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Oxygenases for oxygen sensing*

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Abstract: In animals, cellular and physiological responses to oxygen level variations are regulated via the post-translational modification of the heterodimeric hypoxia-inducible transcription factor (HIF). Hydroxylation of the HIF-α subunit at either of two conserved prolyl residues enables binding to the von Hippel–Lindau protein (pVHL) elongin C/B complex (VCB) which targets HIF-α for degradation via the ubiquitin proteasome pathway. Hydroxylation of an asparaginyl residue in the C-terminal transcriptional activation domain of HIF-α reduces its interaction with the transcriptional coactivator p300. Thus, post-translational hydroxylation is used both to "make" (HIF-VCB) and "break" (HIF-p300) protein–protein interactions in the hypoxic response. The requirement for oxygen of the HIF prolyl and asparaginyl hydroxylases in catalysis links changes in oxygen concentration and transcription of the gene array that enables cells to adapt to hypoxia. All four identified human HIF hydroxylases are members of the Fe(II) and 2-oxoglutarate (2OG)-dependent family of oxygenases. Inhibition of HIF hydroxylases mimics the hypoxic response resulting in the upregulation of erythropoietin (EPO), vascular endothelial growth factor (VEGF), and other proteins of biomedicinal importance. We briefly review biochemical analyses on the HIF hydroxylases and discuss how their structural and mechanistic characteristics may make them suited to their oxygen-sensing role.

Keywords: hypoxia; oxygen; hydroxylase; 2-oxoglutarate; hypoxia-inducible factor.

HYPOXIA-INDUCIBLE FACTOR

Hypoxia-inducible factor (HIF) is an α,β-heterodimeric transcription factor that regulates the manner in which cells adapt to hypoxia in most, if not all metazoans, including humans. Both HIF-α and HIF-β are basic helix–loop–helix (bHLH) proteins of the PAS family [PER (period circadian protein), ARNT (aryl-hydrocarbon-receptor nuclear translocator), SIM (single-minded protein)] [1]. The α,β-HIF heterodimer binds to a core DNA motif (G/ACGTG) in hypoxia-response elements (HREs) that are regulatory elements present in the promoter regions associated with a range of genes involved in the hypoxic response [2]. To date, >100 human genes are known to be directly regulated by HIF, and it is predicted that there may be up to 200–300 human HIF target genes [3]. HIF-regulated genes play central roles in both systemic responses to hypoxia, such as cell proliferation, angiogenesis and erythropoiesis, and in intracellular responses, such as regulation of glycolysis (for reviews, see [4–6]). HIF-regulated

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genes include those encoding for proteins that are of medicinal interest such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and the inducible form of nitric oxide synthase (iNOS).

In 2001, it emerged that a predominant oxygen sensing component of the HIF system comprises oxygenase enzymes that catalyze the post-translational modification of HIF-α [7–9]. There is no evidence that human communities living with conditions of unusually low oxygen availability suffer from increased incidence of cancer or other chronic diseases. Thus, it can be argued that therapeutic manipulation of the HIF system is best carried out at the point(s) at which oxygen availability is sensed, i.e., the HIF hydroxylases and/or as yet unidentified sensors.

The biomedicinal roles of HIF-regulated genes have led to significant academic and pharmaceutical interest in manipulating the hypoxic response for therapeutic benefit, including in the fields of anemia, ischemic disease, and oncology (for reviews, see [10–12]). Artificial induction of the natural HIF-mediated hypoxic response might be used to treat ischemic disease or anemia; inhibition of the hypoxic response might be used for the treatment of tumors via inhibition of angiogenesis. Given the extensive precedent for enzymes, including metalloenzymes such as cyclooxygenases, as targets for therapeutic intervention using small molecules, inhibition of the HIF hydroxylases is of particular interest. Below we summarize studies on the biochemistry of the HIF system (for an overview, see Fig. 1), with particular emphasis on the HIF hydroxylases (for other relevant reviews, see [5,6,13–15]).

