakanishi, S. W. Kim, T. Wakeshi, and H. Shoun, “Cloning and characterization of two flavohaemoglobins from *Aspergillus oryzae*,” *Biochemical and Biophysical Research Communications*, vol. 381, no. 1, pp. 7–11, 2009.

[113] S. Zhou, T. Narukami, M. Nameki et al., “Heme-biosynthetic porphobilinogen deaminase protects *Aspergillus nidulans* from nitrosative stress,” *Applied and Environmental Microbiology*, vol. 78, no. 1, pp. 103–109, 2012.

[114] A. N. Hvitved, *NO dioxygenation in mammalian myoglobin and microbial flavohaemoglobins* [Ph.D. thesis], Rice University, Houston, Tex, USA, 2008.

[115] M. Boccara, C. E. Mills, J. Zeier et al., “Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host,” *Plant Journal*, vol. 43, no. 2, pp. 226–237, 2005.

[116] D. Mastronicoila, F. Testa, E. Forte et al., “Flavohaemoglobin and nitric oxide detoxification in the human protozoan parasite *Giardia intestinalis*,” *Biochemical and Biophysical Research Communications*, vol. 399, no. 4, pp. 654–658, 2010.

[117] S. Rafferty, B. Luu, R. E. March, and J. Yee, “*Giardia lamblia* encodes a functional flavohaemoglobin,” *Biochemical and Biophysical Research Communications*, vol. 399, no. 3, pp. 347–351, 2010.

[118] S. Gupta, S. Pawaria, C. Lu et al., “An unconventional hexacoordinated flavohaemoglobin from *Mycobacterium tuberculosis*,” *The Journal of Biological Chemistry*, vol. 287, no. 20, pp. 16435–16446, 2012.
B. J. Smagghe, J. T. Trent III, and M. S. Hargrove, “NO dioxygenation in group I truncated hemoglobin,” *Journal of the American Chemical Society*, vol. 132, no. 9, pp. 2968–2982, 2010.

P. Ascenzi, A. Bocedi, M. Bolognesi, G. Fabozzi, M. Milani, and P. Visca, “Nitric oxide scavenging by *Mycobacterium leprae* GlbO involves the formation of the ferric heme-bound peroxynitrite intermediate,” *Biochemical and Biophysical Research Communications*, vol. 339, no. 1, pp. 450–456, 2006.

M. Brunori, A. Giuffrè, K. Nienhaus, G. U. Nienhaus, F. M. Scandurra, and B. Vallone, “Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 24, pp. 8483–8488, 2005.

H. Ouellet, L. Juszczak, D. Dantsker et al., “Reactions of *Mycobacterium tuberculosis* truncated hemoglobin O with ligands reveal a novel ligand-inclusive hydroxide bond network,” *Biochemistry*, vol. 42, no. 19, pp. 5764–5774, 2003.

S. Herold and A. Pupo, “Oxytheglobin scavenges nitrogen monoxide and peroxynitrite: a possible role in functioning nodules?” *Journal of Biological Inorganic Chemistry*, vol. 10, no. 8, pp. 935–945, 2005.

K. H. Hebelstrup, M. van Zanten, J. Mandon et al., “Haemoglobin modulates NO emission and hypoxaemia under hypoxia-related stress in *Arabidopsis thaliana*,” *Journal of Experimental Botany*, vol. 63, no. 15, pp. 5581–5591, 2012.

M. Perazzolli, P. Dominici, M. C. Romero-Puertas et al., “*Arabidopsis* nonsymbiotic hemoglobin AHB1 modulates nitric oxide bioactivity,” *Plant Cell*, vol. 16, no. 10, pp. 2785–2794, 2004.

H. Ouellet, Y. Ouellet, C. Richard et al., “Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 9, pp. 5902–5907, 2002.

R. Pathania, N. K. Navani, A. M. Gardner, P. R. Gardner, and K. L. Dikshit, “Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin, HbN in *Escherichia coli*,” *Molecular Microbiology*, vol. 45, no. 5, pp. 1303–1314, 2002.

R. Pathania, N. K. Navani, A. M. Gardner, P. R. Gardner, and K. L. Dikshit, “Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin, HbN in *Escherichia coli*,” *Molecular Microbiology*, vol. 45, no. 5, pp. 1303–1314, 2002.

A. U. Igamberdiev, C. Seregélyes, N. Manac’h, and R. D. Hill, “NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley hemoglobin,” *Planta*, vol. 219, no. 1, pp. 95–102, 2004.

A. U. Igamberdiev, N. V. Bykova, and R. D. Hill, “Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin,” *Planta*, vol. 223, no. 5, pp. 1033–1040, 2006.

B. J. Smagghe, J. T. Trent III, and M. S. Hargrove, “NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction in vivo,” *PLoS ONE*, vol. 3, no. 4, Article ID e2039, 2008.

K. E. Halligan, F. L. Jourd’heuil, and D. Jourd’heuil, “Cytochrome P450 is expressed in the vasculature and regulates cell respiration and proliferation nitric oxide dioxygenation,” *The Journal of Biological Chemistry*, vol. 284, no. 13, pp. 8539–8547, 2009.

A. M. Gardner, M. R. Cook, and P. R. Gardner, “Nitric-oxide dioxygenase function of human cytoglobin with cellular reductants and in rat hepatocytes,” *The Journal of Biological Chemistry*, vol. 285, no. 31, pp. 23850–23857, 2010.

X. Liu, D. Follmer, J. R. Zweier et al., “Characterization of the function of cytoglobin as an oxygen-dependent regulator of nitric-oxide concentration,” *Biochemistry*, vol. 51, no. 25, pp. 5072–5082, 2012.

U. Flögel, M. W. Merx, A. Gödecke, U. K. M. Decking, and J. Schrader, “Myoglobin: a scavenger of bioactive NO,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 2, pp. 735–740, 2001.

N. L. Scott, Y. Xu, G. Shen et al., “Functional and structural characterization of the 2/2 hemoglobin from *Synecococcus* sp. PCC 7002,” *Biochemistry*, vol. 49, no. 33, pp. 7000–7011, 2010.

J. J. Stewart and K. J. Coyne, “Analysis of raphidophyte assimilatory nitrate reductase reveals unique domain architecture incorporating a 2/2 hemoglobin,” *Plant Molecular Biology*, vol. 77, no. 6, pp. 565–575, 2011.

P. K. Witting, D. J. Douglas, and A. G. Mauk, “Reaction of human myoglobin and nitric oxide. Heme iron or protein sulphydryl (s) nitrosation dependence on the absence or presence of oxygen,” *The Journal of Biological Chemistry*, vol. 276, no. 6, pp. 3991–3998, 2001.

K. J. Gupta, J. K. Shah, Y. Brotman et al., “Inhibition ofaconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids,” *Journal of Experimental Botany*, vol. 63, no. 4, pp. 1773–1784, 2012.
cells by nitric oxide,” Free Radical Biology and Medicine, vol. 36, no. 4, pp. 507–516, 2004.

[179] M. Juckett, Y. Zheng, H. Yuan et al., “Heme and the endothelium. Effects of nitric oxide on catalytic iron and heme degradation by heme oxygenase,” The Journal of Biological Chemistry, vol. 273, no. 36, pp. 23388–23397, 1998.

[180] J. Wang, S. Lu, P. Moënne-Loccoz, and P. R. Ortiz de Montellano, “Interaction of nitric oxide with human heme oxygenase-1,” The Journal of Biological Chemistry, vol. 278, pp. 2341–2347, 2003.

[181] V. A. Szalai and G. W. Brudvig, “Reversible binding of nitric oxide to tyrosyl radicals in photosystem II. Nitric oxide quenches formation of the S3 EPR signal species in acetate-inhibited photosystem II,” Biochemistry, vol. 35, no. 47, pp. 15080–15087, 1996.

[182] E. Meilhoc, Y. Cam, A. Skapski, and C. Bruand, “The response of nitric oxide on the nitrogen-fixing symbiont Sinorhizobium meliloti,” Molecular Plant-Microbe Interactions, vol. 19, no. 4, pp. 441–450, 2006.

[183] Y. Shimoda, F. Shimoda-Sasakura, K. I. Kucho et al., “Overexpression of class I plant hemoglobin genes enhances symbiotic nitrogen fixation activity between Mesorhizobium loti and Lotus japonicus,” Plant Journal, vol. 57, no. 2, pp. 254–263, 2009.

[184] J. C. Trinchant and J. Rigaud, “Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids,” Applied and Environmental Microbiology, vol. 44, no. 6, pp. 1385–1388, 1982.

