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Oxygen Sensing Strategies in Mammals and Bacteria

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Abstract

The ability to sense and adapt to changes in pO_2 is crucial for basic metabolism in most organisms, leading to elaborate pathways for sensing hypoxia (low pO_2). This review focuses on the mechanisms utilized by mammals and bacteria to sense hypoxia. While responses to acute hypoxia in mammalian tissues lead to altered vascular tension, the molecular mechanism of signal transduction is not well understood. In contrast, chronic hypoxia evokes cellular responses that lead to transcriptional changes mediated by the hypoxia inducible factor (HIF), which is directly controlled by post-translational hydroxylation of HIF by the non-heme Fe(II)/αKG-dependent enzymes FIH and PHD2. Research on PHD2 and FIH is focused on developing inhibitors and understanding the links between HIF binding and the O_2 reaction in these enzymes. Sulfur speciation is a putative mechanism for acute O_2-sensing, with special focus on the role of H_2S. This sulfur-centered model is discussed, as are some of the directions for further refinement of this model. In contrast to mammals, bacterial O_2-sensing relies on protein cofactors that either bind O_2 or oxidatively decompose. The sensing modality for bacterial O_2-sensors is either via altered DNA binding affinity of the sensory protein, or else due to the actions of a two-component signaling cascade. Emerging data suggests that proteins containing a hemerythrin-domain, such as FBXL5, may serve to connect iron sensing to O_2-sensing in both bacteria and humans. As specific molecular machinery becomes identified, these hypoxia sensing pathways present therapeutic targets for diseases including ischemia, cancer, or bacterial infection.

Keywords

HIF; hypoxia; oxygen sensing; cysteine; FNR; FixL

1. Introduction

Oxygen is the terminal electron acceptor used during aerobic respiration, making it essential for the survival of nearly all plants, animals, and single-celled organisms. During respiration, the reduction of O_2 into H_2O is coupled to the production of cellular energy in the form of ATP. Too little oxygen results in inefficient respiration and a switchover to

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anaerobic metabolism or cell death if the proper adaptive response is not elicited. Conversely, too much oxygen can lead to oxidative stress and the production of reactive oxygen species (ROS) that can damage the cellular machinery and cause cellular death. As a result of the necessity to regulate O_2 availability, organisms have evolved complex and elegant systems for sensing and maintaining O_2 homeostasis. O_2 sensing pathways typically utilize metalloproteins to either reversibly bind O_2 or to catalyze irreversible O_2-dependent reactions, leading to transcriptional and metabolic changes in response to varied pO_2. With few exceptions, disparate O_2-sensing pathways are utilized by mammals and bacteria, leading us to treat O_2-sensing in these organisms separately in this review.

The atmosphere is composed of ~ 21 % oxygen, leading to an appreciable concentration of dissolved O_2 in water under ambient conditions (288 μM at 20 °C)[1]. However, due to the limits of diffusion and metabolic demands, intracellular O_2 levels are often less than 10% of this ambient value in mammalian tissues. There can be further variation according to the tissue, cell type, and developmental stage of the mammal[2]. It's estimated that O_2 concentrations are ~5% of the ambient level in typical tissues from adults [3], which leads to a number of finely tuned responses to variations over a narrow range of pO_2. Like mammals, bacteria must also adapt to changes in $[O_2]$ (or, equivalently, pO_2) in order to maintain proper cellular function. As many bacteria are facultative anaerobes and their entire metabolism can change in response to the availability of terminal electron acceptors, in many ways bacterial responses are more extreme than those of mammals. Furthermore, pathogenic bacteria must distinguish changes in pO_2 from changes in ROS, as they experience wide variations in pO_2 as well as large pulses of ROS due to host immune response.

Biological literature typically refers to pO_2 sensing from the perspective of a norm in which $[O_2]$ is sufficient – making detection a matter of sensing O_2 deficiency, or hypoxia. Importantly, hypoxia has a significant effect on the pathogenesis of many human diseases, including chronic heart and lung disease, myocardial ischemia, pulmonary hypertension, and cancer, all of which are major contributors to the mortality rate worldwide [3-7]. Similarly, due to the important role of O_2 in bacterial metabolism, bacterial hypoxia sensing plays a significant role in bacterial proliferation. Having an understanding of the different strategies and cellular mechanisms to sense and regulate pO_2 can help improve therapeutic development targeting these pathways, either for treating diseases related to poor oxygenation or for combating pathogens.

2.1 Acute Hypoxia Sensing by Mammalian Tissue

Mammals follow distinct sensing strategies for acute hypoxia (time scale of minutes) and chronic hypoxia (time scale of hours to days). Acute hypoxia sensing starts at the tissue level with specialized cells responding to changes in pO_2 through the effects of potassium and calcium ion channels on the membrane potential of the cells [2, 8, 9]. The two dominant physiological responses to acute hypoxia are hypoxic systemic vasodilation and hypoxic pulmonary vasoconstriction, which change vascular tension to modify blood flow. Vasodilation is a response that increases the perfusion of blood to the affected tissue while vasoconstriction is a regulatory mechanism that balances perfusion to ventilation. Although

these acute responses are crucial for tissues experiencing hypoxia, and are thought to be mediated within the cell by ion channel signaling pathways, the specific molecular mechanisms linking changes in pO_2 to ion channel activity are not well understood.

2.1.1 Neuroepithelial bodies (NEBs) and type 1 glomus cells—The first step in acute hypoxia response is facilitated by specific chemosensory cells termed neuroepithelial bodies (NEBs), and type 1 glomus cells of the carotid body. NEBs are the first responders to pO_2 as they line the mucosa of mammalian airways. NADPH oxidase (NOX) has been experimentally demonstrated to mediate the hypoxic response in NEBs via ROS generation [10]. Activation of NOX by protein kinase C (PKC) leads to superoxide (O_2^-) production which may be the signal to close K^+ channels. Counter intuitively, hypoxia results in increased production of O_2^- by NOX [11]. Whether the chemical signal is O_2^- or H_2O_2 is ambiguous as O_2^- can dismutate to H_2O_2 and direct addition of H_2O_2 boosts the whole cell K^+ channel current in NEBs (Fig 1.) [12]. The shift in membrane potential due to K^+ channel closure causes an influx of Ca^{2+}, triggering neurotransmitter release (serotonin and neuropeptides) from NEBs (Fig. 2) [13]. Although activation of NOX by PKC led to hypoxia sensitive K^+ channel closure in the immortalized model NEB H146 cell line [14] NOX inhibitors failed to fully suppress this response, suggesting that multiple mechanisms may be utilized by these cells to sense O_2 [15].

Type 1 glomus cells are specialized chemoreceptor cells belonging to the carotid body, and are similar to NEBs. In type 1 glomus cells, hypoxia results in closure of K^+ channels, which depolarizes the cellular membrane. The resultant membrane depolarization triggers an influx of Ca^{2+} and the release of neurotransmitters (dopamine) to the carotid-sinus nerve that relays the information to the central nervous system and stimulates ventilation (Fig. 2) [9, 16]. In contrast to the role NOX plays in NEBs, NOX suppresses K^+ channel closure in type 1 glomus cells [11, 17], indicating that the mechanisms for O_2-sensing is subtly tweaked from tissue to tissue. As the same ion channel types can be either potentiated or inhibited depending on cell types, this would allow individual tissues to fine tune the hypoxic response.

2.1.2 Smooth muscle cells (SMCs)—SMCs work in concert with NEBs and type 1 glomus cells to facilitate tissue-specific responses to acute hypoxia. In pulmonary artery SMCs, acute hypoxia closes the K^+ channels which leads to an increase in intracellular [Ca^{2+}], triggering neurotransmitter release and vasoconstriction [18]. In contrast, the hypoxic response in many other tissues is vasodilation due to the function of systemic SMCs. In systemic SMCs, hypoxia causes ATP sensitive K^+ (K_{ATP}) channels to open and hyperpolarize the membrane, inhibiting Ca^{2+} influx and causing muscle cell relaxation [18, 19]. These findings and the observation that inhibiting oxidative phosphorylation drastically influences O_2 sensing in NEBs and SMCs led to the model that the energy demands of the cell ultimately govern the response to hypoxia, however the scientific literature contains conflicting data regarding this model. For example, myocite relaxation can occur at pO_2 levels that don't compromise energy metabolism, suggesting that decreased energy metabolism is not a requirement for hypoxia sensing by ion channels [8]. For a more in

depth discussion of this evidence the reader is directed to more focused reviews on that subject [2, 8].