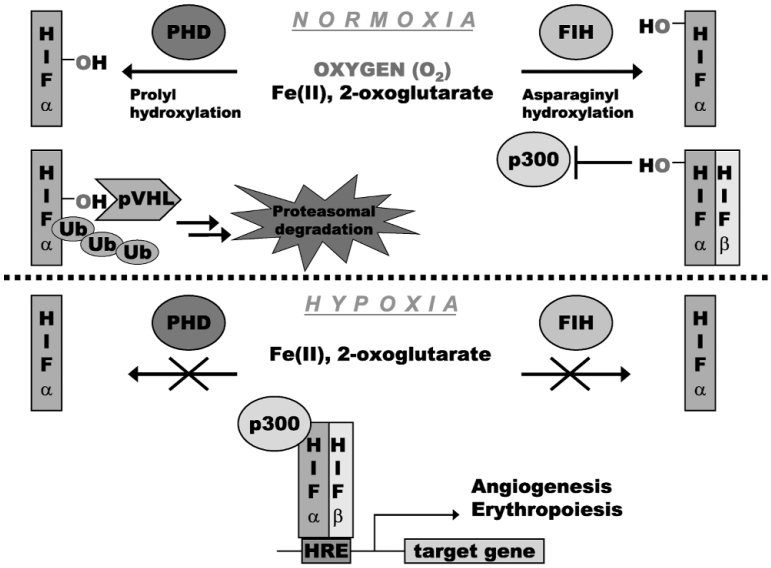

Fig. 1 Dual regulation of HIF-α subunits in normoxia by prolyl and asparaginyl hydroxylation. In the presence of oxygen (*normoxia*), active HIF hydroxylases (PHDs and FIH) downregulate and inactivate HIF-α subunits. The PHDs hydroxylate a prolyl residue in the N- and C-terminal ODDDs. This enhances binding to pVHL, with subsequent ubiquitin-(Ub)-mediated proteolysis, resulting in the degradation of HIF-α subunits. FIH hydroxylates an asparaginyl residue in the C-terminal activation domain, which blocks p300 coactivator recruitment and results in the transcriptional inactivation of HIF-α subunits. In *hypoxia*, HIF hydroxylase activity is suppressed, allowing the formation of a transcriptionally active complex by dimerization of HIF-α with HIF-β subunits and translocation into the nucleus. Subsequent binding to HREs on HIF target genes activates their expression.

Both levels of the HIF-α subunits and their transcriptional activity are regulated by oxygen availability. There are three related forms of human HIF-α (HIF-1α, HIF-2α, and HIF-3α), each encoded by a separate genetic locus [16,17]. HIF-1α and HIF-2α have closely related domain architectures,

whereas HIF-3α is less closely related, and not as well studied. One splice variant of murine HIF-3α, inhibitory PAS domain protein (IPAS), inhibits the induction of HIF-mediated transcription via dimerization with HIF-1α, and high IPAS levels in the cornea may inhibit angiogenesis there [18]. In contrast to HIF-α, HIF-β (ca. 90 kDa), which is identical to ARNT, apparently has no direct sensing role in the hypoxic response.

Providing that oxygen is not limiting, both the HIF-1α and HIF-2α subunits (ca. 95 kDa) are degraded by the proteasome: HIF-1α and HIF-2α both have a central oxygen-dependent degradation domain (ODDD), composed of two subdomains (NODDD and CODDD), each of which is sufficient to enable degradation of HIF-α [19]. The von Hippel–Lindau protein (pVHL) elongin C/B (VCB) complex enables binding of the hydroxylated ODDD of HIF-α to an E3 ubiquitin ligase complex that catalyzes lysyl ubiquitinylation of HIF-α, so marking it for proteasomal degradation [20–22].

