HHS Public Access

Author manuscript

Curr Opin Microbiol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Curr Opin Microbiol. 2015 August ; 26: 41–47. doi:10.1016/j.mib.2015.04.006.

Oxygen Sensing by Protozoans: How They Catch Their Breath

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Abstract

Cells must know the local levels of available oxygen and either alter their activities or relocate to more favorable environments. Prolyl 4-hydroxylases are emerging as universal cellular oxygen sensors. In animals, these oxygen sensors respond to decreased oxygen availability by up-regulating hypoxia-inducible transcription factors. In protists, the prolyl 4-hydroxylases appear to activate E3-SCF ubiquitin ligase complexes potentially to turn over their proteomes. Intracellular parasites respond to decreased oxygen by utilizing both types of oxygen-sensing pathways. Since parasites are exposed to diverse oxygen tensions during their life cycle, oxygen sensing is likely a critical process and this review will discuss how these oxygen-sensing mechanisms contribute to the behavior of these unicellular eukaryotes.

O_2 sensing is important for cellular life in an aerobic atmosphere

As an atmospheric gas that dissolves in aqueous biological solvents, O_2 is a thermodynamically favorable acceptor for electrons and protons in mitochondrial respiration and other metabolic pathways. Its consumption by aerobic metabolism, combined with its limited solubility and slow diffusion in water, leads to concentration gradients of O_2 within cells, tissues, and the local environments [1–4]. Cells have evolved multiple autonomous mechanisms to rapidly reorganize their metabolism to cope with O_2 depletion [5,6]. These range from the rapid formation of short-lived reactive oxygen species (ROS) to longer-term changes in a cell's transcript and protein repertoire. Together these alterations impact the cell's metabolism and other activities such as motility to enable a cell to migrate to areas of higher O_2 availability. As an example, when the soil amoeba *Dictyostelium* is exposed to low O_2 it migrates toward higher O_2 levels where it can continue its developmental program and differentiate into a fruiting body [7].

Cells express several types of O_2 sensors. One class directly binds O_2 effecting a conformational change that modifies the protein's function [8]. A second class includes proteins such as oxidases and non-heme dioxygenases that utilize O_2 as a substrate for the biochemical reactions that they catalyze. The non-heme dioxygenases have an Fe center that coordinates and splits O_2, distributing one of the O-atoms to the target substrate [9], and the other to α-ketoglutarate (αKG or 2-OG), which decomposes into succinate and CO_2 (Fig. 1A). These dioxygenases include a variety of enzymes such as demethylases and

hydroxylases that can modify several types of amino acids and nucleic acids. This review will focus primarily on non-heme dioxygenases and in particular prolyl 4-hydroxylases (P4Hs) since they are the best characterized O_2 sensors in protozoans and animals and have potential roles in other eukaryotes and bacteria [10].

Transcription-dependent O_2-sensing in animals

Transcriptome rewiring is a key mechanism by which animals (and many fungi) respond to decreased O_2-availability and PHDs (prolyl hydroxylase domain proteins) are the O_2 sensing P4Hs most important in regulating these O_2-regulated transcription factors. They function by hydroxylating two conserved prolines in the α subunit of the heterodimeric hypoxia inducible factor (HIF) transcription factor, resulting in the formation of (*4S,2R*)-hydroxyproline, (Hyp) [6,9,11]. Prolyl hydroxylated HIFα is recognized by and targeted for proteasome-dependent degradation by the VHL ubiquitin ligase complex. Thus, HIFα is rapidly degraded under O_2 replete conditions. However, the PHDs have low affinity for O_2 and when O_2 becomes limiting, as can occur in tissue environments, HIFα accumulates, translocates to the nucleus, binds HIFβ (also called ARNT), and activates target gene expression. Many of these genes, such as glycolytic enzymes and vascular endothelial growth factor, are important to coordinate a cell's response to decreased O_2 availability. HIFα is regulated by another non-heme dioxygenase, Factor-inhibiting-HIF (FIH), which hydroxylates an asparagine in the protein's C-terminal transcriptional coactivator domain [12,13]. This O_2-dependent process diminishes transactivation activity, which reinforces the action of PHDs in regulating HIF1α activity. Besides HIF1α, PHDs regulate other proteins including PKM2 [14] and the TRPA1 [15] ion channels. Thus, hydroxylation of prolines and other amino acid is emerging as a widespread post-translational modification and we expect that these lists will continue to grow.

