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2-Oxoglutarate-Dependent Oxygenases

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Abstract

2-Oxoglutarate (2OG)-dependent oxygenases (2OGXs) catalyze a remarkably diverse range of oxidative reactions. In animals, these comprise hydroxylations and *N*-demethylations proceeding via hydroxylation; in plants and microbes, they catalyze a wider range including ring formations, rearrangements, desaturations, and halogenations. The catalytic flexibility of 2OGXs is reflected in their biological functions. After pioneering work identified the roles of 2OGXs in collagen biosynthesis, research revealed they also function in plant and animal development, transcriptional regulation, nucleic acid modification/repair, fatty acid metabolism, and secondary metabolite biosynthesis, including of medicinally important antibiotics. In plants, 2OGXs are important agrochemical targets and catalyze herbicide degradation. Human 2OGXs, particularly those regulating transcription, are current therapeutic targets for anemia and cancer. Here, we give an overview of the biochemistry of 2OGXs, providing examples linking to biological function, and outline how knowledge of their enzymology is being exploited in medicine, agrochemistry, and biocatalysis.

Contents

INTRODUCTION

Following pioneering studies identifying their roles in collagen (1) and small-molecule biosynthesis, 2-oxoglutarate (2OG; also known as α-ketoglutarate)-dependent oxygenases (2OGXs) have emerged as a widely distributed superfamily in aerobic biology (2). Most (likely >95%) 2OGXs employ Fe(II) as a cofactor and 2OG and O_2 as cosubstrates, producing CO_2 and succinate as coproducts (**Figure 1**). In terms of both substrate and product selectivity, 2OGXs are among the most biochemically flexible enzymes (**Figure 2**) (3). 2OGXs catalyze oxidation of both oligomeric substrates (proteins, nucleic acids, lipids) and small molecules, the latter in isolated form and when tethered to peptides (4). The most common 2OGX-catalyzed reactions are hydroxylations, including *N*-methyl, and, to a lesser extent, *O*-methyl demethylations, which proceed via initial hydroxylation (**Figure 2**; **Supplemental Table 1**) (5, 6). 2OGXs also catalyze more exotic transformations, commonly in secondary metabolism, where their roles are medicinally relevant, e.g., during β-lactam biosynthesis. This review provides a concise overview of current knowledge of structural, mechanistic, and functional studies on 2OGXs, guides the reader to specialist reviews, and identifies avenues for future research.

Supplemental Material

2OG: 2-oxoglutarate

2OGX: 2-oxoglutarate-dependent oxygenase

Oxygenase: enzyme that catalyzes substrate oxidation with oxygen transfer from dioxygen

Double-stranded β-helix (DSBH): core fold of 2OGX; also characteristic of JmjC and cupin-fold proteins

STRUCTURAL AND MECHANISTIC OVERVIEW OF 2OGXs

Extensive crystallographic analyses reveal 2OGX structures are characterized by a distorted double-stranded β-helix (DSBH; also known as jelly-roll) core fold (**Figure 3**) (6–10). The right-handed class I DSBH fold occurs in many protein classes and is the only one of four possible DSBH folds observed in biology. The DSBH fold is also characteristic of cupin and JmjC proteins, many of which are 2OGXs (9). The DSBH fold in 2OGXs was predicted following studies on isopenicillin N synthase (IPNS), which does not use 2OG, but is structurally homologous with and mechanistically related to 2OGXs. Crystallography of deacetoxycephalosporin C synthase (DAOCS) (11), taurine dioxygenase (12), and clavaminic acid synthase (13) confirmed the presence of the DSBH in 2OGXs and defined subfamily-characteristic features (**Figure 3**) (7–10).