[185] K. Kato, K. Kanahama, and Y. Kanayama, “Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in Lotus root nodules,” Journal of Plant Physiology, vol. 167, no. 3, pp. 238–241, 2010.

[186] M. R. Hyman, L. C. Seefeldt, T. V. Morgan, D. J. Arp, and L. E. Mortenson, “Kinetic and spectroscopic analysis of the inactivating effects of nitric oxide on the individual components of Azotobacter vinelandii nitrogenase,” Biochemistry, vol. 31, no. 11, pp. 2947–2955, 1992.

[187] K. H. Tibelius and R. Knowles, “Hydrogenase activity in Azospirillum brasilense is inhibited by nitrite, nitric oxide, carbon monoxide, and acetylene,” Journal of Bacteriology, vol. 160, no. 1, pp. 103–106, 1984.

[188] J. C. Toledo Jr. and O. Augusto, “Connecting the chemical and biological properties of nitric oxide,” Chemical Research in Toxicology, vol. 25, no. 5, pp. 975–989, 2012.

[189] J. S. Beckman and W. H. Koppenol, “Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly,” American Journal of Physiology, vol. 271, no. 5, pp. C1424–C1437, 1996.

[190] B. Roy and J. Garthwaite, “Nitric oxide activation of guanylyl cyclase in cells revisited,” Proceedings of the National Academy of Sciences of the United States of America, vol. 103, no. 32, pp. 12185–12190, 2006.

[191] E. Mo, H. Amin, I. H. Bianco, and J. Garthwaite, “Kinetics of a cellular nitric oxide/cGMP/phosphodiesterase-5 pathway,” The Journal of Biological Chemistry, vol. 279, no. 25, pp. 26149–26158, 2004.

[192] J. C. Crack, J. Green, M. I. Hutchings, A. J. Thomas, and N. E. Le Brun, “Bacterial iron-sulfur regulatory proteins as biological sensor-switches,” Antioxidants and Redox Signaling, vol. 17, no. 9, pp. 1215–1231, 2012.

[193] A. M. Gardner, C. R. Gessner, and P. R. Gardner, “Regulation of the nitric oxide reduction operon (norRVW) in Escherichia coli. Role of NorR and d54 in the nitric oxide stress response,” The Journal of Biological Chemistry, vol. 278, no. 12, pp. 10081–10086, 2003.

[194] E. T. Yulk, M. A. Elbaz, M. M. Nakano, and P. Moënne-Loccoz, “Transcription factor NsrR from Bacillus subtilis senses nitric oxide with a 4Fe–4S cluster,” Biochemistry, vol. 47, no. 49, pp. 13084–13092, 2008.

[195] E. T. Yulk, A. Ioanoviciu, S. Sivaramakrishnan, M. M. Nakano, P. R. Ortiz de Montellano, and P. Moënne-Loccoz, “Nitric oxide dioxygenation reaction in DevS and the initial response to nitric oxide in Mycobacterium tuberculosis,” Biochemistry, vol. 50, no. 6, pp. 1023–1028, 2011.

[196] N. Manac’h-Little, A. U. Igamberdiev, and R. D. Hill, “Hemoglobin expression affects ethylene production in maize cell cultures,” Plant Physiology and Biochemistry, vol. 43, no. 5, pp. 485–489, 2005.

[197] A. Ledo, J. Frade, R. M. Barbosa, and J. Laranjinha, “Nitric oxide in brain: diffusion, targets and concentration dynamics in hippocampal subregions,” Molecular Aspects of Medicine, vol. 25, no. 1-2, pp. 75–89, 2004.

[198] R. M. Santos, C. F. Lourenço, A. Ledo, R. M. Barbosa, and J. Laranjinha, “Nitric oxide inactivation mechanisms in the brain: role in bioenergetics and neurodegeneration,” International Journal of Cell Biology, vol. 2012, Article ID 391914, 13 pages, 2012.

[199] S. W. Kim, S. Fushinobu, S. Zhou, T. Wakagi, and H. Shoun, “The possible involvement of copper-containing nitrite reductase (NirK) and flavohemoglobin in denitrification by the fungus Cylindrocarpon tonkinense,” Bioscience, Biotechnology and Biochemistry, vol. 74, no. 7, pp. 1403–1407, 2010.

[200] I. C. Anderson, M. Poth, J. Homstead, and D. Burdige, “A comparison of NO and N₂O production by the autotrophic nitrifier Nitrosomonas europaea and the heterotrophic nitrifier Alcaligenes faecalis,” Applied and Environmental Microbiology, vol. 59, no. 11, pp. 3525–3533, 1993.

[201] H. J. E. Beaumont, B. van Scooten, S. I. Lens, H. V. Westerhoff, and R. J. M. van Spanning, “Nitrosomonas europaea expresses a nitric oxide reductase during nitrification,” Journal of Bacteriology, vol. 186, no. 13, pp. 4417–4421, 2004.

[202] H. Yamasaki and Y. Sakihama, “Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species,” FEBS Letters, vol. 468, no. 1, pp. 89–92, 2000.

[203] Y. Sakihama, S. Nakamura, and H. Yamasaki, “Nitric oxide production mediated by nitrate reductase in the green alga Chlamydomonas reinhardtii: an alternative NO production pathway in photosynthetic organisms,” Plant and Cell Physiology, vol. 43, no. 3, pp. 290–297, 2002.

[204] N. J. Gilberthorpe and R. K. Poole, “Nitric oxide homeostasis in Salmonella typhimurium: roles of respiratory nitrate reductase and flavohemoglobin,” The Journal of Biological Chemistry, vol. 283, no. 17, pp. 11146–11154, 2008.

[205] A. U. Igamberdiev and R. D. Hill, “Nitrate, NO and hemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways,” Journal of Experimental Botany, vol. 55, no. 408, pp. 2473–2482, 2004.

[206] N. Gruber and J. N. Galloway, “An Earth-system perspective of the global nitrogen cycle,” Nature, vol. 451, no. 7176, pp. 293–296, 2008.
V. Smil, “Global population and the nitrogen cycle,” *Scientific American*, pp. 76–81, 1997.

N. Takaya and H. Shoun, “Genetic engineering using fungal flavohemoglobin for constructing *Pseudomonas stutzeri* strain emitting less nitrous oxide,” *Journal of Bioscience and Bioengineering*, vol. 94, no. 3, pp. 282–284, 2002.

D. R. Smart and A. J. Bloom, “Wheat leaves emit nitrous oxide during nitrate assimilation,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 14, pp. 7875–7878, 2001.

J. L. Zweier, H. Li, A. Samouilov, and X. Liu, “Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall,” *Nitrergic Oxidative Stress*, vol. 22, no. 2, pp. 83–90, 2010.

M. M. Nakano, “Essential role of flavohemoglobin in long-term anaerobic survival of *Bacillus subtilis*,” *Journal of Bacteriology*, vol. 188, no. 17, pp. 6415–6418, 2006.

A. Rogstam, J. T. Larsson, P. Kjelgaard, and C. von Wachenfeldt, “Mechanisms of adaptation to nitrosative stress in *Bacillus subtilis*,” *Journal of Bacteriology*, vol. 189, no. 8, pp. 3063–3071, 2007.

A. R. Richardson, P. M. Dunman, and F. C. Fang, “The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity,” *Molecular Microbiology*, vol. 61, no. 4, pp. 927–939, 2006.

V. L. Gonçalves, L. S. Nobre, J. B. Vicente, M. Teixeira, and L. M. Saraiva, “Flavohemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*,” *FEBS Letters*, vol. 580, no. 7, pp. 1817–1821, 2006.

S. Zhou, S. Fushinobu, S. W. Kim et al., “Functional analysis and subcellular location of two flavohemoglobin from *Aspergillus oryzae*,” *Fungal Genetics and Biology*, vol. 48, no. 2, pp. 200–207, 2011.

F. Sebbane, N. Lemaitre, D. E. Sturdevant et al., “Adaptive response of *Yersinia pestis* to extracellular effectors of innate immunity during bubonic plague,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 31, pp. 11766–11771, 2006.

Y. Wang, A. K. Dunn, J. Wilneff, M. J. McFall-Ngai, S. Spiro, and E. G. Ruby, “*Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid-*Vibrio fischeri* symbiosis,” *Molecular Microbiology*, vol. 78, no. 4, pp. 903–915, 2010.

E. Parrilli, M. Giuliani, G. Marino, and M. L. Tutino, “Influence of production process design on inclusion bodies protein: the case of an Antarctic flavohemoglobin,” *Microbial Cell Factories*, vol. 9, article 19, 2010.