Adaptation to hypoxia may involve vasodilation or vasoconstriction, depending on the affected tissue; however, both result from changes in membrane channels that cause myocite contraction or relaxation. In this manner, adaptation to acute hypoxia relies on a carefully orchestrated response that involves neuroepithelial cells, type 1 glomus cells, and smooth muscle cells. The cellular machinery that play a role in the acute hypoxic response are just now emerging and understanding of the molecular details awaits further research.

2.1.3 Heme oxygenase (HO)—One challenge in this area of study is distinguishing O_2-sensing processes from those that merely consume O_2. An excellent example of this is HO, an O_2-consuming enzyme. One isoform of HO (HO-2) has been proposed to sense hypoxia, based on data from genetic knockouts in mice, but the literature is conflicting. Heme oxygenase oxidatively cleaves heme into CO and biliverdin, consuming O_2 and NADPH [20], however this does not necessarily mean that HO senses O_2.

There are three isoforms of HO identified of which the first two have been well characterized and reported to have a high affinity for O_2 ($P_{50(O2)}$ values: HO-1 = 0.036 μM , HO-2 = 0.013 μM) [2, 21]. HO-1 is an inducible heme oxygenase that is expressed in response to stresses such as hypoxia [22], whereas HO-2 is constitutively expressed and is proposed to modulate Ca^{2+} activated K^+ channels in the membranes of glomus cells[23]. While the extremely low $P_{50(O2)}$ of HO-1 and -2 indicates that these enzymes will be saturated with respect to [O_2] over the physiological range of O_2, and incapable of a response proportionate to changes in pO_2, HO-2 has intriguing connections to cellular hypoxia responses.

HO-2 was proposed to respond to hypoxia due to the HO-2 produced CO opening K^+ channels [23]. This model requires that hypoxia leads to decreased [CO], thereby closing the K^+ channels and leading to Ca^{2+} influx and neurotransmitter release [24]. While the very low $P_{50(O2)}$ value for HO-2 appears to eliminate a direct link between CO production and physiologically relevant changes in [O_2], a number of observations suggest a connection between HO-2 and responses to hypoxia. At the organismal level, HO-2 deficient mice were reported to be hypoxaemic (decreased pO_2 in the blood) and exhibited signs of vasoconstriction activity [25]. Cell-based assays showed that both HO-2 activity and CO could open K^+ channels, implicating HO-2 in the O_2 sensing pathway for pulmonary artery SMCs [25, 26]. In contrast, other cell-based assays produced conflicting data on the role of HO-2 in glomus cells [25, 27], which may be due to the use of cells from different species in these studies. These findings underscore the need to resolve the mechanisms linking hypoxia to ion channel function, in order to have a more comprehensive understanding of O_2 sensing in mammalian tissue.

An important aspect of acute hypoxia sensing that remains elusive is a molecular view connecting decreased pO_2 with K^+ channel activity. The proposals described above are further muddied by patch-clamp experiments indicating that K^+ ion channels may sense O_2 *directly* as there is an absence of detectable modifications to the cytosolic environment, such

as changes in pH, ATP levels or [Ca^{2+}] [8]. Although NOX regulates ion channels [10-12], the specific molecular players connecting NOX to K^+ channels remain to be clarified. Whether CO, O_2^-, or the recently proposed H_2S (see below), the chemistry underneath acute hypoxia sensing promises to be a fertile field for investigation.

2.2 H_2S as an O_2 Sensor

A recent proposal for hypoxia sensing in higher organisms is that hydrogen sulfide (H_2S), or some other sulfur species, is the direct sensor for acute hypoxia in many tissues of higher organisms [28, 29]. While controversial, there are compelling correlations between O_2 and H_2S biochemistry, suggesting a connection between these gases. H_2S elicits responses similar to those caused by hypoxia in many tissues [28], and the molecular players are more fully identified than for the CO and O_2^- models discussed above. The key features of this hypothesis are: the O_2-sensitive speciation of sulfur into reduced and oxidized pools to signal changes in pO_2; and the transduction of this signal by an unknown mechanism into cellular responses to hypoxia.

At a very basic level, the speciation of sulfur into reduced (H_2S) and oxidized (SO_x) pools depends on the availability of O_2, leading to a correlation between hypoxia and elevated [H_2S] within cells [30]. While a simplified view suggests that this is due to the balance between the cytosolic metabolism of S-containing compounds to produce H_2S and the mitochondrial oxidation of H_2S to SSO_3^{2-} and SO_4^{2-} (Fig. 3), the story is somewhat more complex. In particular, the distribution of various enzymes involved in sulfur metabolism may be more varied than previously thought. As oxidation to form SSO_3^{2-} and SO_4^{2-} are slowed under conditions of low pO_2, the reduced sulfur pool increases under hypoxic conditions. But other factors, such as H_2S consumption by ROS [31-33] and H_2S production promoted by elevated glutathione levels [34] indicate that H_2S levels do not respond solely to changes in pO_2. This interplay between various redox pools, pO_2 and [H_2S], combined with the challenges in measuring different sulfur species [31, 35, 36], makes it difficult to establish a clear causal link between hypoxia and elevated levels of reduced sulfur species.

A simplified view of the production of H_2S centers on the transsulfuration pathway and on cysteine catabolism [31, 32, 37, 38]. In the transsulfuration pathway, H_2S is liberated from cysteine, homocysteine, and cystathionine by the PLP-dependent enzymes cystathione β-synthase (CBS) and cystathione γ-lyase (CSE), which are typically cytosolic enzymes [39]. However, data suggests that CBS and CSE translocate to mitochondria under cellular stress, which may account for cysteine metabolism within the mitochondria [40, 41]. H_2S is also produced from Cys by the sequential action of the enzymes cysteine aminotransferase (CAT), which uses αKG as a co-substrate, and mercaptopyruvate sulfotransferase (MST); while CAT and MST are predominantly cytosolic, MST is also found within the mitochondrion [42]. Whereas oxidation of mitochondrial H_2S to SO_4^{2-} leads to excretion, the fate of cytosolic H_2S is less clear (Fig. 3). Connections between the metabolism of glutathione (GSH), cysteine, and H_2S (as reviewed by Gojon [31]), imply that cytosolic H_2S equivalents are fed into the mitochondria for oxidation. Indeed, it was recently proposed that H_2S oxidation rather than H_2S production is the crucial step in hypoxia sensing [43].

Mitochondrial enzymes oxidize H_2S within the mitochondrial matrix, connecting H_2S catabolism to respiration through the quinone pool in the inner membrane [37]. The membrane enzyme sulfide quino-oxidoreductase (SQR) oxidizes S^{2-} to S^0 using the quinone pool, transferring the S^0 equivalent to glutathione to form a persulfide (GSSH) and sending electrons into the respiratory redox chain via catechols. The S^0 equivalent is then oxidized to the S^{4+} oxidation state (sulfite) by the enzyme persulfide dioxygenase (ETHE1) [37, 38, 44] with subsequent conversion either by sulfite oxidase to sulfate, or by rhodanese, which combines a S^0 equivalent (RSSH) with sulfite to form thiosulfate (SSO_3^{2-}). The non-enzymatic reduction of thiosulfate by GSH has been shown to rapidly produce H_2S under hypoxic conditions using purified reagents or mammalian tissues, suggesting that this may be a reaction that will rapidly increase the H_2S level under acute hypoxia [34].

Cells can both produce and consume H_2S in proportion to varied pO_2, with GSH, thiosulfate, and sulfite as key players in H_2S metabolism. Notably, the rate of H_2S consumption decreases when $[O_2]$ drops below ~ 20 μM in bovine tissues [30], suggesting that acute hypoxia leads to elevated H_2S. Thiosulfate has been proposed to serve as a pool for the rapid reductive release of H_2S by reducing agents such as glutathione (GSH), which could lead to the production of H_2S under hypoxic conditions [34]. The mitochondrial enzyme persulfide dioxygenase (ETHE1) catalyzes a key O_2-consuming step in the catabolism of H_2S, producing sulfite [44]. Precisely how the concentrations of these molecular species vary with pO_2 is crucial for the H_2S-centered model for acute hypoxia sensing. Intriguingly, ETHE1 is a non-heme Fe(II)-dependent oxygenase [44] which binds to the Fe(II) cofactor using a His_2Asp facial triad; this is similar to the cofactor structure for the well-established hypoxia sensors factor inhibiting HIF (FIH) and prolyl hydroxylase (PHD), which control the transcriptional activity of HIF, the hypoxia inducible factor (see below). If H_2S is a transient hypoxia sensor, it would be a remarkable development if yet another non-heme Fe(II) dioxygenase were to sense O_2 in cells. This, however, remains an open question at present.