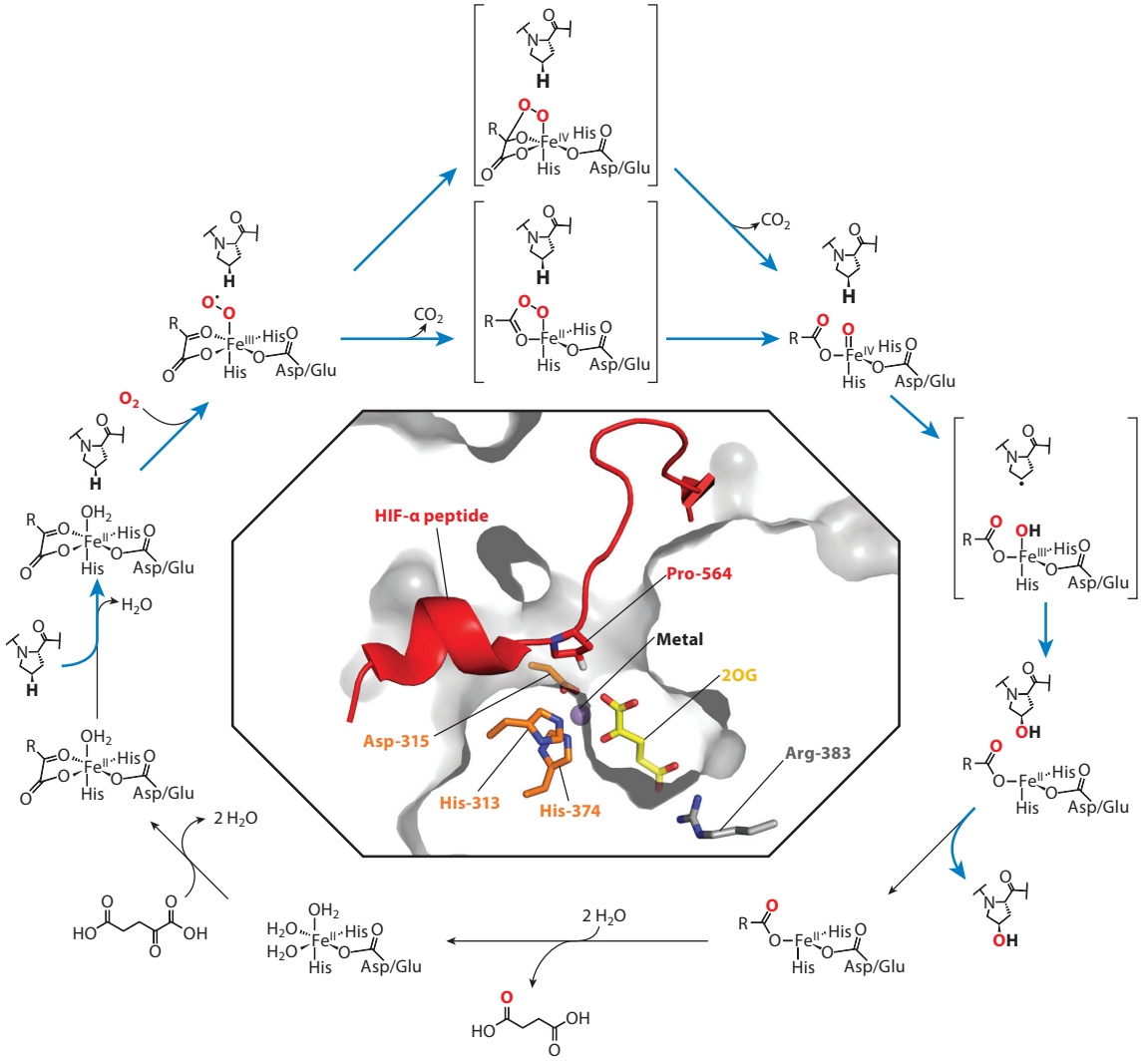


Figure 1

Consensus mechanism for 2OGX-catalyzed hydroxylation. Following bidentate coordination of 2OG to Fe(II), substrate binding (e.g., for a prolyl residue) proximal to Fe(II) normally weakens binding of a metal-ligated water molecule, providing a vacant coordination site for O_2. Oxidative decarboxylation of 2OG forms succinate, CO_2, and an Fe(IV)-oxo intermediate. The latter reacts with substrate C–H to form an alcohol concomitant with reduction of Fe(IV) to Fe(II). Note the coordination position of the 2OG C-1 carboxylate can vary (see text). (*Inset*) View from a structure of human PHD2 complexed with Mn(II) [substituting for Fe(II) in crystallography], 2OG, and a fragment of the HIF-α with the oxidized C–H bond shown projecting toward the metal (PDB ID: 5L9B). Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; HIF-α, hypoxia-inducible factor alpha; PDB, Protein Data Bank; PHD2, prolyl hydroxylase domain 2.

The eight-stranded (I–VIII) 2OGX DSBH core fold has major (I, III, VI, VIII) and minor (II, IV, V, VII) β-sheets forming a squashed barrel, the more open end of which contains Fe(II) and 2OG binding elements that are normally isolated from solution (**Figure 3**) (7–10). The DSBH core is augmented by additional β-strands extending the major and, sometimes, minor β-sheets. α-Helices present at the N terminus of the DSBH play roles in fold stabilization and, sometimes,

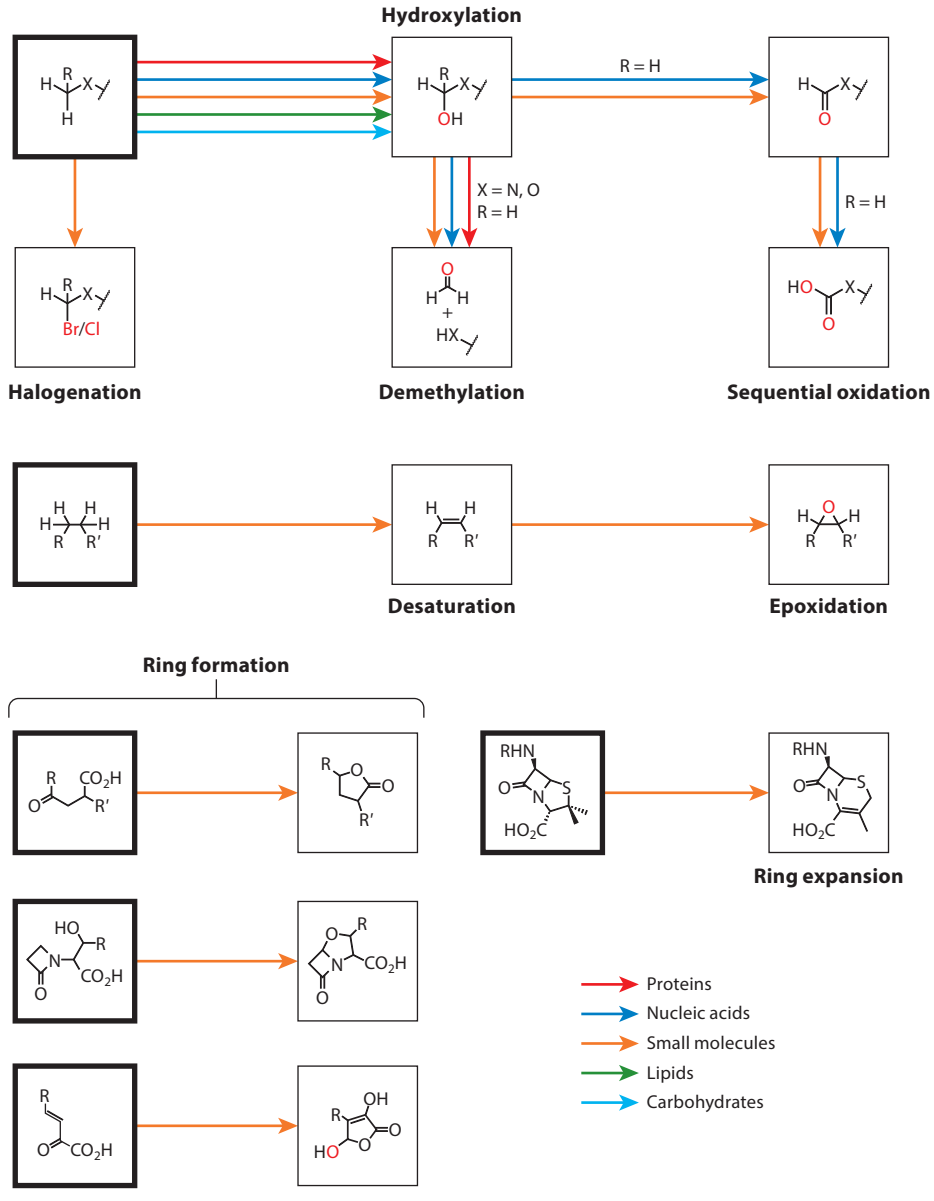


Figure 2

Overview of reactions catalyzed by 2-oxoglutarate-dependent oxygenases (2OGXs): hydroxylation, demethylation, sequential oxidation, ring formation, ring expansion, halogenation, epoxidation, and desaturation. Colored arrows represent reactions on protein (*red*), nucleic acid (*dark blue*), small-molecule (*orange*), lipid (*green*), or carbohydrate (*light blue*) substrates. Only hydroxylation and demethylation reactions are reported on protein and nucleic acid substrates (at least for natural reactions); lipids/carbohydrates undergo hydroxylation. Ring formation/expansion, halogenation, epoxidation, and desaturation reactions are reported (almost) only on small molecules. The scheme is not exhaustive, e.g., there is a 2OGX-catalyzed cyclic peroxidation.