HIF-1α and HIF-2α also possess two transcriptional activation domains: The C-terminal transactivation domain (CTAD), and the N-terminal transactivation domain (NTAD), the latter of which overlaps with the CODDD. Transcriptional activation by the CTAD is reduced in the presence of nonlimiting oxygen by prevention of its interaction with the CH-1 (cysteine/histidine rich) domain of the transcriptional coactivator p300 [9,23].

Formation of the α,β-HIF heterodimer complex together with binding of necessary coactivators including p300, CBP, SRC-1, and TIF2, forms a transcriptionally active complex that binds to the pentanucleoside hypoxic response element sequence (HRE). Dimerization of the α and β subunits occurs via their bHLH and PAS domains [24].

HIF PROLYL AND ASPARAGINYL HYDROXYLATION

Both the HIF-α and HIF-β subunits are produced constitutively, but in normoxia, when oxygen is not limiting, HIF-1α and HIF-2α undergo oxygen-dependent post-translational hydroxylations that deactivate them and signal for their proteasomal degradation (Fig. 2; for reviews, see [5,6,25–27]). Hydroxylation of HIF-1/2α at conserved prolyl residues in NODDD and CODDD (Pro402 and Pro564, respectively, in HIF-1) regulates the interaction of HIF-α with VCB. *trans*-4-Prolyl hydroxylation increases the affinity of HIF-α peptides for the VCB complex by about 1000-fold [28]. Structural analyses of a hydroxylated HIF-1α CODDD peptide fragment complexed to VCB revealed that binding of

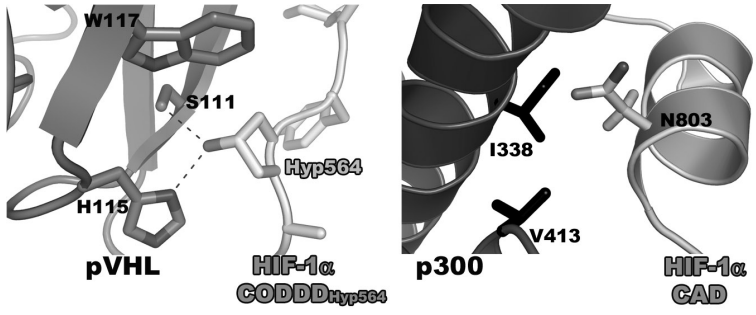

Fig. 2 The effect of hydroxylation on HIF activity. (a) Hydroxylation of HIF-1α CODDD Pro564 (Hyp564) enables hydrogen bonding with the hydroxyl group of Ser111 and the imidazole group of His115 in pVHL. This interaction forms the basis of the interaction between these two proteins, resulting in proteasome-mediated degradation of HIF-α. (b) Hydroxylation of HIF-1α CTAD Asn803 (β-hydrogens modeled in gray) prevents its local secondary structure formation and disrupts binding in a hydrophobic pocket of p300.

hydroxylated HIF-α CODDD is mediated, at least in part, by two optimized hydrogen bonds, occurring between the alcohol of the hydroxylated proline and Ser111 and His115 of pVHL in VCB [28,29]. In these structures, the pyrrolidine ring of CODDD hydroxyprolyl-564 is observed in the C^4-exo conformation. Notably, this is the same conformation as observed for hydroxyprolyl residues in collagen where prolyl hydroxylation serves to stabilize the collagen triple helix via operation of the stereoelectronic gauche effect [30]. In hypoxia, the rate of prolyl-4-hydroxylation is slowed, allowing HIF-α to escape proteasomal degradation and act as a transcriptional activator.

Mutations in the pVHL protein cause VHL syndrome, which is associated with tumor growth and affects one in 35 000 humans (for review, see [4]). Inherited mutations in the *VHL* gene can also cause erythropoiesis, which can occur as part of VHL syndrome or independently. The functional effects of certain mutations in pVHL leading to von Hippel–Lindau disease have been rationalized by the structures of VCB in complex with $HIF_{(Hyp564)}$ peptide [28,29].