Exogenously administered H_2S (or N-acetyl cysteine) mimics the hypoxic response in a variety of tissues, such as vasoconstriction or vasodilation depending on the organism [30]. However, the responses to elevated $[H_2S]$ and low $[O_2]$ may be complementary rather than identical, with much cross-talk between the two stressors [32]. While it has been asserted that this indicates that H_2S and hypoxia are mediated by the same effector pathways within cells [28], the molecular pathways linking H_2S to tissue responses have not yet been identified for testing. An intriguing study from 2011 found that, while HIF-1 was required for the transcriptional effects of chronic H_2S in *C. elegans*, the identity of genes regulated by chronic H_2S administration were distinct from those regulated by hypoxia [45]. It is possible that better discrimination of responses to acute hypoxia from those due to chronic hypoxia would help to clarify the specific effector pathways induced by H_2S.

Although the proposal that H_2S levels report on acute hypoxia is consistent with a number of correlations, the molecular basis for signal transduction is unclear. Two key questions moving forward are: How is sulfur speciation transduced as a signal? How does the mitochondrial sulfur pool connect with the cytosolic sulfur pool? It has been suggested that post-translational protein modification via sulfhydration is the molecular basis for signaling

increased [H_2S] [46]. In sulfhydration, a protein-bound persulfide (R-SSH) is formed by one of several possible mechanisms [31, 47]. There is an oxidant-promoted reaction (RSH + H_2S $\rightarrow$ RSSH + 2 H^+ + 2 e-), or sulfane transfer from an endogenous persulfide (GSSH + RSH $\rightarrow$ GSH + RSSH). Notably, this signaling mode suggests that sulfane sulfur (S^0) may be the sulfur signaling agent rather than H_2S [47]. We were unable to find any tests of sulfhydration connected to hypoxia sensing at the time of this review.

The connections between cytosolic and mitochondrial sulfur speciation are not completely understood. Under some conditions the persulfide form of the cytosolic enzyme MST may be imported into the mitochondria [42], thereby importing S^0 equivalents in limited concentrations. However, in order to excrete the sulfide formed within the cytosol, there must be bulk mitochondrial import followed by oxidation, or else excretion into the circulatory system. Alternatively, O_2-sensing may be solely a function of sulfur speciation within the mitochondrion [43]. Despite these gaps, the proposal that H_2S senses acute hypoxia is much richer in molecular detail than the proposals connecting K^+ ion channels to hypoxia via CO and O_2^- signaling.

2.3 Hypoxia Inducible Factor (HIF) and the HIF hydroxylases (FIH and PHD)

The response to acute hypoxia is largely a matter of vascular tension to modify blood delivery to tissues, however chronic hypoxia leads to responses within cells at the transcriptional level. HIF is the most important monitor of pO_2 as it controls the expression of hundreds of genes over a wide range of physiological O_2 tensions [48]. HIF also links acute and chronic responses to hypoxia by controlling a number of adaptations to pO_2 including erythropoiesis, angiogenesis, glucose metabolism, cell proliferation, and glycolysis [3, 49]. Several isoforms of HIF are known, with HIF-1 being the dominant player in the human hypoxia response.

After the discovery of HIF-1 in the nuclear extracts of hypoxic cells in 1992 [50], it was soon realized that HIF-1 was a heterodimeric protein consisting of α and β subunits [51], and that HIF-1α levels and transcriptional activity increased with decreasing cellular O_2 concentrations [52]. Three isoforms of the HIFα transcription factor have been identified, HIF-1α -3α, which are evolutionarily conserved in all metazoans [53] but absent from bacteria, yeast, and plants. While the abundance of HIF-α increase under hypoxia [49, 51], HIF-1β is a constitutively expressed protein that is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). Although both HIF-1α and HIF-2α are capable of initiating transcriptional activity, HIF-1α is believed to be the key regulator of the hypoxic response as the other two isoforms have varied expression levels depending on cell types [3]. HIF-3α, which is incapable of initiating hypoxia induced transcriptional activity, is not closely related to HIF-1α or HIF-2α and appears to serve no direct role in hypoxic sensing [48, 54].

The transcriptional activity of HIF-1α decreases with increasing pO_2 due to the competition between nuclear translocation and the post-translational hydroxylation of HIF-1α. HIF-1α accumulates during hypoxia, permitting it enough time to translocate into the nucleus. Once there, it can dimerize with HIF-1β to bind the transcriptional co-activator protein p300 at the promoter regions of genes that allow the cell to survive hypoxia. HIF-1α undergoes O_2-

dependent post-translational modifications that either marks it for proteasomal degradation or prevents its interaction with p300, halting HIF-1 mediated gene expression (Fig 4). These post-translational modifications to HIF-1α take the form of hydroxylations to specific Pro and Asn residues, the rates of which are proportional to [O_2]. Consequently, the transcriptional activity of HIF-1α decreases when the hydroxylation rate exceeds the rate of nuclear localization.

The regulatory enzymes that control HIF-1 activity via posttranslational hydroxylation of asparagine and proline residues are the proteins FIH and PHD. Only one isoform of FIH is currently known, but there are 3 isoforms of PHD (PHD1-3) identified to date, of which PHD2 is recognized as the main regulator of HIF-1α [55]. Both PHD2 ($K_{M(O2)}$ = 250 μM) [56] and FIH ($K_{M(O2)}$ = 90 μM) [49] are O_2 sensors as they have an absolute requirement for O_2 in order for chemistry to occur, and their activity is proportional to variations in pO2 over the physiological range. The dual regulation of HIF-1α likely ensures strict control of the hypoxic response over a wide range of pO_2 levels.

FIH and PHD2 belong to a large superfamily of non-heme Fe(II)/αKG-dependent oxygenases. FIH and PHD2 are proposed to follow the consensus ordered sequential mechanism for this superfamily, in which the co-substrate αKG binds first, then substrate (a domain of HIF-1α) binds second, followed by O_2 (Fig. 5). Substrate binding stimulates O_2 reactivity, which is typically attributed to the creation of an open coordination site due to aquo release. Oxidative decarboxylation generates a high-valent ferryl intermediate that abstracts hydrogen from an un-activated carbon, followed by rebound chemistry to hydroxylate the substrate (Fig 5). It is worth noting that the ferryl intermediate has been observed for neither FIH nor PHD2, suggesting that the chemistry of these two enzymes may differ in subtle ways from that of the broader αKG oxygenase superfamily. For more comprehensive reviews on the chemistry and structural conservation of αKG oxygenases see reviews [57] and [58].

PHD2 and FIH hydroxylate specific residues in the O_2-dependent degradation domain (ODD) and the C-terminal transactivation domain (CTAD) of HIF-1α, which leads to decreased transcriptional activity for HIF. Hydroxylation of either of two proline residues (Pro402, Pro564) in the ODD by PHD2 is a prerequisite of HIF-1α recognition by the von Hippel-Lindau protein (pVHL) (Fig. 4) [59], with hydroxylation at Pro564 being ten-fold faster than at Pro402 [60-62]. pVHL is the recognition component of an E3 ubiquitin-protein ligase which targets HIF-1α for rapid proteasomal degradation after successive rounds of ubiquitinylation [63]. Hydroxylation of Asn803 in the CTAD by FIH blocks p300 from binding, stopping expression of HIF-1α target genes (Fig. 4) [64]. O_2 mediated regulation of HIF-1α varies slightly between different cells, depending on the O_2 requirements in specific cell types [65].