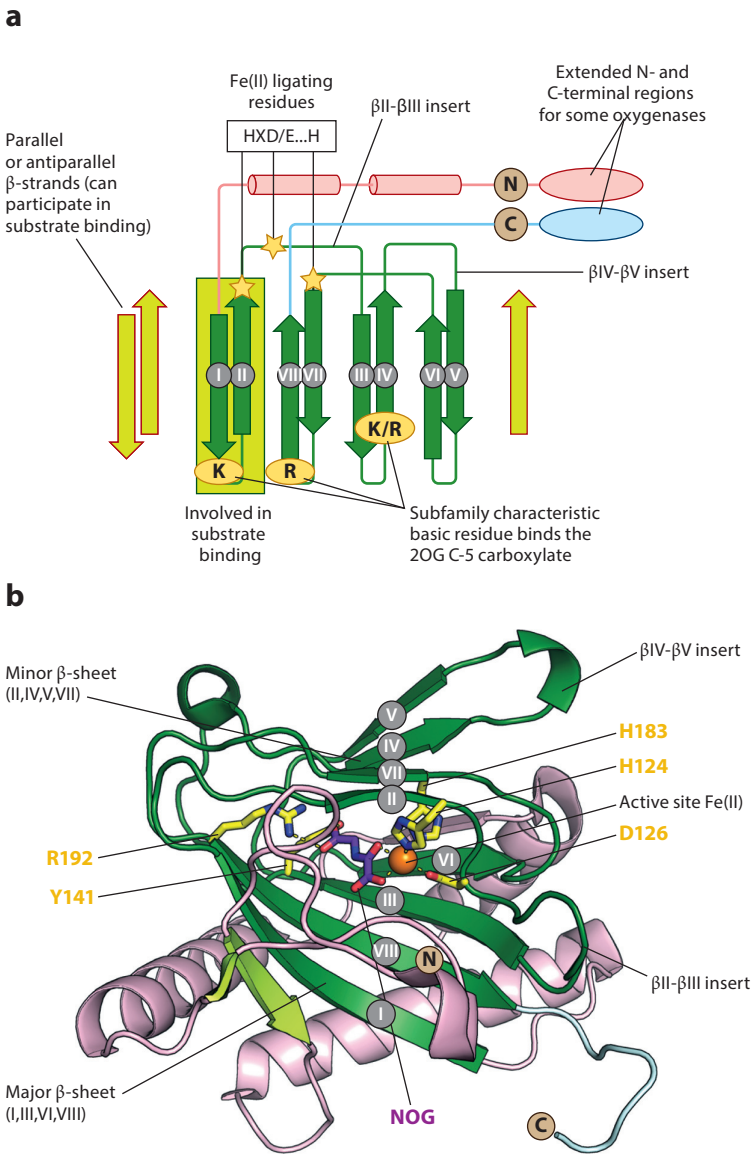


Figure 3

2OGXs contain a core DSBH fold with conserved Fe(II) and 2OG binding sites. (*a*) Composite 2OGX topology diagram showing the core DSBH (*green*) (I-VIII), N-terminal region (*pink*), and C-terminal region (*pale cyan*), as well as iron- (*yellow stars*) and 2OG- (*yellow ovals*) binding residues. βII-βIII and βIV-βV inserts, sometimes involved in substrate binding, are labeled. (*b*) View of a PPHD structure (PDB ID: 4IW3) from *Pseudomonas putida* showing Fe (*orange sphere*) and 2OG (*purple*); other coloring is as in panel *a*. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; DSBH, double-stranded β-helix; NOG, *N*-oxalylglycine; PDB, Protein Data Bank; PPHD, *Pseudomonas* prolyl hydroxylase domain.

dimerization; other α-helices present at the C terminus are involved in substrate recognition and/or dimerization (**Figure 3**; **Supplemental Figure 1**). Loops, which can have extensive secondary structure, play key structural and catalytic roles and are often subfamily characteristic (e.g., the loop/insert linking DSBH β-strands IV and V) (**Figure 3*b***) (7–10).

KDM: N^{ε}-methyl-lysine demethylase

HIF: hypoxia-inducible factor comprising α- and β-subunits

Prolyl hydroxylase domain (PHD): HIF-α prolyl hydroxylase

Many 2OGXs, often including those catalyzing protein/nucleic acid modifications, e.g., the JmjC N^{ε}-methyl-lysine demethylases (KDMs) (14) and ribosomal oxygenases (15), have additional domains (7–10, 16) impacting structure and catalysis. For example, the plant homeodomain of one JmjC KDM, KDM7B/PHF8 (17, 18), binds to H3K4me$_3$ (i.e., the N^{ε}-trimethylated form of histone H3), which directs the 2OGX (JmjC) domain to H3K9me$_2$. Such events likely occur elsewhere; for example, the tetratricopeptide repeat domain of animal procollagen prolyl hydroxylases is likely involved in substrate binding (1, 19).

Following a perceptive proposal (20), an ordered sequential mechanism for 2OGXs has been validated and supported by biophysics and kinetics (**Figure 1**) (6–8, 10, 16, 21). Evidence implies all 2OGX catalysis requires Fe(II), normally bound by three residues forming a highly, but not universally, conserved HXD/E. . .H triad with the proximal histidine on β-II (often a distorted β-strand) and the distal histidine on β-VII (**Figure 3**) (6–10). The third metal-chelating residue is normally an aspartate; reasons for the occasional presence of glutamate are unclear. There are exceptions to this pattern: In Asp/Asn hydroxylase acting on epidermal growth factor (EGF)-like proteins (22) and in 2OG-dependent halogenases (4, 23), a metal chelating residue is substituted for a non-chelating residue (with other changes) to enable halide metal complexation (4, 23). For some apparently non-catalytically active 2OGX-fold proteins (pseudoenzymes), such as the human JmjC protein PHF2, tyrosine substitutes for the distal histidine (24). As-yet unassigned, abnormal catalytic roles for these proteins cannot be excluded. Using only two or three metal ligands may enable catalytic flexibility of 2OGXs, compared with their P_{450} counterparts, as interactions between substrate and metal are limited by the four-coordinate porphyrin.