HIF-α hydroxylation also occurs at human HIF-1α Asn803 (Fig. 2b) [9]. 4*S*-Hydroxylation of Asn803 ablates the binding of HIF-1α to p300/CBP (CREB binding protein), a transcriptional coactivator protein [31]. NMR studies of an unmodified CTAD polypeptide in complex with CH-1 reveal that HIF-1α Asn803 is part of an α-helix buried at the complex interface and suggest that β-hydroxylation of Asn803 may prevent HIF-1α binding to CH-1 by disrupting both the hydrophobic interactions between the molecules and/or formation of the α-helix adopted by CTAD in this complex [32,33].

HIF HYDROXYLASES

Three closely related human Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenases catalyze human HIF prolyl hydroxylation—the prolyl hydroxylase domain enzymes (PHD)1, PHD2, and PHD3 (also known as EGLN 1-3 enzymes) [34,35]. The three human PHDs exhibit different degrees of selectivity for CODDD over NODDD, with PHD3 being the most selective and apparently only accepting CODDD in studies with isolated protein and HIF fragment peptides [35–38].

In the consensus mechanism for 2OG oxygenases (Fig. 3), they catalyze the oxidative decarboxylation of 2OG to give succinate (into which one of the atmospheric dioxygen atoms is incorporated [39]), carbon dioxide, and an enzyme-bound Fe(IV)=O ferryl intermediate that effects the two-electron oxidation (hydroxylation) of the substrate [40–42]. Crystallographic analyses on PHD2 (Fig. 4), thought to be the most important of the PHDs involved in the hypoxic response in the majority of normoxic tissues, has revealed the archetypal double-stranded β-helix core fold of the 2OG oxygenase family, and a triad of conserved iron-binding residues [43]. The narrow active site opening apparent in the structure also suggests why PHD2 has an unusually high affinity for iron and 2OG compared to other studied family members [44].

Various kinetic analyses have been carried out on the HIF hydroxylases; their oxygen-binding capabilities appear to be within the normal range for 2OG oxygenases, making them suited to act as sensing enzymes under physiological conditions [37,45,46]. Recent kinetic studies also indicate that the preferred substrates for the PHDs are significantly longer than the relatively short peptides (<20–40 residues) used in some analyses [45,47]. Coupled with the structural analyses, it seems likely that the substrate specificity of the PHDs, in part, is determined by regions relatively remote from the iron center, with biochemical assays suggesting the involvement of a substrate binding "lid" [48,49].

Two identified inherited mutations in PHD2, Pro317Arg and Arg371Pro, are linked with familial erythrocytosis [50,51]. Pro317 is located two residues from the iron-binding aspartyl residue (Asp315) in a β-turn and is located close to the active site entrance. Arg371 of PHD2 is also located close to another metal-binding residue, His374. These mutations may therefore affect metal binding or catalysis, or as they involve proline residues and are thus likely to affect protein structure, may also cause disease by perturbing NODDD or CODDD binding.