HIF controls the expression of several genes that help mediate the adaptive response to fluctuating pO_2, such as NOX [66], HO-1 [22], GLUT-1 [7] and PHD2 [55], placing it at the center of O_2–sensing and homeostasis. Although the transcriptional activity of HIF is linked to both acute and chronic hypoxia sensing, the true sensors are FIH and PHD2, which use molecular oxygen to hydroxylate HIFα. Interestingly, HIF controls the expression of PHD2,

which is induced under hypoxia. As the $K_{M(O2)}$ for PHD2 is high in relation to physiological pO_2 within cells [49, 56], PHD2 has low activity during hypoxia, but its expression would ensure that the proper balance to HIF-1α levels are restored upon reoxygenation of the cell. Whilst the critical players (FIH and PHD2) in HIF mediated pO_2 sensing have been identified, key questions that remain to be answered include: How is the chemistry of O_2 activation stimulated by enzyme/HIF-1α binding? Are there physiological effectors of PHD2/FIH activity, including other gases such as H_2S, CO, and NO? How can we selectively inhibit or increase PHD2/FIH activity? These would facilitate therapeutic targeting of these enzymes, as well as fundamental insight into the chemistry of this superfamily of non-heme Fe(II) αKG-dependent hydroxylases.

The O_2-activation by FIH and PHD2 is significantly stimulated by substrate binding [67], as seen for other aKG hydroxylases. This is crucial to their role as O_2-sensors because O_2-activation in the absence of HIF leads to enzyme inactivation [68]. The best supported model for substrate-stimulated O_2-activation in these enzymes focuses on the coordination geometry of the Fe(II) cofactor. The Fe(II) switches from predominantly 6-coordinate to 5-coordinate upon substrate binding due to aquo release, as shown by electronic spectroscopy and computational results for several enzymes from the aKG-dependent hydroxylase family [67, 69, 70]. By creating a coordination site on Fe(II), O_2 binding is then forced to follow substrate binding. Although the chemical mechanism of O-O bond cleavage is unclear, back bonding from αKG to the metal likely plays a role in stimulating O_2 activation at Fe(II) [69, 70].

Despite the coordination changes observed under non-turnover conditions, certain features of the above model need to be refined. For example, the kinetics of ligand exchange from the Fe(II) cofactor is not known – unless aquo exchange from the Fe(II) is very slow, O_2 would be able to access the metal prior to substrate binding. Further, the redox potential of the Fe(II) has been proposed to shift upon substrate binding, thereby facilitating its reaction with O_2 [71]. These two points suggest that substrate binding may, in fact, simply increase the equilibrium binding affinities for O_2 relative to that of H_2O. Another broader question about this model that remains under study is how substrate binding alters contacts within the active site to stimulate O_2 activation. Answering these questions will elucidate much about the specific chemistry involved in O-O cleavage as related to hypoxia sensing.

Recent work on the HIF hydroxylases has provided some insight into the molecular mechanism of pO_2 activation by the HIF hydroxylases. The mechanical linkage between HIF-1α binding and O_2-activation remain unclear in FIH, however this is central to O_2 sensing. Although FIH will inactivate *in vitro* through an auto-oxidation reaction in the absence of HIF-1α [68, 72], HIF-1α binding stimulates the O_2 reactivity of FIH many-fold. MCD and CD data of FIH in solution revealed that the Fe(II) cofactor geometry shifts from 6-coordinate to a mixture of 5/6-coordinate upon HIF-1α binding [67], in agreement with the X-ray crystal structure of HIF-1α bound FIH [73] and suggesting that aquo release may limit the O_2 reactivity [74]. As the consensus mechanism for this family of enzymes requires that the Fe(II) be 5-coordinate prior to binding O_2 [58], the MCD data showing only partial formation of the 5-coordinate Fe(II) suggests that O_2 binding or activation may be sluggish in FIH. Further support for this notion comes from a suite of mechanistic data which points

to rate-limiting O_2 activation for FIH under accessible pO_2 [75], which would lead to a direct correlation between enzyme activity and pO_2. Notably, modeling and mutation studies demonstrate that the second coordination sphere residues are pivotal for directing O_2 reactivity in FIH [67, 76]. The picture which emerges is one in which HIF-1α binding induces structural changes near the Fe(II), including aquo release, which stimulates O_2-activation.

PHD2 activity also appears to be limited by the rate of O_2-activation, consistent with its role as an O_2-sensor. Pre-steady state kinetics indicated that PHD2 reacted with O_2 very slowly when compared to other αKG oxygenases, and related freeze-quench experiments failed to isolate the putative ferryl intermediate [77], which was consistent with O_2-activation as the rate-limiting step in PHD2. Steady-state kinetics further supports rate-limiting O_2-activation in PHD2 [78]. In order for the HIF hydroxylases to be good O_2 sensors, O_2 binding or a step subsequent to O_2 binding should be rate limiting as this would lead to proportionate changes in activity with pO_2. The $K_{M(O2)}$ being higher than the physiologically relevant pO_2 is also consistent with the notion of the HIF hydroxylases being O_2 sensors as this would allow the rate of their chemistry be proportionate to pO_2 near physiological levels.

A number of endogenously produced molecules, such as NO, CO, and H_2S, are intriguing as putative effectors of the HIF hydroxylases as they may bind in place of O_2 and are known to regulate pathways related to hypoxia responses. Nitric oxide (NO) regulates vascular tone, and has been shown to inhibit PHD2 [79] and FIH [80], hinting that this and other transient species could be physiological effectors of the HIF hydroxylases. Although CO binding to the HIF hydroxylases has yet to be demonstrated, endogenous CO has been shown to exhibit anti-apoptotic, anti-inflammatory, and protective responses against hyperoxia-induced lung injury [27]. Unpublished results show that H_2S inhibits FIH (IC_{50} ~ 150 μM) (Taabazuing and Knapp, unpublished) – although this level of H_2S is likely to be supraphysiological, it nevertheless further implicates H_2S in the overall hypoxia response.

Methods to inhibit the HIF hydroxylases would be highly desired, as they would permit therapeutic targeting of the hypoxia sensing machinery. Although a number of synthetic compounds have been reported to inhibit these enzymes [81-83], challenges include improving both selectivity [81] and the relatively low affinity of the inhibitors (IC_{50} > 1μM). Inhibitors for FIH and PHD2 are typically structural mimics of αKG, able to bind to the Fe(II) cofactor via one or two ligation points, opening up the likelihood of cross-reactivity with other αKG oxygenases. Inclusion of a chiral center near the Fe(II) binding group improved inhibitor specificity in one study [84], suggesting that further improvements in inhibitors may be eminently achievable with further synthesis and screening efforts.

3. Bacterial O_2 Sensing

Bacterial responses to changes in pO_2 are crucial for balancing central metabolism as well as inducing cellular machinery needed for specialized metabolic pathways. These pathways include nitrogen fixation, hydrogen production and sulfate reduction, which utilize enzymes with metal cofactors that are highly sensitive to O_2. Bacteria rely largely on sensory proteins containing [Fe-S] clusters or hemes to regulate gene expression in response to changes in

pO_2. In contrast to the oxygenase chemistry of FIH and PHD in higher organisms, which lead to altered protein/protein binding affinities, bacterial sensors typically report on the presence of O_2 by changing the sensor/DNA binding affinity.

Bacterial sensor proteins bind O_2 to the iron cofactor leading to altered downstream effects. Reversible binding of O_2 to the heme protein sensors induces structural changes that affect binding to DNA or other proteins, allowing for reversible and dynamic gene regulation based on the O_2-binding equilibrium. Heme-based sensors have been classified by their heme-binding-domains into four families [85]. Well studied heme-sensors include FixL [86], *Ec*Dos [87], *Ax*PDEA1 [88], HemAT [89], and the CO-binding sensor CooA [90]. In contrast, O_2 binding to [Fe-S] clusters leads to cluster degradation and diminished DNA binding affinity of the sensory proteins, making O_2 sensing responsive to multiple steps, such as sensor protein production, cluster degradation, and metallocluster formation. [Fe-S] proteins such as FNR [91], NreB [92], ArnR [93], and WhiB [94] all sense O_2; other reactive species such as CO, NO, and reactive oxygen species are sensed in a similar way.

Two modes of bacterial O_2 sensing are direct transcriptional control and two-component signaling cascades. The well studied O_2-sensor FNR exemplifies direct transcriptional control, as the $[Fe_4S_4]^{2+}$ cofactor is degraded by O_2, leading to attenuated DNA binding affinity [91]. The classic example of a two component signal transduction pathway for O_2 sensing is the FixL/FixJ system, in which the O_2 sensing protein (FixL) relays its response to varying pO_2 through a histidine kinase (HK) domain to the response regulator protein (FixJ). The sensing proteins in these latter systems are typically heme-based sensing proteins, with exceptions to this including the [Fe-S] two component systems NreBC [92, 95, 96] and AirSR [97].