Supplemental Material

2OG occupies a discrete pocket that does not overlap with that of substrate (6–10) and complexes iron in a bidentate manner via its C-1 carboxylate and ketone oxygens (**Figures 1** and **4**). The 2OG C-1 carboxylate oxygen forms a complex *trans* to either the proximal or distal histidine (6–10). The 2OG methylenes and C-5 carboxylate are generally observed in an extended approximately coplanar form. This arrangement may hinder fragmentation of 2OG-derived intermediates (for one unusual case, see the section titled 2OGX-Catalyzed Small-Molecule Oxidations) (**Figures 1** and **4**) (6–8, 10). Residues involved in 2OG side-chain binding are subfamily characteristic, a feature exploited in developing selective inhibitors (25). Binding of the 2OG C-5 carboxylate normally involves at least two polar residues—one with a basic (Lys or Arg) and one with a polar neutral (Ser or Tyr) side chain (7–10). Variations in residues binding 2OG may reflect binding/kinetic properties. For example, 2OG is bound in a tight pocket in the hypoxia-inducible factor (HIF) prolyl hydroxylase domains (PHDs), which slowly catalyze substrate hydroxylation (7, 26, 27). In other cases, such as the KDM4 family of JmjC KDMs, the 2OG binding pocket is larger, though its functional significance is unclear (28).

Following from work on taurine dioxygenase (29), formation of an enzyme Fe(II)-2OG-substrate complex is supported by kinetics, spectroscopy, and biophysics (**Figures 1** and **4**) (5, 21, 30). Substrate binding (normally) weakens binding of Fe(II)-ligated water, opening a coordination site for O_2 binding (5, 21, 30). In some cases, O_2 channels leading to Fe(II) are proposed, but to date, these are unvalidated. Whether initial O_2 binding to the metal occurs *trans* to either the proximal or the distal histidine is also unclear; there is crystallographic evidence that it varies (6–10).

2OG decarboxylation may occur via a cyclic peroxy intermediate, not yet definitively observed spectroscopically, to produce succinate; CO_2 (which, at least in some cases, leaves at this stage); and

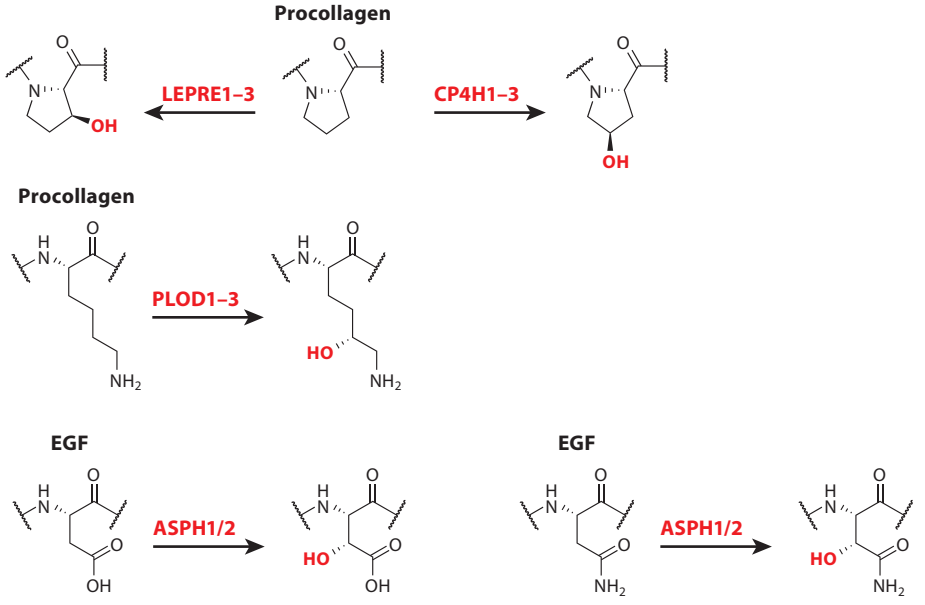


Figure 4

Hydroxylation reactions in collagen biosynthesis and EGFs catalyzed by 2OGXs. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; ASPH, aspartyl/asparaginyl hydroxylase; CP4H, procollagen C-4 prolyl hydroxylase; EGF, epidermal growth factor; LEPRE, procollagen C-3 prolyl hydroxylase; PLOD, procollagen C-5 lysyl hydroxylase.

an Fe(IV)=O (ferryl) intermediate, which has been spectroscopically characterized (**Figure 1**) (6, 7, 9, 21). Recently, a peroxysuccinate intermediate has been reported for an arginine hydroxylase (31). Hydroxylation likely occurs either by a concerted process or by hydrogen abstraction followed by rapid reaction of the substrate radical and an Fe(III)-OH species (6, 7, 9, 21). The protonation state of the ferryl intermediate during this stage is uncertain. Loss of the oxidized product is followed by succinate, which can be rate limiting. Variations on the consensus mechanism occur, e.g., in the order of succinate/product release, but evidence indicates it operates for most 2OGXs (31–33). In addition to O_2 activation, mechanistic gaps include how the Fe(IV)=O intermediate is generated and details of how the more exotic 2OG oxygenase-catalyzed transformations, e.g., oxidative rearrangements, occur, for which radical intermediates are likely (32).

Given their diverse reactions (5), 2OGX substrates have more variations in binding modes than do Fe(II)/2OGs (**Figure 4**) (7, 16). Substrate binding typically involves DSBH strands (βI, βII, βVII, and βVIII) at the active site entrance, loops linking DSBH strands (βI/II, βII/III, and βIV/V), and other elements (e.g., C-terminal helices). Although precise interactions (e.g., electrostatic or hydrophilic) depend on the subfamily and substrate (**Figures 1** and **4**) (9, 16), they position the substrate in a stereochemically favorable manner with respect to the Fe(IV)=O intermediate. Failure to do so can result in uncoupling of 2OG and substrate oxidation (with potential for oxidative damage) (5, 35); uncoupling is observed with nonoptimal substrate analogs or when correct substrate binding is disrupted by mutation.

In 2OGX reactions other than simple hydroxylation, complex kinetics and dynamics can manifest. Current crystallographic evidence likely underestimates the extent of conformational changes in catalysis, though some (26, 27, 36–39) implies substantial movement during substrate

binding and product release (40). Nuclear magnetic resonance (NMR) studies on AlkB and the HIF PHD2 demonstrate Fe(II) binding and 2OG preorganization of the protein for substrate binding that triggers substantial conformational changes (**Figure 4**) (26, 41). Few structures are reported for 2OGXs complexed with intact oligomeric protein substrates. Structures of a PHD homolog from *Pseudomonas putida* in complex with its intact substrate, GTP-dependent elongation factor thermally unstable (EF-Tu), reveal substrate binding induces major changes in both enzyme and substrate conformations: Crucial for its function as a GTPase, the EF-Tu switch I loop moves >20 Å upon *Pseudomonas* PHD binding (**Figure 3**) (39). Structures for asparaginyl-hydroxylase factor inhibiting HIF (FIH) in complex with intact versions of its ankyrin repeat domain (ARD) substrates have not been reported. However, evidence from structures of uncomplexed ARD proteins and of FIH complexed with ARD fragments implies substrates undergo conformational changes away from their stereotypical conserved fold to bind productively (42). Overall, further work that employs solution-based methods is needed to determine the precise nature of the conformational changes in 2OGX catalysis.