O_2-sensing in amoebae

While HIFα is restricted to animals, P4Hs are evolutionarily conserved and found not only in all animals [16] but fungi, amoebazoa, and, evidently, prokaryotes [10]. Thus P4Hs appear to predate the emergence of HIFα in animals, raising the question of what their targets are in other organisms and whether they are involved in O_2-sensing. The discovery of a *Dictyostelium* P4H (DdPhyA), which hydroxylates Pro143 in the Skp1 subunit of the SCF class of E3-Ub ligases, raised the possibility that this P4H enzyme is an O_2 sensor [17] (Fig. 1B).

Dictyostelium resides in the soil where O_2 availability is governed by the rate of infusion from the atmosphere, and depletion by microbial and root metabolism. The amoebae use bacteria as a nutrient source and under starvation the amoeba differentiate into dormant spores. This involves a developmental program resulting in cell aggregation to form a polarized multicellular slug that migrates to the soil surface and transforms into a fruiting body consisting of dormant spores that survive until activated by a nutrient rich environment. O_2 is one sensory modality used by *Dictyostelium* to assess whether they are above ground at a site appropriate for fruiting body formation. Culmination therefore occurs in an O_2 dependent manner and the O_2 set point for culmination, which is well above that

required for metabolism, can be raised or lowered by forced under- or over-expression of the DdPhyA [18*]. Since purified DdPhyA has a K_m for O_2 above 21%, this suggests that O_2 control of culmination is mediated via PhyA activity. This mechanism is supported by pulse-labeling studies with ^{35}S-Met, that showed that Skp1 hydroxylation lags behind synthesis at low, but not normal, O_2 levels [19*]. When *Dictyostelium* is cultured under standard submerged conditions, development requires high atmospheric levels of O_2 (70–100%) by a mechanism that depends on PhyA and Skp1 hydroxylation [20]. The data suggest that O_2 is limiting for hydroxylation of newly synthesized Skp1, which might have a privileged role in forming regulatory SCF complexes composed of nascent FBPs.

Not only is Skp1 a substrate for PhyA it is a functional mediator of PhyA activity in cells. Skp1 *under*-expression has the same effect on the O_2-setpoint for culmination as PhyA *over*-expression, suggesting that hydroxylation reduces Skp1 activity [21*]. Conversely, Skp1 overexpression raises the O_2-setpoint similar to disrupting *phyA*. As expected, the activity of Skp1 is suppressed by forced overexpression of PhyA, and is not additive with genetic depletion of *phyA* [21*]. Paradoxically, Skp1 with a point mutation at the position of Pro143 exhibits less activity, though this might reflect separate effects on protein folding and function. Over-expression of Skp1 also inhibits sporulation, a subsequent developmental step that occurs as prespore cells rise to the top of the fruiting body. In contrast to culmination, inhibition depends on its Skp1 hydroxylation. Thus in addition to being the major if not only functional mediator of PhyA action, Skp1 contributes multiple functions in development that vary in their dependence on its hydroxylation.

Skp1 prolyl hydroxylation renders it a substrate for Gnt1 [22], the first of three glycosyltransferases (GTs) that assemble a linear pentasaccharide on Hyp143 (Fig. 1B). This raises the question of whether hydroxylation alone controls Skp1 function, or simply enables subsequent glycosylation. Both answers are correct. Genetic disruption of *gnt1* results in an O_2 requirement for culmination that lies between that of *phyA*-KO and w/t cells, and also yields morphologically defective fruiting bodies at any O_2 level [22]. A KO of the second GT (*pgtA*), which allows for only a single sugar (GlcNAc) to be added, has an O_2 requirement that is very similar to that of *gnt1*-KO cells, but rescues the defective fruiting body morphology. Remarkably, KO of the third GT (AgtA), allowing for the trisaccharide to be assembled, reverts the O_2-dependence almost back to that of *phyA*-KO cells and has unexpectedly delayed early development [23]. Accumulating evidence suggests that AgtA has a second function as a Skp1 binding protein, via its WD40 repeat domain, that dampens its function regardless of its glycosylation status [24]. Consistent with this, the high O_2-requirement of *agtA*-KO cells is ameliorated by genetic reduction of Skp1 levels [21*]. Thus glycosylation and the three GTs modulate the signal generated by prolyl hydroxylation of Skp1.