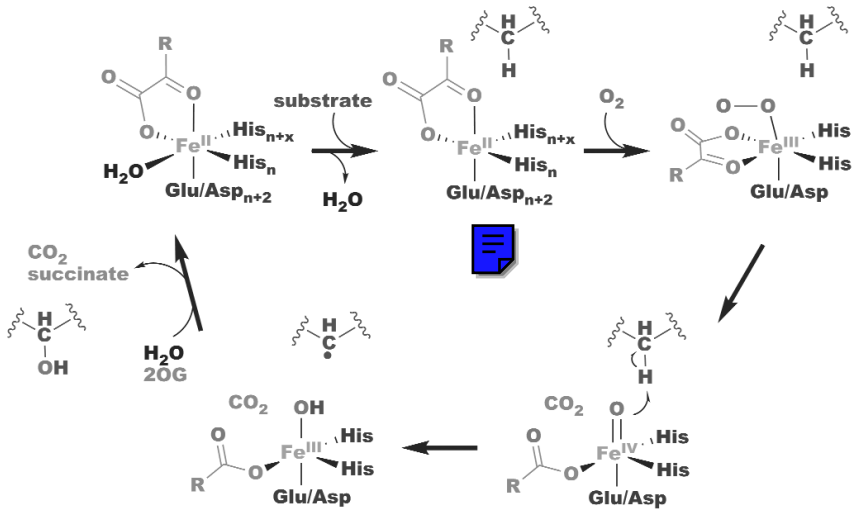

Fig. 3 Mechanistic proposal for the HIF hydroxylases. Fe^{2+} is bound to the enzyme through the HXD/E…H motif, with the remaining three coordination sites being occupied by the cosubstrate 2OG and a water molecule. Substrate binding displaces a water molecule from Fe^{2+}, which allows the productive binding of triplet-state molecular oxygen. Subsequent oxidative decarboxylation of 2OG generates carbon dioxide, succinate, and a ferryl (Fe^{IV}=O) species. The latter is responsible for hydroxylation of the substrate by hydrogen abstraction and subsequent rebound of a hydroxyl group. The point at which carbon dioxide leaves the active site is unclear.

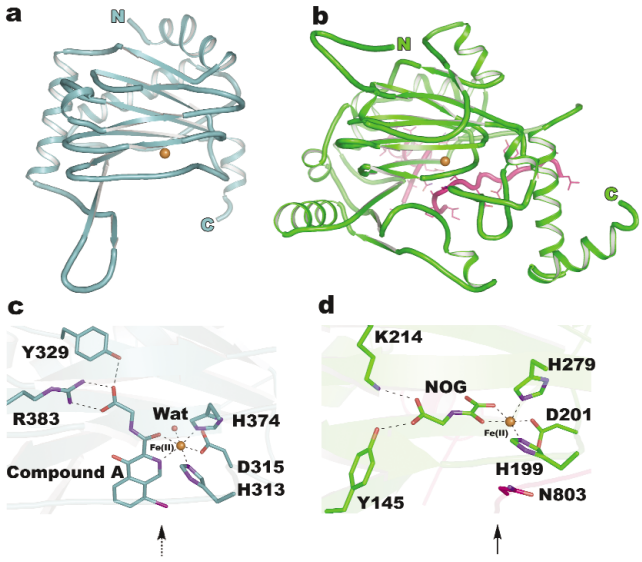

Fig. 4 Views from the crystal structures of the HIF prolyl (a, PHD2) and asparaginyl (b, FIH) hydroxylases. Close up view of the active sites of PHD2 (c) and FIH (d) showing octahedral coordination of Fe(II) with three protein ligands (HXD…H), the 2-oxoglutarate analogs and a water molecule. Solid/dotted arrows represent observed/putative sites for substrate entrance. A metal-bound water molecule is sometimes observed in Fe(II)/2OG oxygenases in the absence of substrate representing a potential oxygen binding site.

HIF asparaginyl hydroxylation is catalyzed by factor-inhibiting HIF (FIH) [52,53]. FIH was originally identified as a protein that binds HIF-α [54], and is also a 2OG oxygenase, but one that is significantly different from the PHDs [55–57]. Although the general mechanisms and double-strand β-helix core structures of FIH and PHDs are likely similar, there are significant differences between their active sites and overall protein folds.

Structures have been reported for FIH with peptide substrates [56] (Fig. 4). FIH, which unlike PHD is a homodimer, has been crystallized in the presence of a CTAD peptide, and the resultant structures revealed the existence of two distinct FIH-CTAD interaction sites, one involving the hydroxylation site ($CTAD_{795–806}$) and a second lying to the C-terminal of this site ($CTAD_{813–822}$). At the hydroxylation site, the $CTAD_{795–803}$ residues are bound in a groove and adopt an extended conformation linked to FIH by hydrogen bonds and hydrophobic interactions. Asn803 of the CTAD is completely buried at the active site and its β-carbon is directly adjacent to the Fe(II). CTAD Asn803 and Ala804 form an inverse γ-turn, stabilized by a hydrogen bond between the backbone carbonyl of Val802 and NH of Ala804, which projects the side chain of Asn803 toward the Fe(II).