K. T. Elvers, S. M. Turner, L. M. Wainwright et al., “NsrR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated hemoglobin,” *Molecular Microbiology*, vol. 57, no. 3, pp. 735–750, 2005.

M. S. Pittman, K. T. Elvers, L. Lee et al., “Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NpA and NfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress,” *Molecular Microbiology*, vol. 63, no. 2, pp. 575–590, 2007.

J. L. Turrion-Gomez, A. P. Eslava, and E. P. Benito, “The flavohemoglobin BCFH1G1 is the main NO detoxification system and confers protection against nitrosative conditions but is not a virulence factor in the fungal necrotroph *Botrytis cinerea*,” *Fungal Genetics and Biology*, vol. 47, no. 5, pp. 484–496, 2010.

G. Fabozzi, P. Ascenzi, S. D. Renzi, and P. Visca, “Truncated hemoglobin GlbO from *Mycobacterium leprae* alleviates nitric oxide toxicity,” *Microbial Pathogenesis*, vol. 40, no. 5, pp. 211–220, 2006.

Z. L. Qu, N. Q. Zhong, H. Y. Wang, A. P. Chen, G. L. Jian, and G. X. Xia, “Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHBD1 triggers defense responses and increases disease tolerance in *Arabidopsis*,” *Plant and Cell Physiology*, vol. 47, no. 8, pp. 1058–1068, 2006.

K. T. Elvers, G. Wu, N. J. Gilberthorpe, R. K. Poole, and S. F. Park, “Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in *Campylobacter jejuni* and *Campylobacter coli*,” *Journal of Bacteriology*, vol. 186, no. 16, pp. 5332–5341, 2004.

C. Lu, T. Egawa, L. M. Wainwright, R. K. Poole, and S. R. Yeh, “Structural and functional properties of a truncated hemoglobin from a food-borne pathogen *Campylobacter jejuni*,” *The Journal of Biological Chemistry*, vol. 282, no. 18, pp. 13627–13636, 2007.

H. K. Smith, M. Shepherd, C. Monk, J. Green, and R. K. Poole, “The NO-responsive hemoglobins of *Campylobacter jejuni*: concerted responses of two globins to NO and evidence in *vitro* for globin regulation by the transcription factor NssR,” *Nitrergic Oxidative Stress*, vol. 25, no. 2, pp. 234–241, 2011.

F. C. Fang, “Antimicrobial reactive oxygen and nitrogen species: concepts and controversies,” *Nature Reviews Microbiology*, vol. 2, no. 10, pp. 820–832, 2004.

P. Mukhopadhyay, M. Zheng, L. A. Bedzyk, R. A. LaRossia, and G. Storz, “Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 3, pp. 745–750, 2004.

D. Schnappinger, S. Ehrt, M. I. Voskuil et al., “Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment,” *Journal of Experimental Medicine*, vol. 198, no. 5, pp. 693–704, 2003.

M. C. Justino, J. B. Vicente, M. Teixeira, and L. M. Saraiva, “New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide,” *The Journal of Biological Chemistry*, vol. 280, no. 4, pp. 2636–2643, 2005.

M. P. Nittler, D. Hocking-Murray, C. K. Foo, and A. Sil, "Identification of Histoplasma capsulatum transcripts induced in response to reactive nitrogen species," *Molecular Biology of the Cell*, vol. 16, no. 10, pp. 4792–4813, 2005.

J. E. Karlinsey, J. S. Bang, L. A. Becker et al., “The NsrR regulon in nitrosative stress resistance of *Salmonella enterica* serovar Typhimurium,” *Molecular Microbiology*, vol. 85, no. 6, pp. 1179–1193, 2012.

C. M. Moore, M. M. Nakano, T. Wang, R. W. Ye, and J. D. Helmann, “Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside,” *Journal of Bacteriology*, vol. 186, no. 14, pp. 4655–4664, 2004.

J. Hemish, N. Nakaya, V. Mittal, and G. Enikolopov, “Nitric oxide activates diverse signaling pathways to regulate gene expression,” *The Journal of Biological Chemistry*, vol. 278, no. 43, pp. 42321–42329, 2003.

A. Sarver and J. DeRisi, “Fzl1p regulates an inducible response to nitrosative stress in *Saccharomyces cerevisiae*,” *Molecular Biology of the Cell*, vol. 16, no. 10, pp. 4781–4791, 2005.

E. D. Chow, O. W. Liu, S. O’Brien, and H. D. Madhani, “Exploration of whole-genome responses of the human AIDS-associated yeast pathogen *Cryptococcus neoformans* var grubii: nitric oxide stress and body temperature,” *Current Genetics*, vol. 52, no. 3–4, pp. 137–148, 2007.
p53 are regulated by distinct threshold concentrations of nitric oxide," Proceedings of the National Academy of Sciences of the United States of America, vol. 101, no. 24, pp. 8894–8899, 2004.

[303] C. A. Hundahl, H. Luuk, S. Ilmjärv et al., "Neuroglobin-deficiency exacerbates Hif1A and c-FOS response, but does not affect neuronal survival during severe hypoxia in vivo," PLoS ONE, vol. 6, no. 12, Article ID e28160, 2011.

[304] M. Iijima, H. Shimizu, Y. Tanaka, and H. Urushihara, "Identification and characterization of two flavohemoglobin genes in Dictostelium discoideum," Cell Structure and Function, vol. 25, no. 1, pp. 47–55, 2000.

[305] S. Baidya, J. W. Cary, W. S. Grayburn, and A. M. Calvo, "Role of nitric oxide and flavohemoglobin homolog genes in Aspergillus nidulans sexual development and mycotoxin production," Applied and Environmental Microbiology, vol. 77, no. 15, pp. 5524–5528, 2011.

[306] T. M. Stevanin, R. C. Read, and R. K. Poole, "The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: Links with swarming motility," Gene, vol. 398, no. 1–2, pp. 62–68, 2007.

[307] L. A. Mur, A. Sivakumaran, J. Mandon, S. M. Cristescu, F. I. Harren, and K. H. Hebelstrup, "Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens," Journal of Experimental Botany, vol. 63, no. 12, pp. 4375–4387, 2012.

[308] Y. Shimoda, M. Nagata, A. Suzuki et al., "Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in Lotus japonicus," Plant and Cell Physiology, vol. 46, no. 1, pp. 99–107, 2005.

[309] A. D. Frey, T. Koskenkorva, and P. T. Kallio, "Vitreoscilla hemoglobin promoter is not responsive to nitrosative and oxidative stress in Escherichia coli," FEMS Microbiology Letters, vol. 224, no. 1, pp. 127–132, 2003.

[310] Y. Sasaki, N. Takaya, A. Nakamura, and H. Shoun, "Isolation of flavohaemoglobin from the actinomycete Streptomyces antibioticus grown without external nitric oxide stress," Bioscience, Biotechnology and Biochemistry, vol. 68, no. 5, pp. 1106–1112, 2004.

[311] M. I. Hutchings, N. Mandhana, and S. Spiro, "The NorR protein of Escherichia coli activates expression of the flavoredoxin gene NorV in response to reactive nitrogen species," Journal of Bacteriology, vol. 184, no. 16, pp. 4640–4643, 2002.

[312] A. Pohlmann, R. Cramm, K. Schmelz, and B. Friedrich, "A novel NO-responding regulator controls the reduction of nitric oxide in Ralstonia eutropha," Molecular Microbiology, vol. 38, no. 3, pp. 626–638, 2000.

[313] B. D'Autréaux, N. P. Tucker, R. Dixon, and S. Spiro, "A non-heme iron centre in the transcription factor NorR senses nitric oxide," Nature, vol. 437, no. 7059, pp. 769–772, 2005.

[314] H. Cruz-Ramos, J. Crack, G. Wu et al., "NO sensing by FNR: regulation of the Escherichia coli NO-detoxifying flavohaemoglobin, Hmp," EMBO Journal, vol. 21, no. 13, pp. 3235–3244, 2002.

[315] B. D'Autréaux, D. Touati, B. Bersch, J. M. Latour, and I. Michaud-Soret, "Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron," Proceedings of the National Academy of Sciences of the United States of America, vol. 99, no. 26, pp. 16619–16624, 2002.

[316] M. J. Crawford and D. E. Goldberg, "Regulation of the Salmonella typhimurium flavohemoglobin gene: a new pathway for bacterial gene expression in response to nitric oxide," The Journal of Biological Chemistry, vol. 273, no. 51, pp. 34028–34032, 1998.

[317] J. Membrillo-Hernández, S. O. Kim, G. M. Cook, and R. K. Poole, "Paraquat regulation of hmp (flavohaemoglobin) gene expression in Escherichia coli K-12 is SoxRS independent but modulated by α(s)," Journal of Bacteriology, vol. 179, no. 10, pp. 3164–3170, 1997.