The primary function of Skp1 as part of the E3-SCF ubiquitin complex is to bind the F-box domain of F-box proteins (FBP) (Fig. 2). FBPs are heterogeneous multidomain proteins that serve as receptors for substrates to be poly-ubiquitinated and subsequently degraded [25]. *Dictyostelium* is predicted to have 55 FBPs and the mechanism(s) controlling binding specificity to Skp1 is complex but important since $E3^{SCF}$Ub-ligases likely control the fates of hundreds if not thousands of cellular proteins. Co-immunoprecipitation studies in

Dictyostelium suggest that two distinct FBPs (FbxD and FbxA) preferentially associate with fully glycosylated Skp1 compared to unmodified Skp1 [19*]. In addition, FbxD binds better to the trisaccharide form of Skp1 (in agtA KO strains) than unmodified Skp1 although not as well as it does to fully modified Skp1. While, structural data indicate that addition of GlcNAc promotes increased α-helical content in Skp1 [26], further glycosylation has little on Skp1 structure suggesting that the peripheral sugars of the pentasaccharide regulate FBP binding through different mechanisms. Taken together these data indicate that O_2 regulated Skp1 hydroxylation/glycosylation has various novel effects on Skp1 and serves to complement other SCF regulatory mechanisms that include the neddylation of cullin-1 which controls the exchange activity of Cand1 at the Cullin-1/Skp1 interaction site [27,28]. Further studies are needed to identify target substrates of *Dictyostelium* FBPs to determine how changes to the cell's proteome mediate O_2-dependent culmination and development. In addition, we have identified putative Skp1-modification genes in other disease-causing amoebazoa, such as *Acanthamoeba*, suggesting that O_2-sensing may contribute to virulence in ocular keratitis.

O_2-sensing in *Toxoplasma*

Toxoplasma gondii is an unrelated protozoan that can infect almost all known mammals. It is passed between hosts by digestion of either infectious oocysts shed in feline (the parasite's definitive host) fecal material or tissue cysts found in undercooked meat from other infected mammals [29]. The acidic environment of the stomach releases parasites from oocysts or tissue cysts and once these parasites reach the anaerobic environment of the gut they infect intestinal epithelial cells, transform into replicative tachyzoites, and use recruited inflammatory monocytes and dendritic cells to disseminate via a Trojan Horse mechanism to diverse tissues throughout the body. In tissues, tachyzoites can develop into tissue cysts that are impervious to either anti-parasitic drugs or the resulting immune response.

Thus, like *Dictyostelium*, *Toxoplasma* is exposed to diverse O_2 tensions during its life cycle and how the parasite senses and responds to changing O_2 levels has been an open area of investigation. A clue about how *Toxoplasma* senses O_2 came from the discovery that homologues of the *Dictyostelium* Skp1 modification pathway genes are conserved in *Toxoplasma* [30] (Fig. 1C). *Toxoplasma* PhyA (TgPhyA) is 40% similar in sequence to DdPhyA and can complement Dd*phyA*-KO mutants in O_2-dependent culmination [31*]. Furthermore, TgSkp1, which contains the equivalent of Pro143, is glycosylated in a TgPhyA-dependent fashion, and purified TgPhyA is capable of hydroxylating TgSkp1 *in vitro* [31*]. Genetic disruption of the Tg*phyA* gene reveals that while TgPhyA is not essential its loss does reduce parasite growth at atmospheric O_2 and this growth defect is intensified at 0.5% O_2, a low but physiologically relevant O_2 tension. Slow growth suggests that, in the absence of an O_2 sensor, the parasite is unable to accommodate to the stress of low O_2. Based on studies in *Dictyostelium*, these results suggest that Skp1 prolyl hydroxylation and/or glycosylation allows *Toxoplasma* to adapt to decreased O_2 availability by altering the parasite's proteome.