3.1 Sensing of O_2 by transcriptional regulators

Direct O_2 sensing at the transcriptional level occurs when the sensor serves the dual roles of O_2 binding and DNA binding. FNR is one of the most extensively studied O_2-sensing DNA binding proteins. *E. coli* FNR is a 53 kDa homodimeric protein [98] under anaerobic conditions, with each monomer containing a $[Fe_4S_4]^{2+}$ cluster coordinated by four cysteine residues [91, 98, 99]. Dimeric FNR binds to DNA to promote the expression of genes for proteins involved in anaerobic metabolism and to repress those involved in aerobic metabolism [100].

The cofactor of FNR is highly sensitive to O_2 *in vitro*, with ~95% activity at 0.3 μM O_2 but only ~50% activity at 6 μM O_2 [101], which mirrors *in vivo* measurements [102]. Upon exposure to O_2, the $[Fe_4S_4]^{2+}$ cluster of FNR rapidly degrades to $[Fe_2S_2]^{2+}$ (Fig. 6) [100, 103, 104]. The rapid $[Fe_4S_4]^{2+}$ to $[Fe_2S_2]^{2+}$ cluster conversion leads to dissociation of homodimerric FNR into its subunits, ultimately weakening DNA-binding [100, 105]. There are two proposed mechanisms for oxidative decomposition of the $[Fe_4S_4]^{2+}$ clusters: metal oxidation [106-109] and sulfur-based oxidation [99, 110, 111]. Regardless of the mechanism for cluster conversion, when exposed to superoxide, $[Fe_2S_2]^{2+}$ FNR converts to apo FNR (Fig. 6) [104]. *In vivo*, reactivation of apo-FNR to $[Fe_4S_4]^{2+}$ FNR is possible under anaerobic conditions [112], most likely utilizing the Isc pathway proteins [113].

3.2 Two component signal transduction

Two component signal transduction is a fundamental signaling pathway primarily found in prokaryotes, with limited examples in eukaryotic systems [114-116]. The two components referred to in these systems are: 1) a sensory protein; and 2) a response-regulator protein. The sensing protein is a multidomain protein with a sensing domain and a histidine kinase (HK) domain. Ligand binding to the sensing domain induces an ATP dependent autophosphorylation on the HK domain. Subsequent phosphoryl group transfer from the HK domain to the response-regulator protein transduces the signal. As phosphorylation of the response-regulator protein can stimulate or repress gene expression of downstream targets depending on the specific system, two-component signal transduction can lead to a wide variety of sensory responses.

Representative bacterial O_2-sensing two-component signal transduction systems include Aer2 [117], DosS-R, DosT-R [118], and FixL/FixJ [119]. The FixL/FixJ two component signal transduction sensor pathway exemplifies many of the key features needed for O_2 sensing. FixL/FixJ was first identified in *Sinorhizobium meliloti* (*Sm*FixL) [120] but has been characterized in other bacterial systems including *Bradyrhizobium japonicum* (*Bj*FixL) and *Rhizobium etli* (*Re*FixL), with a homologous FixL in the algae *Chlamydomonas reinhardtii* [121]. These two-component regulatory systems are found widely, even in *Mycobacterium tuberculosis*, a pathogen responsible for causing tuberculosis [122].

FixL proteins contain a HK domain and at least one PAS (Per-ARNT-Sim) domain, which contains the heme that binds O_2 (Fig. 7) [119]. FixL has a relatively low O_2 affinity (K_D ~ 100 μM) [123-125], allowing for physiological changes in pO_2 to alter the transcriptional activity of FixJ. Under hypoxic conditions, the unligated five coordinate high spin Fe(II) of FixL induces gene expression [126-130] as this state of FixL autophosphorylates a conserved His residue within its HK domain. Phosphoryl transfer to a conserved Asp residue in the response regulator FixJ [86] induces a conformational change that leads to dimerization, thereby increasing affinity for the *fixK* promoter and inducing gene expression [131, 132]. Upon binding of O_2, the Fe(II) converts to low spin six coordinate geometry, flattening the heme ring and disrupting hydrogen bonding interactions to the heme propionate groups. Most notable is the distal arginine residue breaking a salt bridge with a heme propionate group to help stabilize O_2, initiating the conformational change of FixL's FG loop needed to inhibit phosphorylation [133-135].

4. Hemerythrin-domain Proteins

The previously discussed O_2-sensing strategies used by bacteria and higher organisms are notable for their distinct cofactor utilization. Heme and iron sulfur cluster based O_2 sensing are the two most prevalent mechanisms utilized by bacteria, whereas non-heme Fe(II), ion channels, and (possibly) H_2S are central to hypoxia sensing in mammalian cells. Recent research shows that hemerythrin-like diiron proteins are emerging as O_2 sensors in both bacteria and humans. While hemerythrin (Hr) is a classic O_2 binding protein from invertebrates, proteins containing an Hr-like domain have been found in bacteria and mammalian cells [136-138] where they are proposed to sense O_2 and/or Fe levels. Two

notable examples are bacterial hemerythrin DcrH-Hr found in *Desulfovibrio vulgaris*, and FBXL5 found in human cells.

DcrH-Hr is thought to function as an O_2-sensor, using the [Fe(II) Fe(II)] cofactor found within its Hr-like domain. Invertebrate Hrs reversibly bind O_2 to the reduced form of the cofactor to form oxy-Hr: [Fe(II) Fe(II)] + $O_2 \Leftrightarrow$ [Fe(III) Fe(II) O_2^-] [139]. As invertebrate oxy-Hr is relatively stable, O_2 can be delivered before oxy-Hr undergoes the much slower auto-oxidation process which forms met-Hr: [Fe(II) Fe(III) O_2^-] $\rightarrow$ [Fe(III) Fe(III)] + H_2O_2. In contrast, oxy DcrH-Hr rapidly auto-oxidizes to form an extremely stable met-DcrH-Hr, suggesting a role in O_2-sensing rather than reversible O_2 delivery [140-142]. Structural features identified in DcrH-Hr that are proposed to promote rapid auto-oxidation and lead to its function as a sensor include a large solvent channel that would facilitate O_2 and water access [142].

The signal transduction mechanism for O_2-sensing for proteins containing a bacterial Hr domain likely relies on an oxidation-state dependent conformational change in the Hr domain. Recently, O_2-sensing was demonstrated for the protein known as Bhr-DGC, a bacterial hemerythrin from *Vibrio cholerae* [143]. The rate for producing cyclic di-GMP, a secondary messenger in bacteria, by Bhr-DGC decreased by 10-fold when the protein was oxidized to the [Fe(III) Fe(III)] oxidation state, indicating that oxidation by O_2 to form met Bhr-DGC led to a detectable change in an important signaling molecule [143].

F-box and leucine-rich repeat protein 5 (FBXL5) contains a HR-like domain that imparts O_2-dependent stabilization to the protein. Although it is not clear as to whether or not FBXL5 serves to sense physiological changes in [O_2], this protein does connect Fe homeostasis to O_2 levels. FBXL5 is an adaptor protein that binds to IRP2 and an SCF E3 ubiquitin ligase, imparting O_2 and Fe sensitivity to the IRP2 protein in human cells. This leads to the proteasomal degradation of IRP2 in the presence of Fe and O_2 [136, 144] as a cellular signal that [Fe] levels are sufficiently high.

The N-terminal HR domain of FBXL5 reversibly binds a [Fe(II) Fe(II)] cofactor which closely resembles the structure of the canonical Hr [145, 146]. As the reduced form of FBXL5 is in equilibrium with unfolded FBXL5, its degraded by the cell, allowing IRP2 to accumulate. Upon exposure to O_2, the cofactor oxidizes to the [Fe(III) Fe(III)] oxidation state which locks in the structure of FBXL5, thereby stabilizing this protein. Oxidized FBXL5 binds IRP2 and an SCF E3 ubiquitin ligase, leading to IRP2 ubiquitinylation and degradation. Consequently, FBXL5 responds to both [Fe] and [O_2], with IRP2 degradation as the signal for sufficient levels of these essential compounds. Although it is not clear whether FBXL5 responds to physiological-relevant changes in [O_2], it is clear that this protein connects Fe and O_2 homeostasis.