[318] J. Membrillo-Hernández, M. D. Coopamah, A. Channa, M. N. Hughes, and R. K. Poole, "A novel mechanism for upregulation of the Escherichia coli K-12 hmp (flavohaemoglobin) gene by the "NO releaser", S-nitrosogluthathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region," Molecular Microbiology, vol. 29, no. 4, pp. 1101–1112, 1998.

[319] S. Spiro, "Regulators of bacterial responses to nitric oxide," FEMS Microbiology Reviews, vol. 31, no. 2, pp. 193–211, 2007.

[320] H. Arai, M. Hayashi, A. Kuroi, M. Ishii, and Y. Igarashi, "Transcriptional regulation of the flavohaemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa," Journal of Bacteriology, vol. 187, no. 12, pp. 3960–3968, 2005.

[321] J. M. Baptista, M. C. Justino, A. M. P. Melo, M. Teixeira, and L. M. Saraiva, "Oxidative stress modulates the nitric oxide defense promoted by Escherichia coli flavoredoxin," Journal of Bacteriology, vol. 194, no. 14, pp. 3611–3617, 2012.

[322] D. M. Bodenmiller and S. Spiro, "The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator," Journal of Bacteriology, vol. 188, no. 3, pp. 874–881, 2006.

[323] S. Kommineni, A. Lama, B. Popescu, and M. M. Nakano, "Global transcriptional control by NorS in Bacillus subtilis," Journal of Bacteriology, vol. 194, no. 7, pp. 1679–1688, 2012.

[324] P. R. Gardner, "Aconitase: sensitive target and measure of superoxide," Methods in Enzymology, vol. 349, pp. 9–23, 2002.

[325] H. J. E. Beaumont, S. I. Lens, W. N. M. Reijnders, H. V. Westerhoff, and R. J. M. van Spanning, "Expression of nitrite reductase in Nitrosomonas europaea involves NsrR, a novel nitrite-sensitive transcription repressor," Molecular Microbiology, vol. 54, no. 1, pp. 148–158, 2004.

[326] K. L. Dickshet, R. P. Dickshet, and D. A. Webster, "Study of Vitreoscilla globin (vgb) gene expression and promoter activity in E. coli through transcriptional fusion," Nucleic Acids Research, vol. 18, no. 14, pp. 4149–4155, 1990.

[327] S. J. Kwon, S. Y. Cho, K. M. Lee, J. Yu, M. Son, and K. H. Kim, "Proteomic analysis of fungal host factors differentially expressed by Fusarium graminearum infected with Fusarium graminearum virus-DK21," Virus Research, vol. 144, no. 1-2, pp. 96–106, 2009.

[328] A. U. Igamberdiev, K. Baron, N. Manac’h-Little, M. Stoimenova, and R. D. Hill, "The haemoglobin/nitric oxide cycle: involvement in flooding stress and effects on hormone signalling," Annals of Botany, vol. 96, no. 4, pp. 557–564, 2005.

[329] R. Wang, K. Guegl, S. T. Labrie, and N. M. Crawford, "Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate," Plant Cell, vol. 12, no. 8, pp. 1491–1509, 2000.

[330] Y. Ohwaki, M. Kawagishi-Kobayashi, K. Wakasa, S. Fujihara, and T. Yoneyama, "Induction of class-I non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured..."
rice cells," Plant and Cell Physiology, vol. 46, no. 2, pp. 324–331, 2005.

[331] D. Kim, M. Hong, Y. Lee, M. Lee, and Y. Seo, "Wheat truncated hemoglobin interacts with photosystem I PSK-I subunit and photosystem II subunit PhsS1," Biologia Plantarum. In press.

[332] P. Bustos-Sanmamed, A. Tobar-Méndez, M. Crespi, S. Sato, S. Tabata, and M. Becana, "Regulation of nonsymbiotic and truncated hemoglobin genes of Lotus japonicus in plant organs and in response to nitric oxide and hormones," New Phytologist, vol. 189, no. 3, pp. 765–776, 2011.

[333] Y. He, Y. Hua, W. Liu, H. Hu, R. F. Keep, and G. Xi, "Effects of cerebral ischemia on neuronal hemoglobin," Journal of Cerebral Blood Flow and Metabolism, vol. 29, no. 3, pp. 596–605, 2009.

[334] C. L. Grek, D. A. Newton, D. D. Spyropoulos, and J. E. Baatz, "Hypoxia up-regulates expression of hemoglobin in alveolar epithelial cells," American Journal of Respiratory Cell and Molecular Biology, vol. 44, no. 4, pp. 439–447, 2011.

[335] Y. Sun, K. Jin, Y. O. Mao, Y. Zhu, and D. A. Greenberg, "Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury," Proceedings of the National Academy of Sciences of the United States of America, vol. 98, no. 26, pp. 15306–15311, 2001.

[336] N. Hümmler, C. Schneider, A. Giessl et al., "Acute hypoxia modifies regulation of neuroglobin in the neonatal mouse brain," Experimental Neurology, vol. 236, no. 1, pp. 112–121, 2012.

[337] N. Liu, Z. Yu, S. Xiang et al., "Transcriptional regulation mechanisms of hypoxia-induced neuroglobin gene expression," Biochemical Journal, vol. 443, no. 1, pp. 153–164, 2012.

[338] B. Haines, M. Demaria, X. Mao et al., "Hypoxia-inducible factor-1 and neuroglobin expression," vol. 514, no. 2, pp. 137–140, 2012.

[339] A. Ilari, A. Bonamore, A. Farina, K. A. Johnson, and A. Boffi, "The X-ray structure of ferric Escherichia coli flavohemoglobin reveals an unexpected geometry of the distal heme pocket," The Journal of Biological Chemistry, vol. 277, no. 26, pp. 23725–23732, 2002.

[340] M. Mukai, C. E. Mills, R. K. Poole, and S. R. Yeh, "Flavohemoglobin, a globin with a peroxidase-like catalytic site," The Journal of Biological Chemistry, vol. 276, no. 10, pp. 7272–7277, 2001.

[341] A. M. Gardner, L. A. Martin, P. R. Gardner, Y. Dou, and J. S. Olson, "Steady-state and transient kinetics of Escherichia coli nitric-oxide dioxygenase (flavohemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis," The Journal of Biological Chemistry, vol. 275, no. 17, pp. 12581–12589, 2000.

[342] Y. Dou, D. H. Malett, R. F. Eich, and J. S. Olson, "Myoglobin as a model system for designing heme protein based blood substitutes," Biophysical Chemistry, vol. 98, no. 1-2, pp. 127–148, 2002.

[343] M. A. Marti, A. Crespo, L. Capece et al., "Dioxygen affinity in heme proteins investigated by computer simulation," Journal of Inorganic Biochemistry, vol. 100, no. 4, pp. 761–770, 2006.

[344] P. R. Gardner, "Assay and characterization of the NO dioxygenase activity of flavohemoglobins," Methods in Enzymology, vol. 436, pp. 217–237, 2008.

[345] R. Daigle, J. A. Rousseau, M. Guertin, and P. Lagüe, "Theoretical investigations of nitric oxide channeling in Mycobacterium tuberculosis truncated hemoglobin N," Biophysical Journal, vol. 97, no. 11, pp. 2967–2977, 2009.

[346] A. Bidon-Chanal, M. A. Marti, A. Crespo et al., "Ligand-induced dynamical regulation of NO conversion in Mycobacterium tuberculosis truncated hemoglobin-N," Proteins, vol. 64, no. 2, pp. 457–464, 2006.

[347] A. Crespo, M. A. Marti, S. G. Kalko et al., "Theoretical study of the truncated hemoglobin HbN: exploring the molecular basis of the NO detoxification mechanism," Journal of the American Chemical Society, vol. 127, no. 12, pp. 4433–4444, 2005.

[348] A. Bidon-Chanal, M. A. Marti, D. A. Estrin, and F. J. Luque, "Dynamical regulation of ligand migration by a gate-opening molecular switch in truncated hemoglobin-N from Mycobacterium tuberculosis," Journal of the American Chemical Society, vol. 129, no. 21, pp. 6782–6788, 2007.

[349] P. Y. Savard, R. Daigle, S. Morin et al., "Structure and dynamics of Mycobacterium tuberculosis truncated hemoglobin N: insights from NMR spectroscopy and molecular dynamics simulations," Biochemistry, vol. 50, no. 51, pp. 11121–11130, 2011.