RELATIONSHIP OF THE HIF HYDROXYLASES TO OTHER HUMAN 2OG OXYGENASES

2OG oxygenases other than the HIF hydroxylases play important roles in human physiology (Fig. 5). They have long been known to catalyze the post-translational hydroxylation of procollagen prolyl and lysyl residues [58]: Prolyl-4-hydroxylation stabilizes the collagen triple helix whilst lysyl hydroxylation serves as an attachment point for glycosylation. 2OG oxygenases catalyze two hydroxylation steps in the biosynthesis of carnitine, hydroxylation of trimethyllysine, and γ-butyrobetaine [59]. Phytanoyl CoA hydroxylase plays a central role in the degradation of the fatty acid side chain of chlorophyll [60,61]. One member of the family, the epidermal growth factor (EGF) hydroxylase, catalyzes the β-hydroxylation of asparaginyl, and unlike FIH, also aspartyl residues in EGF domains [62,63]. The physiological role of the EGF hydroxylases is not well understood, but it has been linked with cancer [64]. 2OG oxygenases can repair certain forms of methylated nucleic acids by an indirect demethylation via hydroxylation of the methyl groups followed by a retro-aldol reaction. In bacteria, there is clear evidence that AlkB plays a repair role [65]. In humans, 8 AlkB homologs are apparent from sequence analyses, but at the time of writing only two have been shown to be nucleic acid demethylases [66].

In Oxford, we have been interested in attempting to identify new substrates for the PHDs and FIH. Various alternative substrates have been proposed for the PHDs, including RNA polymerase II and IκB kinase-β (which is negatively regulated by PHD1) [67,68], however, to date none of these substrates have been verified by direct demonstration of hydroxylation using mass spectrometric analyses. In contrast, FIH has been shown to also catalyze hydroxylation of ankyrin repeat domain (ARD) proteins from the NFκB (nuclear factor κB) and Notch family at highly conserved asparaginyl residues [13,69]. ARD proteins are ubiquitous, with >200 human members of the family being predicted. Recent evidence suggests that ARD hydroxylation may be common in human cells. If correct, this proposal is important as it has been widely perceived that post-translational hydroxylation of cytoplasmic proteins in eukaryotes is rare [70]. The significance of ARD hydroxylation on signaling in non-HIF pathways is unclear. ARD hydroxylation may serve to stabilize the ARD fold in a similar way that prolyl-4-hydroxylation stabilizes the collagen triple helix [30]. Hydroxylated ARD proteins bind less tightly to FIH than their unhydroxylated FIH substrates. It is proposed [71] that the hydroxylation status of the pool of ARD proteins capable of interacting with FIH regulates the amount of FIH that is "free" to hydroxylate HIF-α. If the idea of regulation by the post-translational modification status of a pool of proteins is correct for the HIF system, it would seem possible that it would apply to other regulatory systems.

Biomedicinal interest in the HIF hydroxylases has helped to stimulate functional assignment studies for the putative 50–100 members of the 20G oxygenase family predicted from human genome sequence analyses [72,73]. The PHDs, which appear to form a tight family of three (possibly four) human proteins [35], are well conserved at least from worms through to humans, with the possibility of closely