Biochemical analysis of TgPhyA activity revealed an unexpectedly high affinity for O_2 [31*], which was in contrast to DdPhyA and the PHDs that have apparent low affinities for

O_2. Thus, only low O_2 levels, like those encountered in the gut, would be expected to impact TgPhyA activity. Nevertheless, TgPhyA can function in place of DdPhyA in O_2-dependent culmination [31*] suggesting that TgPhyA is regulated by both O_2 (at very low levels) and αKG, as its K_m for αKG is similar to that of DdPhyA [31*]. Since αKG is generated by the O_2-dependent TCA cycle, it is possible that TgPhyA senses O_2 indirectly via changes in αKG. Whether DdPhyA (and the metazoan PHDs) are similarly regulated awaits metabolomic profiling of *Toxoplasma* and *Dictyostelium* grown at various O_2 tensions.

Other protists and pathogens may employ the Skp1 mechanism to sense O_2

Genome analyses indicate that PhyA, Gnt1, and the target Pro residue in Skp1 co-exist in representatives of all 6 major protist phyla, but are evidently absent from fungi, animals, and higher plants (Fig. 3). Their broad but sporadic presence in diverse protozoans suggest that this is an ancient pathway that was retained during evolution via secondary gene loss. A striking example of this conservation is that the Skp1/PhyA/Gnt1 pathway, which was originally discovered in *Dictyostelium* and is present in other amoebazoae (e.g., *Acanthamoeba*), is conserved in *Toxoplasma* and closely related coccidians (*e.g. Neospora*) as well as in distantly related oomycetes (e.g. *Phytophthora)*. But, the pathway is absent in other amoebazoae (e.g., *Entamoeba* spp.) and apicomplexans including other coccidia (*Eimeria*) and hemosporidia (*Plasmodium*). This is significant because many of the organisms with the Skp1 modification pathway are human and agricultural pathogens and this pathway represents a novel drug target since their hosts do not express it.

Other O_2-sensing mechanisms in fungal and protist pathogens

The secondary loss of Skp1 modification genes may have occurred in organisms that live anaerobically, only experience constant O_2 levels, or acquired other O_2 sensing mechanisms. We identified in apicomplexan, including those lacking PhyA such as *Plasmodium*, a second related P4H we have named PhyB (unpublished data). Further studies are needed to identify PhyB target(s) and determine how PhyB senses O_2.

Trypanosomatids, such as the important human parasites *Trypanosoma cruzi*, *T. brucei*, and *Leishmania* spp., and other diversely related protists (e.g., euglenoids) lack genetic evidence for PhyA-related P4Hs. Thus, other O_2-sensing proteins are likely to be utilized by these organisms and O_2 and α-ketoglutarate utilizing dioxygenases are likely candidates. These organisms have a novel nuclear DNA modification called base J that affects transcription initiation and termination [32,33]. Base J is formed by the action of a DNA-thymine hydroxylase (JBP1/2), which is a non-heme containing O_2 and α-ketoglutarate utilizing dioxygenase, and the resulting 5-hydroxymethyluracil (hmU) is capped with a β-glucose. Thus, the base J pathway parallels the Skp1 modification pathway in which a hydroxylated target serves as a glycosyltransferase substrate. Base J synthesis is reduced in *Trypanosoma cruzi* cells exposed to hypoxia suggesting that the JBP1/2 are O_2 sensors in trypanosomes [34*].

Protozoans may utilize other types of O_2 sensors. For example, non-heme O_2- and αKG-dependent dioxygenases that may function as O_2 sensors include asparagine, histidine and arginine hydroxylases [35] and Jumonji-domain containing proteins that epigenetically regulate gene expression by promoting Lys methylation and histone hydroxylation [9,36,37]. Finally other proteins may sense changes in O_2 availability due to O_2's ability to directly induce structural changes to those proteins that it binds. As an example, O_2 binding to a Fe-heme prosthetic group in an adenylate cyclase of *L. major* stimulates cAMP formation [38*].