Factor inhibiting HIF (FIH): C-3 asparaginyl hydroxylase acting on HIF-α

OVERVIEW OF 2OGX FUNCTIONS

In general, the biological functions of 2OGXs involve either secondary or primary metabolism (5). Their secondary metabolic roles in plants and microbes principally involve biosynthesis or degradation/recycling of small molecules. Their primary metabolic roles include nucleic acid repair, transcriptional/protein biosynthesis regulation, lipid metabolism, hypoxia sensing, and collagen/collagen-related protein biosynthesis (**Figures 2** and **3**) (2).

2OGXs have at least four biologically important cosubstrates/coproducts (2OG, O_2, succinate, CO_2) and use Fe(II) (**Figure 1**). As a result, they appear to be suited to function as metabolic and redox state sensors (43). Limitation of 2OGX catalysis by O_2 availability in animals may facilitate their sensor functions in response to hypoxia (see the section titled Hypoxia-Inducible Factor Hydroxylases and Related Enzymes) (43). 2OG and succinate have pivotal metabolic roles owing to their presence in the tricarboxylic acid (TCA) cycle and other aspects of metabolism (44); variations in 2OG concentrations are linked to important processes including aging and tumor cell metabolism. 2OGX activity may directly impact (local) O_2/2OG/succinate/CO_2 concentrations in cells, and variations in cosubstrate/coproduct availability may regulate their activity. In some tumor cells, succinate, fumarate, or 2-hydroxyglutarate compete for binding with 2OG, thereby inhibiting 2OGXs involved in transcription, thus contributing to genome instability and tumorigenesis (45). Kinetic evidence shows substantial differences in reported K_M (2OG) values (~100 fold) and suggests some 2OGXs are especially susceptible to variations in 2OG availability (and inhibition by TCA cycle–related di-acids) in cells (46, 47).

Fe(II) binding by 2OGXs is reversible (EDTA treatment generates apo-enzymes) and is weaker than metal binding in porphyrin-based oxidative catalysis, indicating 2OGXs could act as sensors of transition metals. Co(II) ions were once used for anemia treatment via a mechanism subsequently proposed to involve inhibition of the 2OG-dependent HIF PHDs (see section titled Hypoxia-Inducible Factor Hydroxylases and Related Enzymes) (48). The processes by which Fe(II) is introduced to, and maintained at, the active sites of 2OGXs in cells are not well understood (49).

Supplemental Material

Below, the biochemical/cellular functions of human 2OGX (**Figures 4–12**; **Supplemental Figure 2**) are described to exemplify current knowledge and to outline the roles of related enzymes in other organisms. Corresponding with the start of 2OGX research, we focus initially on protein hydroxylation. 2OGX roles in secondary metabolism are highlighted, and their roles in biosynthesis of plant signaling metabolites and herbicide degradation are summarized. Finally, we outline applications of 2OGXs in agrochemistry, as pharmaceutical targets, and in biocatalysis.

2OG-DEPENDENT PROTEIN HYDROXYLASES INVOLVED IN COLLAGEN BIOSYNTHESIS AND RELATED ENZYMES

PTM: posttranslational modification

Following the discovery that proteins react with atmospheric O_2 (50), research on collagen biosynthesis identified the first 2OGX as a procollagen prolyl hydroxylase (1). Some procollagen hydroxylases also act on collagen-like proteins, e.g., adiponectin and complement factors, where their activity may regulate function (51). There are three types of 2OG-dependent procollagen hydroxylation: procollagen C-3 and C-4 prolyl hydroxylation and lysyl C-5 hydroxylation (**Figure 4**). CP4H-catalyzed *trans* C-4 hydroxylation of X-Pro-Gly repeat motifs in procollagen (1) occurs in the endoplasmic reticulum and stabilizes the collagen triple helix, likely in part owing to stereoelectronic effects (52).

The domain architecture of animal CP4H is unusual: It has an $\alpha_2\beta_2$ quaternary structure in which the β-subunit is identical in sequence with protein disulfide isomerase (1). The α-subunit of CP4H, like aspartyl/asparaginyl hydroxylase (ASPH), additionally contains a tetratricopeptide repeat domain, which is involved in substrate binding (19). As with many 2OGXs, CP4H catalysis is promoted by ascorbate (1). Ascorbate deficiency leads to scurvy impairing connective tissue formation, likely owing to impaired CP4H activity (1). Despite variable stimulatory effects, ascorbate is often used to promote 2OGX catalysis in vitro. One mechanism likely involves helping to maintain iron in the catalytically active Fe(II) state, but when it is strongly stimulatory (e.g., CP4H), this is probably not its only role.

Procollagen C-3 prolyl hydroxylation is catalyzed by CP3Hs (**Figure 4**). Compared with C-4 prolyl hydroxylation, the role of C-3 prolyl hydroxylation is not as well established, but it may locally destabilize the collagen fold to enable further posttranslational modifications (PTMs) (1). Procollagen also undergoes (5*R*)-hydroxylation on lysines (**Figure 4**), a PTM enabling glycosylation. Some procollagen lysyl hydroxylases also have a glycosyl transferase domain (1), and mutations of these hydroxylases correlate with one form of Ehlers-Danlos disease (1).

Another type of 2OGX protein hydroxylation occurs in the endoplasmic reticulum: ASPH (or BAH) catalyzes C-3 hydroxylation of Asp and Asn residues in domains homologous to those of EGFs including coagulation factors and Notch ligands (**Figure 4**) (22). ASPH has several isoforms and splice variants: Some are linked to cancer, and an antibody to one is used in cancer diagnosis (53); mutations of ASPH are also linked to facial dismorphism (54). The biochemical role of EGF hydroxylation is unclear, but it does not appear to alter the overall substrate fold. However, some hydroxylated residues are involved in Ca(II) binding of the EGF domain (22).