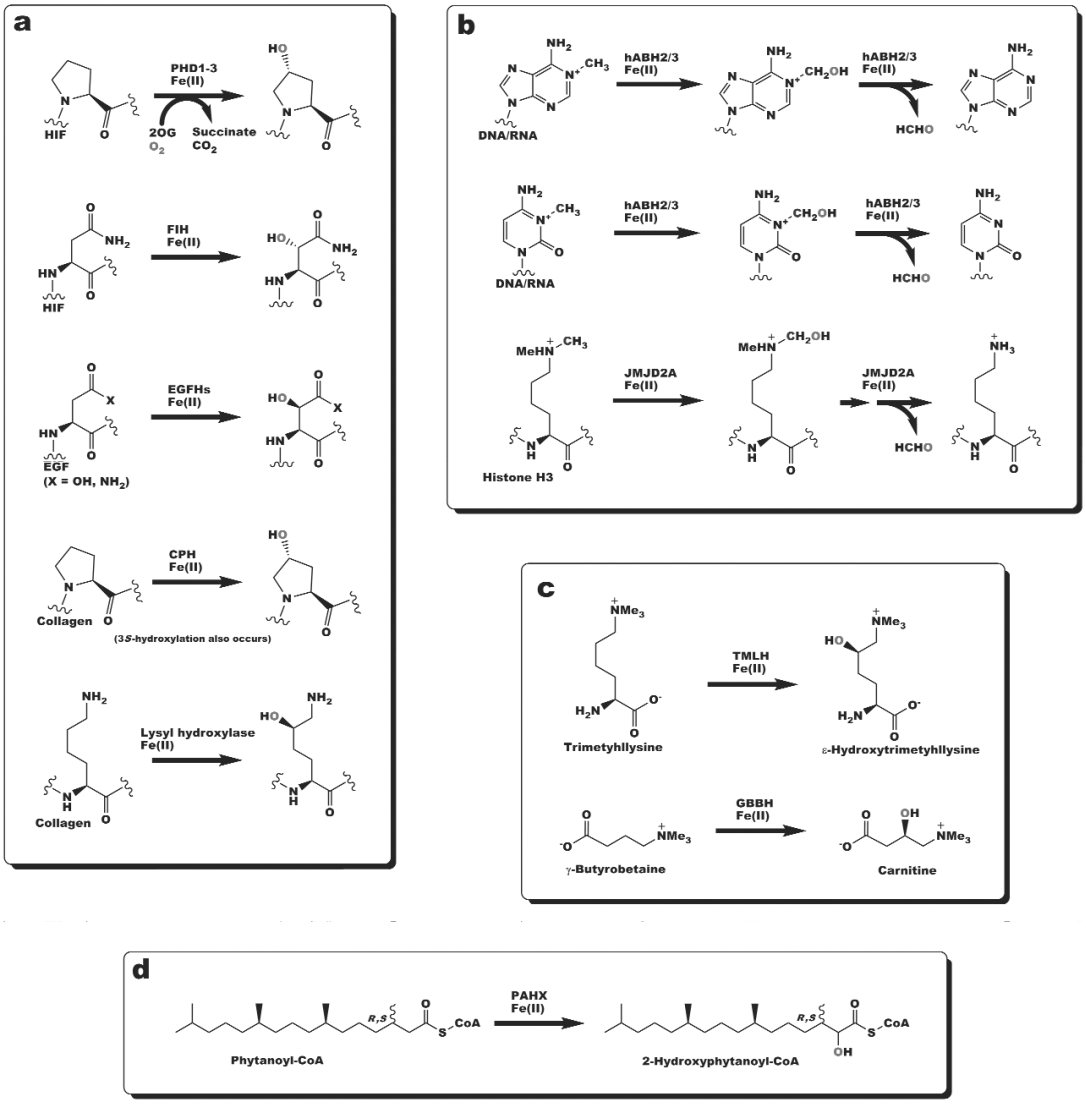

Fig. 5 Reactions catalyzed by the HIF hydroxylases and related human oxygenases. Each reaction is coupled to the conversion of oxygen (O_2) and 2OG to succinate and CO_2, and its substrate is indicated. (a) Hydroxylation reactions utilizing protein substrates, catalyzed by PHDs, FIH, endothelial growth factor hydroxylases (EGFHs), collagen prolyl hydroxylase (CPH), and collagen lysyl hydroxylase. Interestingly, EGFHs catalyze 4*R*-asparaginyl/aspartyl hydroxylation as opposed to 4*S*-asparaginyl hydroxylation of HIF-α substrates by FIH. (b) Hydroxylation-induced demethylation reactions on DNA/RNA and protein substrates, including those catalyzed by alkylated DNA repair protein homologs 2 and 3 (ABH2/3) and JmjC domain-containing protein 2A (JMJD2A). (c) Hydroxylation reactions occurring on amino acid derivatives, catalyzed by trimethyllysine hydroxylase (TMLH) and γ-butyrobetaine hydroxylase (GBBH). (d) Hydroxylation reaction of a fatty acid metabolism intermediate by phytanoyl-CoA α-hydroxylase (PAHX). See text for references.

related enzymes in a limited range of bacteria [43]. In contrast, analysis of the FIH sequence, especially in the light of its crystal structure, revealed it was part of a much wider family of 2OG oxygenases present in most types of life [52,56]. In fact, enzymes related to FIH were already the subject of bioinformatic analysis which has identified them as zinc-dependent transcription factors or transcription/chro-

matin-associated proteins [74]. The assigned role of FIH, together with structural analyses, implied that many of these proteins, termed the Jmj family, were very likely to be Fe(II)-dependent oxygenases involved in transcription [52]. This proposal was verified in an exciting manner when Zhang and colleagues discovered that one of the JmjC proteins (JHDM1) was a histone lysyl demethylase [75,76]. This was only the second histone lysyl demethylase to be identified, the first one being the lysyl specific demethylase (LSD) enzyme [77]. These discoveries opened a new subfield in chromatin biology, and a series of papers describing new members of the Jmj histone lysyl demethylases have recently been published (for review, see [73]). One challenge within this field is to define how individual Jmj enzymes achieve selectivity for different histone sequences and lysyl methylation status.