[350] Y. Ouellet, M. Milani, M. Couture, M. Bolognesi, and M. Guertin, "Ligand interactions in the distal heme pocket of Mycobacterium tuberculosis truncated hemoglobin N: roles of TyrB10 and GlnE11 residues," Biochemistry, vol. 45, no. 29, pp. 8770–8781, 2006.

[351] S. Mishra and M. Meuwly, "Nitric oxide dynamics in truncated hemoglobin: docking sites, migration pathways, and vibrational spectroscopy from molecular dynamics simulations," Biophysical Journal, vol. 96, no. 6, pp. 2105–2118, 2009.

[352] F. Spyarakis, S. Faggiano, S. Abbruzzetti et al., "Histidine E7 dynamics modulates ligand exchange between distal pocket and solvent in AHb1 from Arabidopsis thaliana," Journal of Physical Chemistry B, vol. 115, no. 14, pp. 4138–4146, 2011.

[353] J. J. Weiss, "Nature of the iron-oxygen bond in oxyhaemoglobin," Nature, vol. 202, no. 4927, pp. 83–84, 1964.

[354] J. B. Wittenberg, B. A. Wittenberg, J. Peisach, and W. E. Blumberg, "On the state of the iron and the nature of the ligand in oxyhemoglobin," Proceedings of the National Academy of Sciences of the United States of America, vol. 67, no. 4, pp. 1846–1853, 1970.

[355] R. E. Hui and S. P. Mada, "The reaction of NO with superoxide," Free Radical Research Communications, vol. 18, no. 4, pp. 195–199, 1993.

[356] H. P. Misra and I. Fridovich, "The generation of superoxide radical during the autoxidation of hemoglobin," The Journal of Biological Chemistry, vol. 247, no. 21, pp. 6960–6962, 1972.

[357] E. B. Jensen, "Comparative analysis of autoxidation of haemoglobin," Journal of Experimental Biology, vol. 204, no. 11, pp. 2029–2033, 2001.

[358] X. Zhao, K. Vyas, B. D. Nguyen et al., "A double mutant of sperm whale myoglobin mimics the structure and function of elephant myoglobin," The Journal of Biological Chemistry, vol. 270, no. 35, pp. 20763–20774, 1995.

[359] A. Fago, C. Hundahl, S. Dewilde, K. Gilany, L. Moens, and R. E. Weber, "Allosteric regulation and temperature dependence of oxygen binding in human hemoglobin and cytoglobin: molecular mechanisms and physiological significance," The Journal of Biological Chemistry, vol. 279, no. 43, pp. 44417–44426, 2004.

[360] A. Fago, C. Hundahl, H. Malte, and R. E. Weber, "Functional properties of neuroglobin and cytoglobin. Insights into the ancestral physiological roles of globins," IUBMB Life, vol. 56, no. 11-12, pp. 689–696, 2004.

[361] S. Goldstein, G. Merenyi, and A. Samuni, "Kinetics and mechanism of NO3 reactivity with various oxidation states of NO3 autofluorescence in the rat retina," Experimental Eye Research, vol. 77, no. 1, pp. 79–87, 2003.
myoglobin," Journal of the American Chemical Society, vol. 126, no. 48, pp. 15694–15701, 2004.

[362] E. T. Yukl, S. de Vries, and P. Moënne-Loccoz, "The millisecond intermediate in the reaction of nitric oxide with oxyhemoglobin is an iron(III)-nitrato complex, not a peroxynitrite," Journal of the American Chemical Society, vol. 131, no. 21, pp. 7234–7235, 2009.

[363] S. Herold, M. Exner, and T. Nauser, "Kinetic and mechanistic studies of the NO•-mediated oxidation of oxyhemoglobin and oxyhemoglobin," Biochemistry, vol. 40, no. 11, pp. 3385–3395, 2001.

[364] J. S. Olson, E. W. Foley, C. Rogge, A. L. Tsai, M. P. Doyle, and M. S. Navati and J. M. Friedman, "The use of glassy matricies to T. S. Kurtikyan, S. R. Eksuzyan, V. A. Hayrapetyan, G. G. J. H. Tsai, J. G. Harrison, J. C. Martine et al., "Role of pre- 

[365] M. Anbar and H. Taube, "Interaction of nitrous acid with hemoglobin with peroxynitrite, Isomerization catalyzed by His64 myoglobin mutants," Journal of the Chemical Society A, p. 90, 1969.

[366] M. Anbar and H. Taube, "Interaction of nitrous acid with hydrogen peroxide and with water," Journal of the American Chemical Society, vol. 76, no. 24, pp. 6243–6247, 1954.

[367] J. H. M. Tsai, J. G. Harrison, J. C. Martin et al., "Role of conformation of peroxynitrite anion (ONOO•) in its stability and toxicity," Journal of the American Chemical Society, vol. 116, no. 9, pp. 4115–4116, 1994.

[368] S. Herold, T. Matsui, and Y. Watanabe, "Peroxynitrite isomerization catalyzed by His64 myoglobin mutants," Journal of the American Chemical Society, vol. 123, no. 17, pp. 4085–4086, 2001.

[369] S. Herold, K. Shivasankar, and M. Mehl, "Myoglobin scavenges peroxynitrite without being significantly nitrated," Biochemistry, vol. 41, no. 45, pp. 13460–13472, 2002.

[370] J. L. Bourassa, E. P. Ives, A. L. Marqueling, R. Shimanovich, and J. T. Groves, "Myoglobin catalyzes its own nitration," Journal of the American Chemical Society, vol. 123, no. 21, pp. 5142–5143, 2001.

[371] J. Su and J. T. Groves, "Mechanisms of peroxynitrite interactions with heme proteins," Inorganic Chemistry, vol. 49, no. 14, pp. 6317–6329, 2010.

[372] J. Lee, J. A. Hunt, and J. T. Groves, "Mechanism of iron porphyrin reactions with peroxynitrite," Journal of the American Chemical Society, vol. 120, no. 30, pp. 7493–7501, 1998.

[373] R. Silaghi-Dumitrescu, "A density functional study of heme-peroxynitrite adducts," Journal of Molecular Structure: THEOCHEM, vol. 722, no. 1–3, pp. 233–237, 2005.

[374] J. Su and J. T. Groves, "Direct detection of the oxygen rebound intermediates, ferryl Mb and NO3, in the reaction of metmyoglobin with peroxynitrite," Journal of the American Chemical Society, vol. 131, no. 36, pp. 12979–12988, 2009.

[375] P. K. Wittig, A. G. Mauk, D. J. Douglas, and R. Stocker, "Reaction of human myoglobin and peroxynitrite: characterizing biomarkers for myoglobin-derived oxidative stress," Biochemical and Biophysical Research Communications, vol. 286, no. 2, pp. 352–356, 2001.

[376] N. Romero, R. Radi, E. Linares et al., "Reaction of human hemoglobin with peroxynitrite. Isomerization to nitrate and secondary formation of protein radicals," The Journal of Biological Chemistry, vol. 278, no. 45, pp. 44049–44057, 2003.

[377] A. I. Alayash, B. A. B. Ryan, and R. E. Cashon, "Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins," Archives of Biochemistry and Biophysics, vol. 349, no. 1, pp. 65–73, 1998.

[378] M. Minetti, G. Scorza, and D. Pietraforte, "Peroxynitrite induces long-lived tyrosyl radical(s) in oxyhemoglobin of red blood cells through a reaction involving CO2 and a ferryl species," Biochemistry, vol. 38, no. 7, pp. 2078–2087, 1999.

[379] M. Minetti, D. Pietraforte, V. Carbone, A. M. Salzano, G. Scorza, and G. Marino, "Scavenging of peroxynitrite by oxyhemoglobin and identification of modified globin residues," Biochemistry, vol. 39, no. 22, pp. 6689–6697, 2000.

[380] B. S. Jursic, L. Klasinc, S. Pecur, and W. A. Pryor, "On the mechanism of HOONO to HONO2 conversion," Nitric Oxide, vol. 1, no. 6, pp. 494–501, 1997.

[381] D. S. Bohle and B. Hansert, "O-atom scrambling in the aqueous isomerization of pernitrous acid," Nitric Oxide, vol. 1, no. 6, pp. 502–506, 1997.

[382] W. H. Koppenol, "The chemistry of peroxynitrite, a biological toxin," Quimica Nova, vol. 21, no. 3, pp. 326–331, 1998.

[383] H. Taube, "The production of atomic iodine in the reaction of oxymyoglobin with peroxynitrite," Journal of the American Chemical Society, vol. 54, pp. 463–497, 1906.

[384] W. Bray, "Beiträge zur Kenntnis der Halogensauerstoffverbind- ung I. und II," Zeitschrift für Physikalische Chemie, vol. 183, pp. 113–132, 1895.