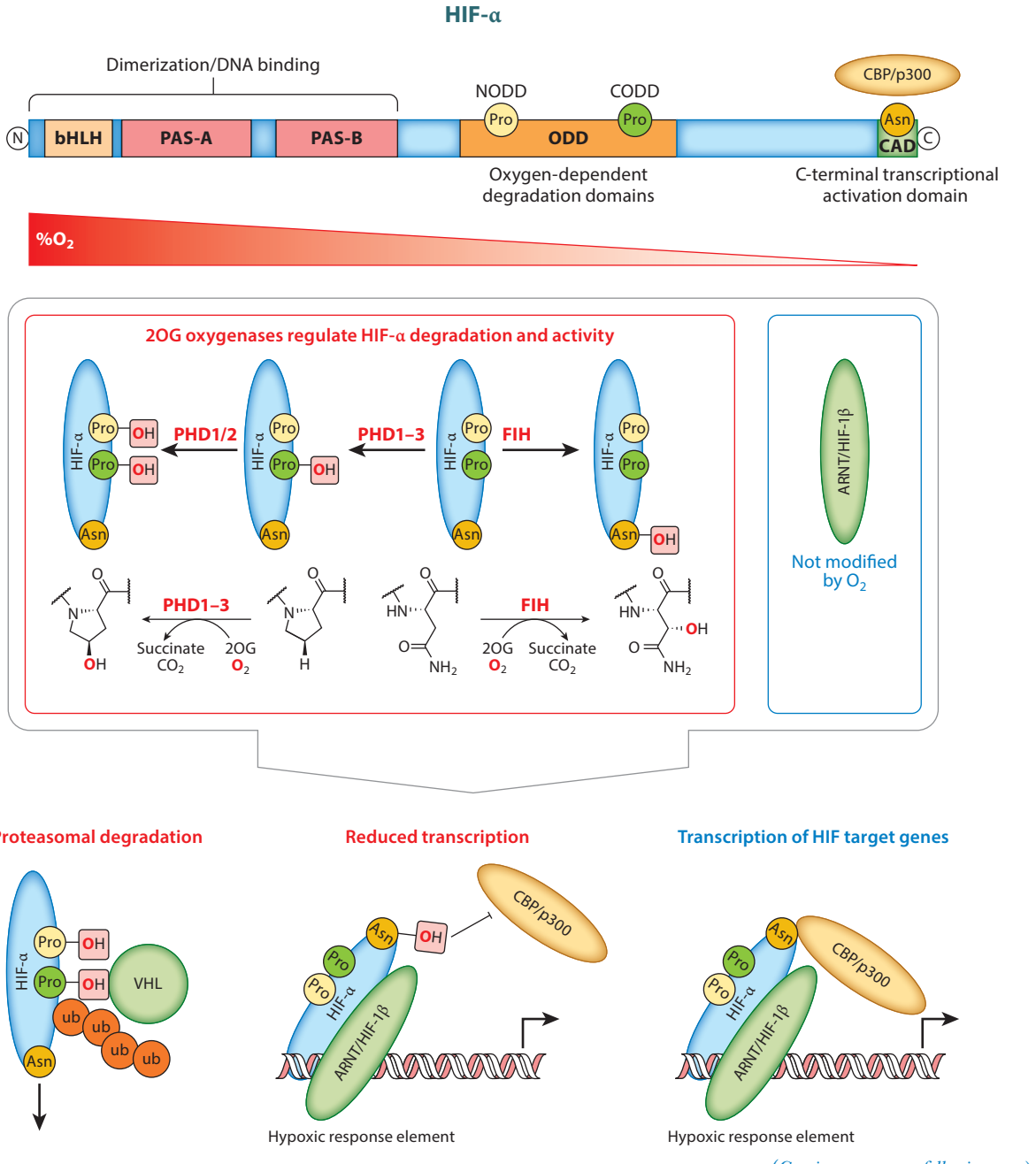
The roles of 2OGXs in collagen biosynthesis illustrate the effectiveness of postoligomerization hydroxylation in triggering physiologically relevant functions. Hydroxylation is a unique postoligomerization modification: The 16-Da oxygen atom is added from the energy-rich, but relatively biologically inert, triplet-state O_2 (55). Hydroxylations at unactivated positions introduce polarity and induce stereoelectronic effects. Procollagen modifications exemplify the diverse consequences that result: C-4 and C-3 prolyl hydroxylations stabilize and destabilize the fold, respectively, and lysyl C-5 hydroxylation enables further PTMs. Other 2OGXs involved in protein or nucleic acid modifications play similar enzymological roles (2), but the biological consequences of the oligomer modifications are often less clear.

HYPOXIA-INDUCIBLE FACTOR HYDROXYLASES AND RELATED ENZYMES

In animals, the HIFs mediate cellular adaptation to chronic limits in O_2 availability. These factors promote transcription of hundreds of genes in a tissue/cell- and environment/context-dependent

manner and indirectly regulate many other genes (43, 56, 58). The HIF system thus enables profound cellular reprogramming to ameliorate the effects of hypoxia (**Figure 5**).

HIF is an α,β-heterodimeric transcription factor containing a basic helix-loop-helix region and PAS domain. HIF binds to hypoxic response elements associated in the promoter regions of its target genes. HIF-α, but not HIF-β (which is identical in sequence to the aryl



(*Caption appears on following page*)

Figure 5 (*Figure appears on preceding page*)

Roles of 2OGXs in regulating HIF-α and -β. HIF-α, but not HIF-β, is regulated by 2OGXs. In humans, prolyl hydroxylation of the three HIF-α isoforms, as catalyzed by PHD1–3, signals for HIF-α degradation by the ubiquitin proteasome system. HIF-α undergoes O_2-regulated prolyl hydroxylation in its NODD and CODD. These modifications strongly (~1,000-fold) promote HIF-α binding to the VHL protein, a targeting component of a ubiquitin E3 ligase system. Asn hydroxylation in the CAD is a second type of HIF-α hydroxylation. It is less sensitive to hypoxia and reduces interaction between HIF-α and transcriptional coactivator proteins (CBP/p300), which are histone acetyltransferases, thereby reducing HIF-promoted transcription (43). Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix region; CAD, C-terminal transcriptional activation domain; CBP, CREB-binding protein; CODD, C-terminal oxygen-dependent degradation domain; FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; NODD, N-terminal oxygen-dependent degradation domain; PHD, prolyl hydroxylase domain; VHL, von Hippel-Lindau.

hydrocarbon receptor nuclear translocator protein), is efficiently degraded in an O_2-regulated manner (**Figure 5**). In hypoxia, as in some tumor cells, HIF-α levels are elevated owing to slowed O_2-dependent degradation (57–60). Some HIF target genes are important for biomedicine. For example, regulating expression of the genes for erythropoietin (EPO) and vascular EGF (VEGF) by manipulating HIF levels has potential to provide therapeutic benefits, e.g., by promoting EPO production to treat anemia or by downregulating vascular EGF production in tumors.