Intracellular parasites utilize both parasite and host O_2 sensing

As a parasite traverses through the body, oxygen sensors such as PhyA and JBP1/2 are likely important for parasite survival and virulence. An infection is, however, a complex environment consisting of multiple types of cells (e.g. parasites, resident tissue cells, and recruited leukocytes) and each must properly function in order for the host and/or parasite to survive and thrive. *Toxoplasma* and other obligate intracellular parasites must ensure that both it and its host cell are in a metabolic state permissive for parasite growth. The first clue that host oxygen sensing plays a role in establishing this state was the finding that *Toxoplasma* activates host HIF1α [39]. Surprisingly, this was not simply a consequence of establishing a hypoxic microenvironment due to parasite oxygen consumption. Rather, *Toxoplasma* specifically activates HIF-1 by a mechanism that involves activin receptor signaling and inactivation of host PHD2 expression and activity [40]. Most significantly, parasite activation of HIF-1 is required for parasite growth under physiological O_2 tensions and does so, in part, by increasing host hexokinase-2 expression (unpublished results).

Other intracellular parasites such as *Leishmania* and *Theileria* activate HIF-1 in the infected host cell although it isn't clear whether this is needed for parasite growth or supports a host defense pathway. In macrophages, *Leishmania* activates HIF-1 through a distinct mechanism involving transcriptional upregulation of HIF-1α and depletion of intracellular Fe to cause reduced PHD activity [41]. This mechanism is reminiscent to how HIF-1 is activated to promote resistance to pathogenic bacteria [42] suggesting that HIF-1 acts to promote resistance to *Leishmania*.

Summary and implications for the future

O_2-sensing is important for protists to grow in the varied environments that they encounter. We are beginning to see that multiple mechanisms have evolved to sense and respond to changes in O_2-availability, and even to perhaps manipulate the O_2-consumption of host cells for their benefit. We highlighted here recent progress in the elucidation of biochemical mechanisms involving a homolog of the animal PHD O_2-sensor whose function is not to regulate the stability of a single transcriptional factor but rather the entire proteome itself. The restriction of this pathway to unicellular parasites and other protists implies that rapid protein turnover in response to altered O_2-levels may be more adaptive for single cells than the slower-acting transcriptional mechanism utilized by animals. Clearly much remains to be done to confirm this speculative model, to determine if it is the evolutionary precursor of the animal O_2-sensing mechanism, and to assess its utility as a drug target.

Acknowledgments

Research from the authors' labs summarized in this article was supported by grants from the NIH (R01AI069986, R01GM037539, R01GM084383), the Oklahoma Center for the Advancement of Science and Technology, and the Mizutani Foundation for Glycoscience.

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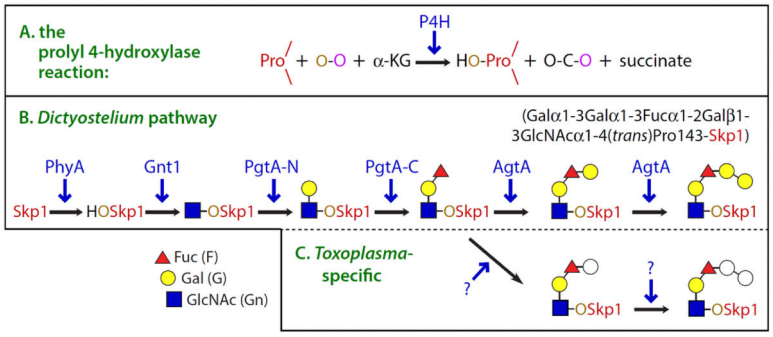

Fig. 1.

The Skp1 modification pathway in *Dictyostelium* and *Toxoplasma*. (A) Summary of the reaction catalyzed by the P4H class of non-heme dioxygenases, in which a protein-linked Pro residues is subject to hydroxylation at its 4-position by an O-atom derived from O_2 (O-O). The other O-atom is transferred to α-KG, which decomposes into CO_2 and succinate. (B) The hydroxyl group generated by the action of the P4H PhyA on Skp1 provides the site for glycosylation by five glycosyltransferase activities encoded by 3 genes in *Dictyostelium*. (C) *Toxoplasma* also assembles a pentasaccharide with the same Hex-Hex-dHex-Hex-HexNAc– composition (unpublished data). Tg-PhyA mediates Skp1 hydroxylation and is required for glycosylation, which has been confirmed by mass spectrometry (in preparation). *gnt1* and *pgtA* are conserved in *Toxoplasma*, and likely modify *Toxoplasma* Skp1 as they do in *Dictyostelium*. However, the physical order of the N- and C-terminal catalytic domains of Dd-PgtA, which mediate the addition of β-Gal and α-Fuc, respectively, is reversed in *Toxoplasma*.