[385] J. Brode, "Die Oxydation des Jodions zu Hypojodit als Zwischenstufe einiger Reaktion," Zeitschrift für Physikalische Chemie, vol. 99, pp. 208–216, 1904.

[386] M. A. Marti, A. Bidon-Chanal, A. Crespo et al., "Mechanism of product release in NO detoxification from Mycobacterium tuberculosis truncated hemoglobin N," Journal of the American Chemical Society, vol. 130, no. 5, pp. 1688–1693, 2008.

[387] A. A. Noyes and W. O. Scott, "Beitrag zur Kenntnis der Gesetze der Geschwindigkeit von polymolekularen Reaktion," Zeitschrift für Physikalische Chemie, vol. 130, no. 5, pp. 1688–1693, 1895.

[388] J. Brode, "Die Oxydation des Jodions zu Hypojodit als Zwischenstufe einiger Reaktion," Zeitschrift für Physikalische Chemie, vol. 99, pp. 208–216, 1904.

[389] B. A. Haines, D. A. Davis, A. Zykovich et al., "Comparative protein interactomics of neuroglobin and myoglobin," Journal of Neuroscience, vol. 123, no. 1, pp. 192–198, 2012.

[390] E. El Hammi, C. Houee-Levin, J. Rezac et al., "New insights into the mechanism of electron transfer within flavohemoglobins: tunnelling pathways, packing density, thermodynamic and kinetic analyses," Physical Chemistry Chemical Physics, vol. 14, no. 40, pp. 13872–13880, 2012.
[395] A. M. Gardner, S. F. Sanders, and P. R. Gardner, “The flavohemoglobin F7 lysine ammonium group is essential for electron-transfer gating and nitric-oxide dioxygenase function,” submitted.

[396] A. Hausladen, A. Gow, and J. S. Stamler, “Flavohemoglobin denitrosylase catalyzes the reaction of a nitrosyl equivalent with molecular oxygen,” Proceedings of the National Academy of Sciences of the United States of America, vol. 98, no. 18, pp. 10108–10112, 2001.

[397] A. Hausladen and J. S. Stamler, “Is the flavohemoglobin a nitric oxide dioxygenase?” Free Radical Biology and Medicine, vol. 53, no. 5, pp. 1209–1210, 2012.

[398] A. Hausladen, A. J. Gow, and J. S. Stamler, “Nitrosative stress: metabolic pathway involving the flavohemoglobin,” Proceedings of the National Academy of Sciences of the United States of America, vol. 95, no. 24, pp. 14100–14105, 1998.

[399] M. Shepherd, P. V. Bernhardt, and R. K. Poole, “Globin-mediated nitric oxide detoxification in the foodborne pathogenic bacterium Campylobacter jejuni proceeds via a dioxygenase or denitrosylase mechanism,” Nitric Oxide, vol. 25, no. 2, pp. 229–233, 2011.

[400] L. S. Nobre, V. L. Gonçalves, and L. M. Saraiva, “Flavohemoglobin of Staphylococcus aureus,” Methods in Enzymology, vol. 436, pp. 203–216, 2008.

[401] M. T. Forrester and M. W. Foster, “Response to, ‘is the flavohemoglobin a nitric oxide dioxygenase?’” Free Radical Biology and Medicine, vol. 53, pp. 1211–1212, 2012.

[402] C. E. Immoos, J. Chou, M. Bayachou, E. Blair, J. Greaves, and P. J. Farmer, “Electrocatalytic reductions of nitrite, nitric oxide, and nitrous oxide by thermophilic cytochrome P450 CYP119 in film-modified electrodes and an analytical comparison of its catalytic activities with myoglobin,” Journal of the American Chemical Society, vol. 126, no. 15, pp. 4934–4942, 2004.

[403] S. Herold and G. Röck, “Mechanistic studies of the oxygen-mediated oxidation of nitrosylhemoglobin,” Biochemistry, vol. 44, no. 16, pp. 6223–6231, 2005.

[404] A. M. Gardner and P. R. Gardner, “Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, Escherichia coli: evidence for a novel inductible anaerobic nitric oxide-scavenging activity,” The Journal of Biological Chemistry, vol. 277, no. 10, pp. 8166–8171, 2002.

[405] D. J. Kliebenstein, R. A. Monde, and R. L. Last, “Superoxide dismutase in Arabidopsis: an ecletic enzyme family with disparate regulation and protein localization,” Plant Physiology, vol. 118, no. 2, pp. 637–650, 1998.

[406] M. Couture, H. Chamberland, B. St-Pierre, J. Lafontaine, and M. Guertin, “Nuclear genes encoding chloroplast hemoglobins in the unicellular green alga Chlamydomonas eugametos,” Molecular and General Genetics, vol. 243, no. 2, pp. 185–197, 1994.

[407] R. Pathania, N. K. Navani, G. Rajamohan, and K. L. Dikshit, “Mycobacterium tuberculosis” hemoglobin HBO associates with membranes and stimulates cellular respiration of recombinant Escherichia coli,” The Journal of Biological Chemistry, vol. 277, no. 18, pp. 15293–15302, 2002.

[408] C. Liu, Y. He, and Z. Chang, “Truncated hemoglobin O of Mycobacterium tuberculosis: the oligomeric state change and the interaction with membrane components,” Biochemical and Biophysical Research Communications, vol. 316, no. 4, pp. 1163–1172, 2004.

[409] Ramandeep, K. W. Hwang, M. Raje et al., “Vitreoscilla hemoglobin: intracellular localization and binding to membranes,” The Journal of Biological Chemistry, vol. 276, no. 27, pp. 24781–24789, 2001.

[410] A. Di Giulio and A. Bonamore, “Globin interactions with lipids and membranes,” Methods in Enzymology, vol. 436, pp. 239–253, 2008.

[411] B. Ertas, L. Kiger, M. Blank, M. C. Marden, and T. Burmester, “A membrane-bound hemoglobin from gills of the green shore crab Carcinus maenas,” The Journal of Biological Chemistry, vol. 286, no. 5, pp. 3185–3193, 2011.

[412] N. Cassanova, K. M. O’Brien, B. T. Stahl, T. McClure, and R. O. Poyton, “Yeast flavohemoglobin, a nitric oxide oxidoeductase, is located in both the cytosol and the mitochondrial matrix: effects of respiration, anoxia, and the mitochondrial genome on its intracellular level and distribution,” The Journal of Biological Chemistry, vol. 280, no. 9, pp. 7645–7653, 2005.

[413] E. J. H. Ross, L. Shearman, M. Mathiesen et al., “Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types,” Protoplasma, vol. 218, no. 3–4, pp. 125–133, 2001.

[414] B. J. Reeder, D. A. Svistunenko, and M. T. Wilson, “Lipid binding to cytoglobin leads to a change in haem co-ordination: a role for cytoglobin in lipid signalling of oxidative stress,” Biochemical Journal, vol. 434, no. 3, pp. 483–492, 2011.

[415] T. Jayaraman, J. Tejero, B. B. Chen et al., “14-3-3 binding and phosphorylation of neuroglobin during hypoxia modulate six-to-five heme pocket coordination and rate of nitride reduction to nitric oxide,” The Journal of Biological Chemistry, vol. 286, no. 49, pp. 42679–42689, 2011.

[416] M. Tiso, J. Tejero, S. Basu et al., “Human neuroglobin functions as a redox-regulated nitrite reductase,” The Journal of Biological Chemistry, vol. 286, no. 20, pp. 18277–18289, 2011.

[417] D. Hamdane, L. Kiger, S. Dewilde et al., “The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin,” The Journal of Biological Chemistry, vol. 278, no. 51, pp. 51713–51721, 2003.

[418] D. Maiti, D. H. Lee, A. A. N. Sarjeant et al., “Reaction of a copper-dioxygen complex with nitrogen monoxide (•NO) leads to a copper(II)-peroxynitrite species,” Journal of the American Chemical Society, vol. 130, no. 21, pp. 6700–6701, 2008.

[419] B. R. Li, J. L. R. Anderson, C. G. Mowat, G. A. Reid, and S. K. Chapman, “Rhodobacter sphaeroides haem protein: a novel cytochrome with nitric oxide dioxygenase activity,” Biochemical Society Transactions, vol. 36, no. 5, pp. 992–995, 2008.

[420] C. N. Hall and J. Garthwaite, “Inactivation of nitric oxide by rat cerebellar slices,” Journal of Physiology, vol. 577, no. 2, pp. 549–567, 2006.