Another link involving 2OG oxygenases and disease has recently been uncovered. A genomic search for type 2 diabetes susceptibility genes identified a common variant in the *FTO* (fat mass and obesity associated) gene whose presence is associated with an increased body mass index and which predisposes individuals to diabetes [78]. Recent work has shown that the FTO protein is an Fe(II)- and 2OG-dependent oxygenase that catalyzes the demethylation of methylated nucleic acids [79]. Purified recombinant FTO was shown to catalyze the Fe(II) and 2OG-dependent demethylation of a 3-methylthymine and, to a lesser extent, 1-methyladenine and 3-methylcytosine in DNA concomitant with the production of carbon dioxide, succinate and formaldehyde. FTO activity was inhibited by Fe(II) chelators and known 2OG oxygenase inhibitors. Consistent with its proposed role in nucleic acid demethylation, FTO is localized to the nucleus. It is presently unclear as to how the demethylation activity of FTO is related to obesity.

Simply because an enzyme requires oxygen for catalysis, it does not mean that it acts as an oxygen sensor. Further, since compensatory factors may be induced, it does not even mean that its reaction step is necessarily oxygen-dependent in the cell within normal physiological ranges. Nonetheless, given the roles of the HIF hydroxylases, there would seem to be a reasonable possibility that other human 2OG oxygenases are involved in oxygen sensing.

FUTURE PROSPECTS

Recent years have seen significant advances in our understanding of the molecular mechanisms of the hypoxic response. The HIF hydroxylases have been identified as leading players in the oxygen-dependent regulation of the HIF system and are current targets for therapeutic intervention. One major challenge for molecular studies in the HIF field is whether it will be possible to quantitatively correlate data on isolated components, such as the rate of HIF hydroxylation under different oxygen concentrations, with the physiological response in an intact organism. Such work could involve studies ranging from biochemistry and structural biology to physiological analysis on mutated animals. The technical difficulties are significant and complicated by the many possible factors that can have an impact on the HIF system, but such work will be essential if we are to achieve detailed molecular understanding of oxygen sensing.

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