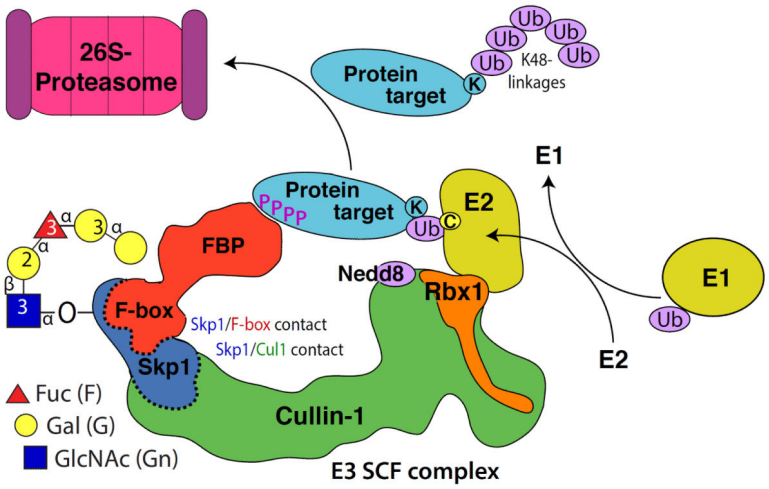

Fig. 2.

Skp1 hydroxylation and glycosylation likely regulate the SCF complex, an E3 ubiquitin ligase that marks target proteins with K48-linked polyubiquitin chains that are recognized by the proteasome for degradation. Polyubiquitination involves successive transfers of Ub from Ub-E2 donors that cycle on, off, and on again after recharging from Ub-E1. The process is controlled by, in some cases, substrate priming such as phosphorylation ($P=PO_4$) as indicated. Ubiquitination is also regulated by neddylation of Cul1, which is under the control of the COP9 signalosome and other factors and promotes flexible tethering of Rbx1 and its associated Ub-E2 to allow access to the Ub target site (K=Lys) on the substrate. Neddylation also excludes Cand1 (not shown), which allows docking of FBP/Skp1 complexes to Cul1 (dashed interface). Skp1 modifications, which occur near its C-terminus, are proposed to promote interactions of FBPs with Skp1 (dashed interface), providing novel SCF-specific regulation. Modified from [42].

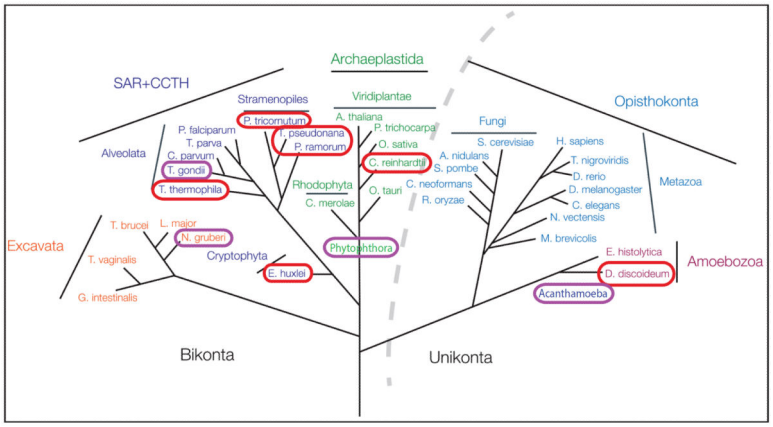

Fig. 3.

Phylogenetic distribution of Skp1 modification genes. Organisms predicted to modify Skp1, based on possession of *phyA*-like and *gnt1*-like genes, and a Skp1 with the equivalent of Pro143 in *Dictyostelium* Skp1, are encircled. Those in violet can cause human or plant disease. Phylogenetic tree of eukaryotic evolution is from [43].