[421] C. N. Hall, R. G. Keynes, and J. Garthwaite, “Cytochrome P450 oxidoeductase participates in nitric oxide consumption by rat brain,” Biochemical Journal, vol. 419, no. 2, pp. 411–418, 2009.

[422] U. N. Westfelt, G. Benthin, S. Lundin, O. Stenqvist, and Å. Wennmalm, “Conversion of inhaled nitric oxide to nitrate in man,” British Journal of Pharmacology, vol. 114, no. 8, pp. 1621–1624, 1995.

[423] C. K. Hallstrom, A. M. Gardner, and P. R. Gardner, “Nitric oxide metabolism in mammalian cells: substrate and inhibitor profiles of a NADPH-cytochrome P450 oxidoeductase-coupled microsomal nitric oxide dioxygenase,” Free Radical Biology and Medicine, vol. 37, no. 2, pp. 216–228, 2004.
[424] K. Schmidt and B. Mayer, “Consumption of nitric oxide by endothelial cells: evidence for the involvement of a NAD(P)H-, flavin- and heme-dependent dioxygenase reaction,” FEBS Letters, vol. 577, no. 1-2, pp. 199–204, 2004.

[425] M. A. Robinson, J. E. Baumgardner, V. P. Good, and C. M. Otto, “Physiological and hypoxic O2 tensions rapidly regulate NO production by stimulated macrophages,” American Journal of Physiology, vol. 294, no. 4, pp. C1079–C1087, 2008.

[426] X. Liu, C. Cheng, N. Zorko, S. Cronin, Y. R. Chen, and J. L. Sweer, “Biophysical modulation of vascular nitric oxide catabolism by oxygen,” American Journal of Physiology, vol. 287, no. 6, pp. H2421–H2426, 2004.

[427] X. Liu, P. Srinivasan, E. Collard et al., “Oxygen regulates the effective diffusion distance of nitric oxide in the aortic wall,” Free Radical Biology and Medicine, vol. 48, no. 4, pp. 554–559, 2010.

[428] M. Palacios-Callender, V. Hollis, M. Mitchison, N. Frakich, X. Liu, P. Srinivasan, E. Collard et al., “Oxygen regulates the effective diffusion distance of nitric oxide in the aortic wall,” Free Radical Biology and Medicine, vol. 48, no. 4, pp. 554–559, 2010.

[429] Y. R. Chen, C. L. Chen, A. Yeh, X. Liu, and J. L. Sweer, “Direct and indirect roles of cytochrome b in the mediation of superoxide generation and NO catabolism by mitochondrial succinate-cytochrome c reductase,” The Journal of Biological Chemistry, vol. 281, no. 19, pp. 13159–13168, 2006.

[430] A. U. Igamberdiev, N. V. Bykova, W. Ens, and R. D. Hill, “Dihydrolipoamide dehydrogenase from porcine heart catalyzes NADH-dependent scavenging of nitric oxide,” FEBS Letters, vol. 568, no. 1–3, pp. 146–150, 2004.

[431] R. Gaupp, N. Ledala, and G. A. Somerville, “Staphylococcal response to oxidative stress,” Frontiers in Cellular and Infection Microbiology, vol. 2, article 33, 2012.

[432] S. Herold and K. Shivashankar, “Metmyoglobin and methemoglobin catalyze the isomerization of peroxynitrite to nitrate,” Biochemistry, vol. 42, no. 47, pp. 14036–14046, 2003.

[433] M. T. Gladwin, “Hemoglobin as a nitrite reductase regulating red cell-dependent hypoxic vasodilation,” American Journal of Respiratory and Critical Medicine, vol. 32, no. 5, pp. 363–366, 2005.

[434] Z. Huang, S. Shiva, D. B. Kim-Shapiro et al., “Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control,” Journal of Clinical Investigation, vol. 115, no. 8, pp. 2099–2107, 2005.

[435] S. Shiva, Z. Huang, R. Grubina et al., “Deoxyhemoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration,” Circulation Research, vol. 100, no. 5, pp. 654–661, 2007.

[436] T. Rassaf, U. Flögel, C. Drezhage, U. Hendgen-Cotta, M. Kelm, and J. Schrader, “Nitrite reductase function of deoxyhemoglobin: oxygen sensor and regulator of cardiac energetics and function,” Circulation Research, vol. 100, no. 12, pp. 1749–1754, 2007.

[437] M. G. Petersen, S. Dewilde, and A. Fago, “Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions,” Journal of Inorganic Biochemistry, vol. 102, no. 9, pp. 1777–1782, 2008.

[438] H. Li, C. Hemann, T. M. Abdelghany, M. A. El-Mahdy, and J. L. Zweier, “Characterization of the mechanism and magnitude of cytooglobin-mediated nitrite reduction and nitric oxide generation under anaerobic conditions,” The Journal of Biological Chemistry, vol. 287, no. 43, pp. 36623–36633, 2012.

[439] R. Sturms, A. A. Dispirito, and M. S. Hargrove, “Plant and cyanobacterial hemoglobins reduce nitrite to nitric oxide under anoxic conditions,” Biochemistry, vol. 50, no. 19, pp. 3873–3878, 2011.

[440] M. Tiso, J. Tejero, C. Kenney, S. Frizzell, and M. T. Gladwin, “Nitrite reductase activity of nonsymbiotic hemoglobins from Arabidopsis thaliana,” Biochemistry, vol. 51, no. 26, pp. 5285–5292, 2012.

[441] A. Sakamoto, S. H. Sakurao, K. Fukunaga et al., “Three distinct Arabidopsis hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration,” FEBS Letters, vol. 572, no. 1–3, pp. 27–32, 2004.

[442] S. Nicolis, E. Monzani, C. Ciaccio, P. Ascenzi, L. Moens, and L. Casella, “Reactivity and endogenous modification by nitrite and hydrogen peroxide: does human neuroglobin act only as a scavenger?” Biochemical Journal, vol. 407, no. 1, pp. 89–99, 2007.

[443] M. Otsuka, S. A. Marks, D. E. Winnica, A. A. Amoscato, L. L. Pearce, and J. Peterson, “Covalent modifications of hemoglobin by nitrite anion: formation kinetics and properties of nitrihemoglobin,” Chemical Research in Toxicology, vol. 23, no. 11, pp. 1786–1795, 2010.

[444] K. J. Gupta, A. U. Igamberdiev, G. Manjunatha et al., “The emerging roles of nitric oxide (NO) in plant mitochondria,” Plant Science, vol. 181, no. 5, pp. 520–526, 2011.

[445] R. Sturms, A. A. DiSpirito, D. B. Fulton, and M. S. Hargrove, “Hydroxylamine reduction to ammonium by plant and cyanobacterial hemoglobins,” Biochemistry, vol. 50, no. 50, pp. 10829–10835, 2011.

[446] A. Bonamore, P. Gentili, A. Illari, M. E. Schinini, and A. Boffi, “Escherichia coli flavohemoglobin is an efficient alkylhydroperoxide reductase,” The Journal of Biological Chemistry, vol. 278, no. 25, pp. 22272–22277, 2003.

[447] G. Wu, H. Corker, Y. Orri, and R. K. Poole, “Escherichia coli Hmp, an “oxygen-binding flavohaemoprotein”, produces superoxide anion and self-destructs,” Archives of Microbiology, vol. 182, no. 2–3, pp. 193–203, 2004.

[448] N. Kawada, D. B. Kristensen, K. Asahina et al., “Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells,” The Journal of Biological Chemistry, vol. 276, no. 27, pp. 25318–25323, 2001.

[449] E. Fordel, L. Thijs, W. Martinet, D. Schrijvers, L. Moens, and S. Dewilde, “Anoxia or oxygen and glucose deprivation in SH-SY5Y cells: a step closer to the unraveling of neuroglobin and cytoglobin functions,” Gene, vol. 398, no. 1-2, pp. 114–122, 2007.

[450] F. Trandaifar, D. Hoogewijs, F. Altieri et al., “Neuroglobin and cytoglobin as potential enzyme or substrate,” Gene, vol. 398, no. 1-2, pp. 103–113, 2007.

[451] F. E. McDonald, J. M. Risk, and N. J. Hodges, “Protection from intracellular oxidative stress by cytoglobin in normal and cancerous oesophageal cells,” PLoS ONE, vol. 7, no. 2, Article ID e30587, 2012.

[452] E. Fordel, L. Thijs, L. Moens, and S. Dewilde, “Neuroglobin and cytoglobin expression in mice: evidence for a correlation with reactive oxygen species scavenging,” FEBS Journal, vol. 274, no. 5, pp. 1312–1317, 2007.

[453] K. G. Paul, “ Peroxidases: past and present,” Journal of Oral Pathology, vol. 16, no. 8, pp. 409–411, 1987.