It appears there are at least two distinct sensing mechanisms among Hr-like domains. Bacterial Hr-like proteins bind O_2 and undergo rapid auto-oxidation [141, 143, 147], forming a stable [Fe(III) Fe(III)] cofactor. In contrast, FBXL5 does not form an observable O_2-adduct, suggesting that cofactor oxidation may involve an alternate chemical mechanism. Comparison of the crystal structures of FBXL5 (PDB 3V5Z, 3V5Y, 3V5X)

[148] and DcrH-Hr (PBD 2AWC) [142] show the binding pocket of FBXL5 is not large enough for O_2 binding, suggesting outer sphere electron transfer between O_2 and the [Fe(II) Fe(II)] center [148]. Unlike DcrH-Hr, which has been demonstrated to directly bind O_2, there is strong experimental evidence that FBXL5 does not bind O_2 at the dinuclear center [145, 148], including NMR studies ruling out a significant conformational changes upon exposure of FBXL5 to O_2 [149].

4. Conclusion

Acute and chronic changes in pO_2 lead to distinct adaptive responses in mammals, ranging in scale from vascular changes through transcriptional changes. While several biochemical pathways for pO_2 sensing are known with varied levels of detail, a number of outstanding research questions remain which focus on the chemical interactions that underlay sensing. Tissue level responses to changed pO_2 appear to be mediated by ion-channels, however the molecular mechanisms for transducing pO_2 into ion channel activity remain to be defined. Similarly, the proteins that control the transcriptional activity of HIF through post-translational hydroxylation (FIH and PHD enzymes) are well defined, leading to questions that largely center on the chemical mechanisms of O-O bond cleavage and the identification of enzyme regulators/inhibitors. Emerging data suggests that the speciation of sulfur compounds report on hypoxia, with two large questions being the connections between cytosolic and mitochondrial sulfur pools, and the mechanisms for transducing the signal. Continuing research in these areas holds promise in treatments for diseases including ischemia and cancer, where vasculature development and anaerobic metabolism play significant roles in cell growth.

Bacterial hypoxia sensing is mediated by direct transcriptional control over gene expression, and is much better understood than hypoxia sensing in mammals. Two component signaling systems allow for signal transduction based on pO_2, classically mediated by O_2 binding directly to heme domains. An alternative approach relies on O_2-mediated degradation of Fe-S clusters to change protein oligomerization, thereby changing transcriptional activity. As O_2 sensing in bacteria is directed toward allowing the organism to adapt metabolically to altered pO_2, these pathways could aid in targeting pathogenic bacteria.

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Abbreviations

ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop-helix
CAT	cysteine aminotransferase
CBS	cystathione β-synthase (CBS)
CSE	cystathione γ-lyase

CTAD	C-terminal transactivation domain
ETHE1	persulfide dioxygenase
FBXL5	F-box and Leucine-rich Repeat Protein 5
FIH	factor inhibiting HIF;
FNR	fumarate and nitrate reduction regulator
GSSH	glutathione persulfide
GST	glutathione S-transferase
hCys	homocysteine
HIF	hypoxia inducible factor;
HK	histidine kinase
HO	heme oxygenase
Hr	hemerythrin
Isc	iron-sulfur cluster
LC-ESI MS	liquid chromatography-electrospray ionization mass spectrometry
MST	mercaptopyruvate sulfurtransferase
NEB	neuroepithelial bodies
NOX	NADPH oxidase
ODD	oxygen degradation domain
PAS	Per-Arnt-Sim
PHD2	HIF prolyl hydroxylase domain 2
PKC	protein kinase C
pVHL	von-Hippel Lindau protein
ROS	reactive oxygen species
SMC	smooth muscle cells
αKG	α-ketoglutarate

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Highlights

Hypoxia sensing leads to important metabolic and transcriptional changes.

Hypoxia sensing occurs by O_2 binding, chemical modifications, and two-component signaling.

A variety of metabolites are implicated in hypoxia sensing.

The HIF pathway is the dominant pathway for long-term hypoxia sensing in human cells.

FIH and PHD are the central players in human hypoxia sensing.

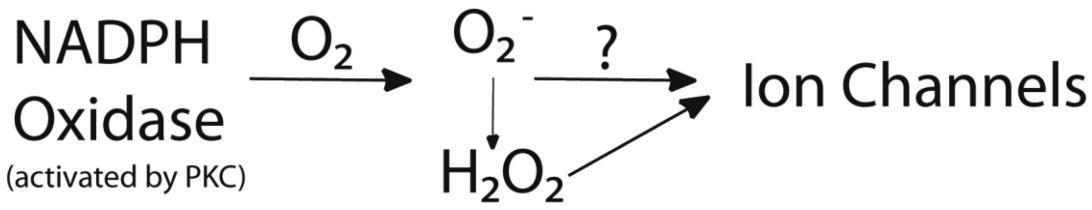

Figure 1. Proposed mechanism for ion channel O_2 sensing in NEBs

NADPH oxidase mediates the hypoxic response in NEBs via O_2^- or H_2O_2.

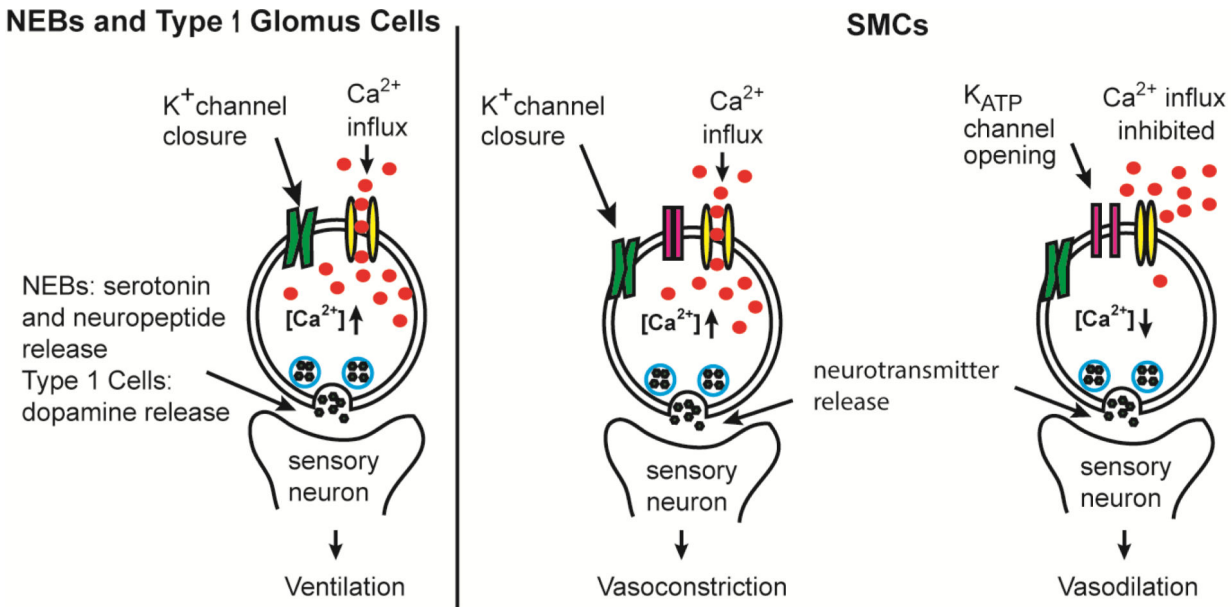

Figure 2. Acute hypoxia sensing in mammalian tissues

Acute hypoxia causes inhibition of K^+ channels in type 1 glomus cells and NEBs. The depolarization increases intracellular [Ca^{2+}], leading to neurotransmitter release and improved ventilation. In systemic SMCs, acute hypoxia causes K_{ATP} channels to open, inhibiting calcium influx and causing vasodilation. Inhibition of K^+ channels in pulmonary artery SMCs causes depolarization and calcium influx that results in vasoconstriction.