In humans, there are three HIF-α isoforms, of which HIF-1α and HIF-2α are the most important. HIF-1α and HIF-2α promote transcription of distinct, sometimes overlapping, sets of genes: Transcription of the EPO gene is upregulated principally by HIF-2α, whereas VEGF and glycolytic enzymes are HIF-1α regulated. To date, 2OGXs provide the only validated direct cellular O_2-sensing mechanism for the hypoxia-sensing system. The three human isoforms of HIF PHDs (PHD1–3) signal for HIF-α degradation by catalyzing the *trans*-4-prolyl hydroxylation of HIF-α isoforms at either, or both, of the two specific sites in the N- or C-terminal oxygen-dependent degradation domain (NODD or CODD, respectively) (**Figure 5**; **Supplemental Figure 2**) (43, 58, 61–64). A single HIF-α asparaginyl hydroxylase (FIH) (65, 66) regulates the transcriptional activity of HIF isoforms (**Figure 5**; **Supplemental Figure 2**).

Supplemental Material

C4-prolyl hydroxylations in the CODD and NODD substantially promote binding between the HIF-α subunit and the von Hippel-Lindau (VHL) protein, a targeting component for a ubiquitin E3 ligase. This process marks HIF-α for proteasomal degradation (**Figure 5**). Human PHDs exhibit different NODD/CODD selectivities: PHD3 is particularly selective for the CODD. By contrast, PHD2, which is the most conserved isoform, contains an MYND zinc finger domain and is indispensable in mice (43, 58). Some clinically observed heterozygous mutations of PHD2 are linked to familial erythrocytosis via HIF-α upregulation and manifest altered NODD/CODD selectivity (67). Crystallographic and solution-state studies, including via NMR spectroscopy, coupled to kinetic and cellular analyses have revealed how wild-type and variant PHDs achieve selectivity through multiple dynamic interactions involving loop and C-terminal regions of the 2OGX domain (7, 26, 27). The triad comprising PHD2, HIF, and VHL is conserved in (nearly) all animals but likely not in earlier eukaryotes (68). The role of PHDs in hypoxia sensing may have originated in prokaryotes, which contain a PHD homolog that catalyzes hydroxylation of EF-Tu (**Supplemental Figure 2**) (39). In early eukaryotes, PHD homologs catalyze hydroxylation of S-phase kinase-associated protein 1, thus enabling glycosylation (echoing the role of procollagen C-5 lysyl hydroxylation). This process may also enable responses to hypoxia and is a possible evolutionary precursor of the HIF system (69).

Another HIF-α hydroxylation comprises FIH-catalyzed asparaginyl hydroxylation of the C-terminal transactivation domain of HIF-1α/HIF-2α (**Figure 5**; **Supplemental Figure 2**) (65, 66). This reaction blocks interaction between HIF-α and CBP/p300 transcription coactivators

(histone acetyltransferases) to inhibit transcription of HIF target genes. FIH was the first of the JmjC proteins identified as a 2OGX (65, 66). Like HIF-α prolyl hydroxylation, the effect of HIF-α asparaginyl-hydroxylation is biochemically profound. Studies with animal models suggest FIH function in the hypoxic response is less important than that of the PHDs (at least PHD2), which is consistent with its sporadic presence in early animals (unlike PHD2) (68). The role of FIH in regulating HIF-responsive gene expression varies substantially (owing to unknown mechanisms), whether upregulating different HIF target genes in the same cell or the same HIF target genes in different cells (70).

To act as hypoxia sensors, HIF hydroxylase activity in cells must be limited by O_2 availability. Compelling evidence indicates that the kinetic properties of PHD2 reflect its sensing role. PHD2 binds Fe(II) unusually tightly and does not catalyze significant substrate-uncoupled 2OG turnover (as observed for many 2OGXs); notably, the isolated PHD2–Fe(II)–2OG–HIF-α complex reacts unusually slowly with O_2 (71). These properties are consistent with the hypoxia-sensing role of PHD2 because they imply its activity is more likely to be limited by O_2, rather than Fe(II) or 2OG, availability. Biophysical analyses indicate the slow reaction with O_2 is due in part to the need for a strongly bound water to vacate the active site Fe(II) so O_2 can bind (27). Crystallographic and kinetic studies suggest that the characteristic kinetic properties of PHD2 are conserved in PHDs from different organisms, including the simplest animal *Trichoplax adhaerens* (68). In contrast with PHD2, FIH has more typical kinetics, including a quicker reaction with O_2, consistent with the observation that FIH is more active than are the PHDs under hypoxic conditions (72, 73). Crystallographic studies reveal the O_2-binding coordination site is more accessible for FIH than the PHDs. Such roles of PHDs in the hypoxic response indicate how directly linked their biochemical properties are to physiology. Other 2OGXs, including demethylases, may also act as hypoxia sensors, though similar direct links to physiology have not been made.

FIH is promiscuous. It accepts HIF-α as well as many proteins from the ARD family, where it catalyzes hydroxylation of residues other than Asn, including Asp and His (74). The PHDs also accept non-HIF substrates, but these functions are less well validated than for FIH. Whereas ARD hydroxylation profoundly affects HIF-α activity, no such effect on Notch or nuclear factor κB signaling, for example, has been found (74). FIH-catalyzed ARD hydroxylation increases the thermal stability of the stereotypical ARD fold, though the biological relevance of this is unclear (42, 75). Unlike the stabilizing effect of C-4 prolyl hydroxylation on collagen, FIH-catalyzed ARD hydroxylation is sporadic and rarely complete, suggesting thermal stabilization is unlikely to be its (only) role. Hydroxylated ARD products bind less tightly to FIH than do unhydroxylated substrates, and many ARDs of different lifetimes interact with FIH. Thus, competition between ankyrins and HIF-α for binding to FIH may modulate the role of FIH and provide a tunable cellular memory of hypoxic events (76).

Another conserved prolyl hydroxylase (OGFOD1 in humans; TPA1 in *Saccharomyces cerevisiae*) catalyzes hydroxylation of the ribosomal protein S23 (RPS23) in eukaryotes (**Figure 6**) (77–79). Structural analyses on ribosomes revealed RPS23 Pro-62 is in close contact with mRNA and that it undergoes *trans* C-3 prolyl hydroxylation in humans, *Drosophila melanogaster*, and likely other animals. In earlier eukaryotes, an additional hydroxylation of Pro-62 occurs, likely at C-4, to give a dihydroxyproline residue (77, 78). In *S. cerevisiae*, RPS23 hydroxylation regulates translation, though it likely has other roles including in stress responses. To date, no evidence indicates OGFOD1-catalyzed hydroxylation has switch-like effects on animal signaling (77, 78, 80).