[454] Y. P. Chen, S. A. Woodin, D. E. Lincoln, and C. R. Lovell, “An unusual dehalogenating peroxidase from the marine terebellid
polychaete *Amphitrite ornata,* *The Journal of Biological Chemistry,* vol. 271, no. 9, pp. 4609–4612, 1996.

[455] H. Yannin, P. D. Butcher, J. A. Mangan, M. A. Rajandream, and A. R. M. Coates, “Regulation of hmp gene transcription in *Mycobacterium tuberculosis*: effects of oxygen limitation and nitrosative and oxidative stress,” *Journal of Bacteriology,* vol. 181, no. 11, pp. 3486–3493, 1999.

[456] K. B. Hota, S. K. Hota, R. B. Srivastava, and S. B. Singh, “Nitroglobin regulates hypoxic response of neuronal cells through Hif-1α and Nrf2-mediated mechanism,” *Journal of Cerebral Blood Flow and Metabolism,* vol. 32, no. 6, pp. 1046–1060, 2012.

[457] Y. Liu and P. R. Ortiz de Montellano, “Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1,” *The Journal of Biological Chemistry,* vol. 275, no. 8, pp. 5297–5307, 2000.

[458] L. R. Kump, “The rise of atmospheric oxygen,” *Nature,* vol. 451, no. 7176, pp. 277–278, 2008.

[459] T. A. K. Freitas, J. A. Saito, S. Hou, and M. Alam, “Globin-coupled sensors, protoglobins, and the last universal common ancestor,” *Journal of Inorganic Biochemistry,* vol. 99, no. 1, pp. 23–33, 2005.

[460] W. Han, J. R. Tuckerman, J. A. Saito et al., “Globin synthesizes the second messenger bis-(3′-5′)-cyclic diguanylic monophosphate in bacteria,” *Journal of Molecular Biology,* vol. 388, no. 2, pp. 262–270, 2009.

[461] K. Wakasugi, T. Nakano, and I. Morishima, “Oxidized human neuroglobin acts as a heterotrimeric G protein guanine nucleotide dissociation inhibitor,” *The Journal of Biological Chemistry,* vol. 278, no. 38, pp. 36505–36512, 2003.

[462] K. Wakasugi and I. Morishima, “Identification of residues in human neuroglobin crucial for guanine nucleotide dissociation inhibitor activity,” *Biochemistry,* vol. 44, no. 8, pp. 2943–2948, 2005.

[463] L. Thijs, E. Vinck, A. Bolli et al., “Characterization of a globin-coupled oxygen sensor with a gene-regulating function,” *The Journal of Biological Chemistry,* vol. 282, no. 52, pp. 37325–37340, 2007.

[464] J. MacMicking, Q. W. Xie, and C. Nathan, “Nitric oxide and macrophage function,” *Annual Review of Immunology,* vol. 15, pp. 323–350, 1997.

[465] M. U. Shiloh and C. F. Nathan, “Reactive nitrogen intermediates and the pathogenesis of *Salmonella* and *Mycobacteria,*” *Current Opinion in Microbiology,* vol. 3, no. 1, pp. 35–42, 2000.

[466] C. T. Privalle, *Methods for the Synthesis of a Modified Hemoglobin Solution,* APEX Bioscience, US, European Patent Office, 2010.

[467] R. P. Gardner, *Nitric Oxide Dioxygenase Inhibitors,* Cincinnati Children’s Hospital Medical Center, US, United States Patent Application Publication, 2008.

[468] G. Bauer, *Method for Inducing Tumor Apoptosis by Increasing Nitric Oxide Levels,* Universitätsklinik Freiburg, DE. United States Patent Application Publication, 2009.

[469] R. A. Helmick, A. E. Fletcher, A. M. Gardner et al., “Imidazole antibiotics inhibit the nitric oxide dioxygenase function of microbial flavohemoglobin,” *Antimicrobial Agents and Chemotherapy,* vol. 49, no. 5, pp. 1837–1843, 2005.

[470] E. El Hammi, E. Warkentin, U. Demmer et al., “Structure of *Ralstonia eutropha* flavohemoglobin in complex with three antibiotic azole compounds,” *Biochemistry,* vol. 50, no. 7, pp. 1255–1264, 2011.

[471] L. S. Nobre, S. Todorovic, A. F. N. Tavares et al., “Binding of azole antibiotics to *Staphylococcus aureus* flavohemoglobin increases intracellular oxidative stress,” *Journal of Bacteriology,* vol. 192, no. 6, pp. 1527–1533, 2010.

[472] A. Basra, M. Edgerton, G. J. Lee et al., *Plants Containing Heterologous Flavohemoglobin Gene and Methods of Use Thereof,* Monsanto Company, US, United States Patent Application Publication, 2012.

[473] H. Kahrman, E. Aytan, and A. G. Kurt, “Production of methionine g-lyase in recombinant *Citrobacter freundii* bearing the hemoglobin gene,” *BMB Reports,* vol. 44, pp. 590–594, 2011.

[474] X. Wang, Y. Sun, X. Shen et al., “Intracellular expression of *Vitreoscilla* hemoglobin improves production of *Yarrowia lipolytica* lipase LIP2 in a recombinant *Pichia pastoris,*” *Enzyme and Microbial Technology,* vol. 50, no. 1, pp. 22–28, 2012.

[475] T. Sanny, M. Arnaldos, S. A. Kunkel, K. R. Pagilla, and B. C. Stark, “Engineering of ethanolic *E. coli* with the *Vitreoscilla* hemoglobin gene enhances ethanol production from both glucose and xylene,” *Applied Microbiology and Biotechnology,* vol. 88, no. 5, pp. 1103–1112, 2010.

[476] X. Xu, Z. Rao, H. Xu et al., “Enhanced production of L-arginine by expression of *Vitreoscilla* hemoglobin using a novel expression system in *Corynebacterium crenatum,*” *Applied Biochemistry and Biotechnology,* vol. 163, no. 6, pp. 707–719, 2011.

[477] H. Zhu, S. Sun, and S. Zhang, “Enhanced production of total flavones and exopolysaccharides via *Vitreoscilla* hemoglobin biosynthesis in *Phellinus igniarius,*” *Bioresource Technology,* vol. 102, no. 2, pp. 1747–1751, 2011.

[478] X. C. Wu, Y. M. Chen, D. LiY et al., “Constitutive expression of *Vitreoscilla* haemoglobin in *Sphingomonas elodea* to improve gellan gum production,” *Journal of Applied Microbiology,* vol. 110, no. 2, pp. 422–430, 2011.

[479] T. E. Pablos, E. M. Mora, S. Le Borgne, O. T. Ramirez, G. Gosset, and A. R. Lara, “*Vitreoscilla* hemoglobin expression in engineered *Escherichia coli*: improved performance in high cell-density batch cultivations,” *Biotecnology Journal,* vol. 6, no. 8, pp. 993–1002, 2011.

[480] J. Shen, H. Zheng, X. Zhi et al., “Improvement of amorpha-4,11-diene production by a yeast-conform variant of *Vitreoscilla* hemoglobin,” *Zeitschrift für Naturforschung C,* vol. 67, no. 3–4, pp. 195–207, 2012.

[481] Y. Luo, X. Kou, X. Ding et al., “Promotion of spinosad biosynthesis by chromosomal integration of the *Vitreoscilla* hemoglobin gene in *Saccharopolyspora spinosa,*” *Science China Life Sciences,* vol. 55, no. 2, pp. 172–180, 2012.

[482] J. J. Stewart and K. J. Coyne, *Novel Nitrate Reductase Fusion Proteins and Uses Thereof,* University of Delaware, US, United States Patent Application Publication, 2012.

[483] M. T. Forrester, C. E. Eyler, and J. N. Rich, “Bacterial flavohemoglobin: a molecular tool to probe mammalian nitric oxide biology,” *Biotechniques,* vol. 50, no. 1, pp. 41–45, 2011.

[484] C. Seregélyes, B. Barna, J. Hennig et al., “Phytohgblobins can interfere with nitric oxide functions during plant growth and pathogenic responses: a transgenic approach,” *Plant Science,* vol. 165, no. 3, pp. 541–550, 2003.

[485] T. E. Mishina, C. Lamb, and J. Zeier, “Expression of a nitric oxide degrading enzyme induces a senescence programme in *Arabidopsis,*” *Plant, Cell and Environment,* vol. 30, no. 1, pp. 39–52, 2007.

[486] F. L. Hämefeld, *Der Chemismus in der tierischen Organisation,* F. A. Brockhaus, Leipzig, Germany, 1840.