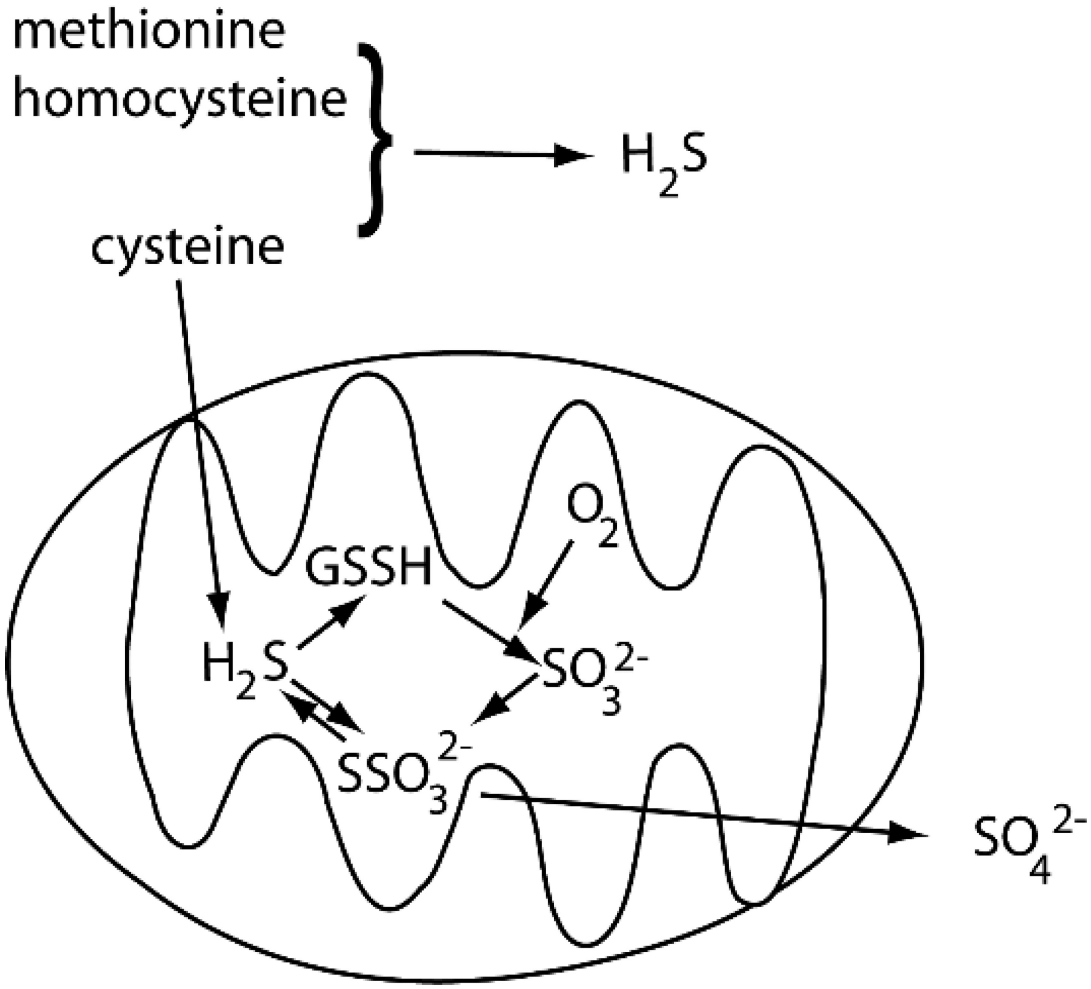

Figure 3. H_2S production and oxidation in the cytosol and mitochondria

Key compounds are cysteine (Cys), homocysteine (hCys), methionine (Met), and glutathione persulfide (GSSH).

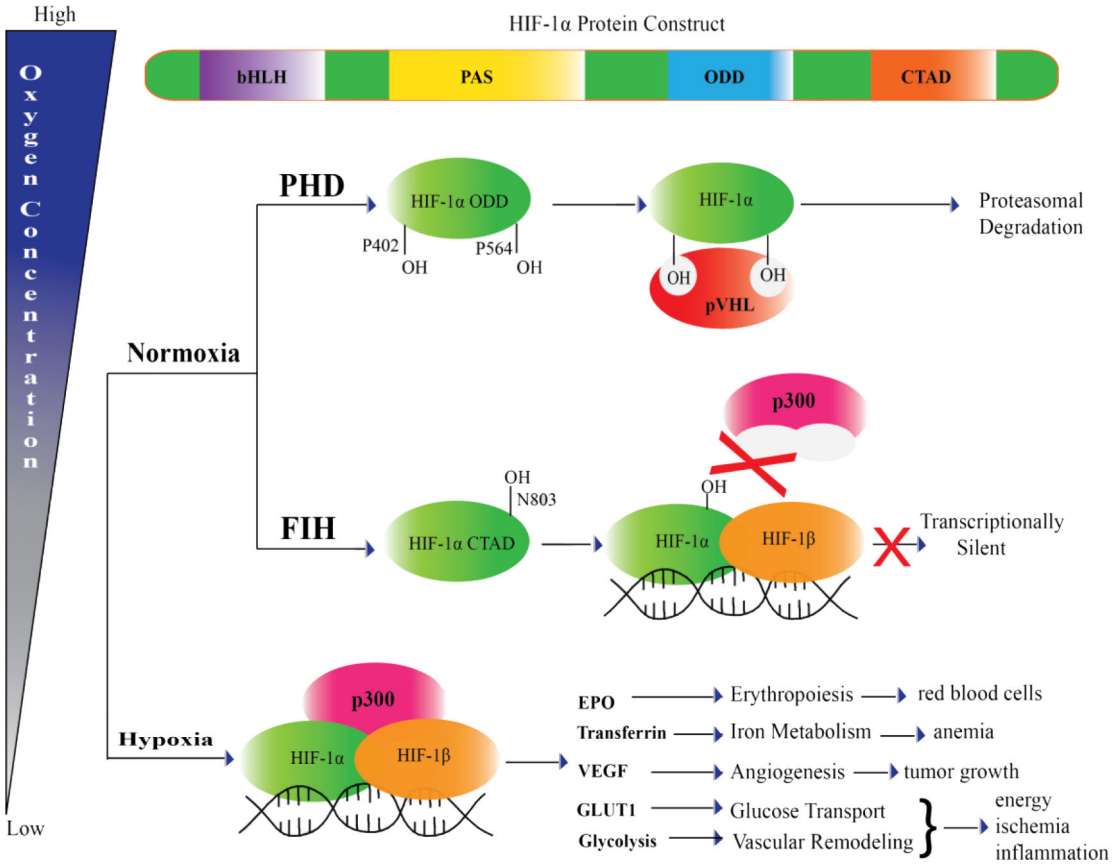

Figure 4. Regulation of HIF-1α by FIH and PHD2

Posttranslational regulation of HIF-1α by FIH and PHD2 control HIF-1α transcriptional activity and stability under normoxic conditions. During hypoxia, HIF-1α forms a transcription complex with HIF-1β and p300, and initiates target gene expression.

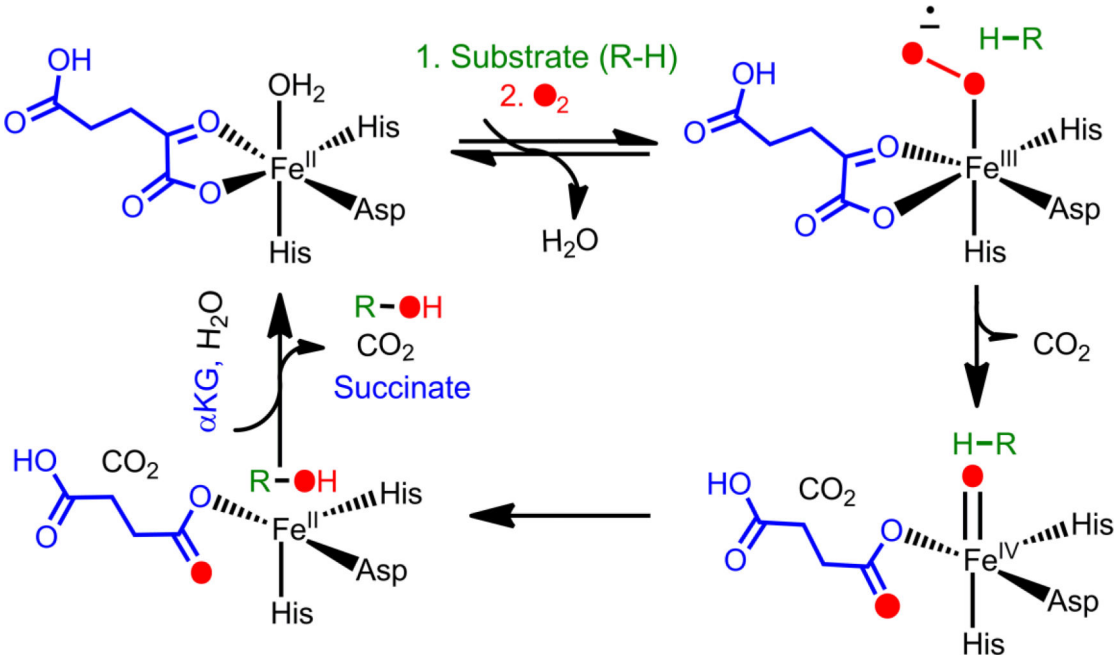

Figure 5.
Proposed consensus mechanism of the αKG-dependent hydroxylases.

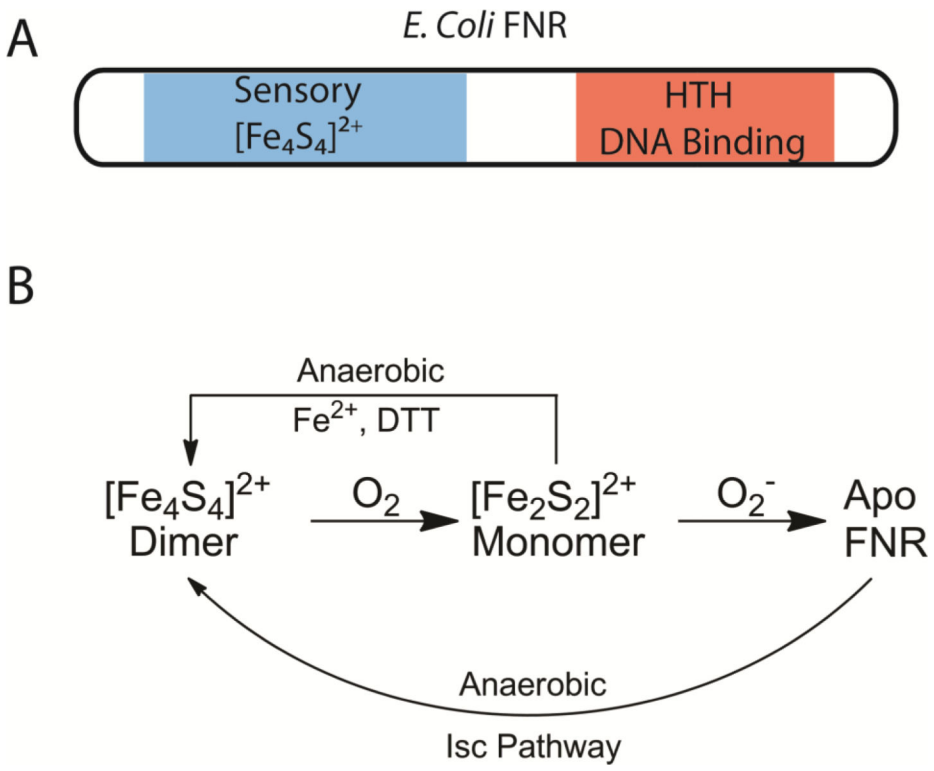

Figure 6. O_2 sensing by FNR

A) FNR protein domain arrangement. B) FNR $[Fe_4S_4]^{2+}$ cluster degradation. In the presence of O_2, the FNR $[Fe_4S_4]^{2+}$ cofactor is rapidly oxidized to $[Fe_2S_2]^{2+}$, leading to dissociation of dimeric FNR. Reconstitution of the $[Fe_4S_4]^{2+}$ cluster in FNR occurs through the Isc pathway *in vivo*, but can also be reconstituted *in vitro* by exogenous Fe^{2+} and DTT [111].

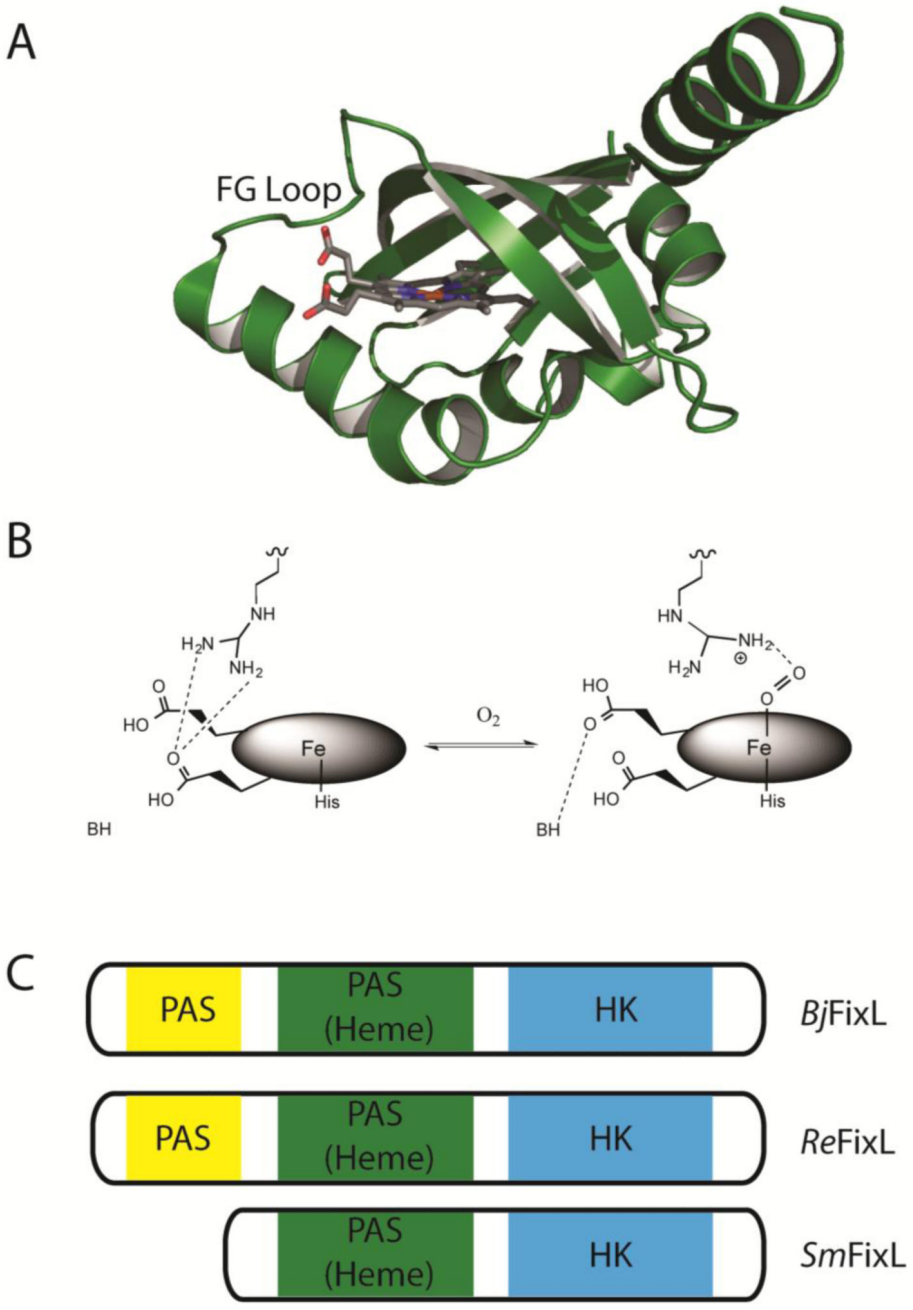

Figure 7.

A) Protein crystal structure of the heme binding PAS domain of FixL from *B. japonicum* in the unliganded Fe^{3+} oxidation state (PDB 1LSW) [134], with the conformationally mobile FG loop labelled in black. B) The heme active site of *Bj*FixL. Hydrogen bond interactions to the heme propionate groups have a significant role in shifting the FG loop conformation for oxy-FixL, slowing autophosphorylation [124, 133-135, 150-152]. C) Protein constructs of

*Bj*FixL, *Re*FixL and *Sm*FixL. The empty N-terminal PAS domain likely tunes O_2 affinity and influences signal transduction [153-156].