Several JmjC protein hydroxylases related to FIH have been characterized. Like FIH, JMJD6 appears promiscuous. It catalyzes (5*S*)-hydroxylation of lysine residues in arginine-serine-rich regions of splicing regulatory proteins (81) and of histone tails (**Figure 7**). More controversially, it has been designated a histone *N*-methyl-arginine demethylase (see the section titled 2OGXs as

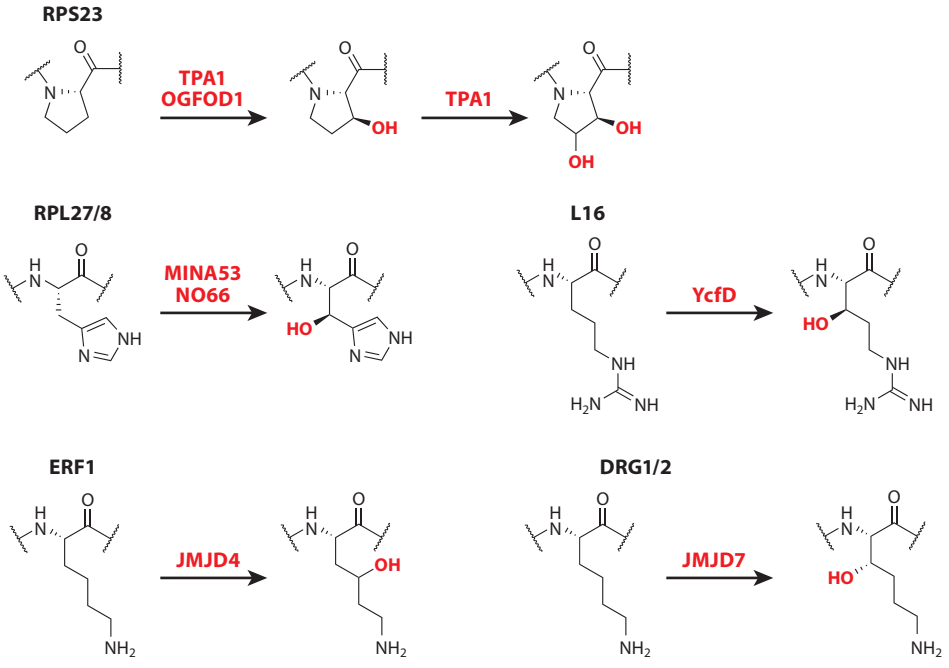


Figure 6

Hydroxylation reactions in ribosomal proteins catalyzed by 2OGX. The human oxygenase OGFOD1 and its *Saccharomyces cerevisiae* homolog, TPA1, catalyze hydroxylation of a prolyl residue in RPS23. Biochemical evidence indicates TPA1 catalyzes sequential hydroxylations at the C-3 and C-4 positions. Originally considered histone methyl-lysine demethylases, MINA53 and NO66 catalyze hydroxylation of His residues in the ribosomal proteins. YcfD is closely related to MINA53 and NO66 in *Escherichia coli* and catalyzes C-3 arginyl hydroxylation in L16. JMJD4 catalyzes C-4 lysyl hydroxylation of ERF1. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; DRG, developmentally regulated GTP-binding protein; ERF, eukaryotic release factor; MINA, myc-induced nuclear antigen; NO66, nucleolar protein 66; RPL27/28 and RPS23, ribosomal protein L27/28 and S23; TPA, termination and polyadenylation protein.

Protein Demethylases) (82). JMJD6-catalyzed protein modification is involved in RNA splicing (hydroxylation of splicing regulatory proteins), carcinogenesis (via modification of tumor suppressor p53), the hypoxic response, regulation of estrogen levels (estrogen receptor alpha), and regulation of heat-shock protein 70 kDa (81).

Like OGFOD1, the ribosomal oxygenases myc-induced nuclear antigen 53 and nucleolar protein 66 target ribosomal proteins but catalyze C-3 hydroxylation of His residues in RPL27 and RPL8, respectively (**Figure 6**) (15, 83). These oxygenases are related to the bacterial 2OG oxygenase YcfD (15, 84), which catalyzes C-3 Arg-hydroxylation of L16, and from which the eukaryotic enzymes may have evolved (**Figure 6**) (15, 83). The precise functions of the ribosomal oxygenases remain unclear, but they are related to growth, possibly via nucleic acid interactions. Their tertiary structures are strikingly conserved from bacteria to humans: They have characteristic dimerization modes and both JmjC and winged-helix domains, the latter of which may enable nucleic acid binding (15).

JMJD4 is involved in translational termination. It catalyzes C-4 lysyl hydroxylation of an NIKS motif in eukaryotic release factor 1, a PTM that promotes translational termination efficiency

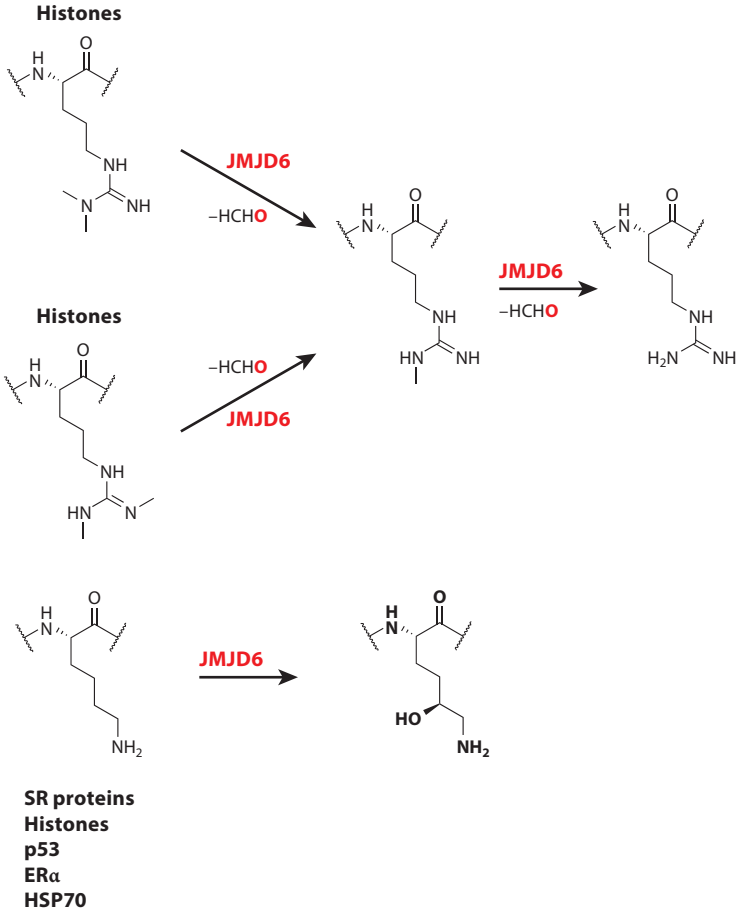


Figure 7

JMJD6-catalyzed reactions. JMJD6 catalyzes C-5 lysyl hydroxylation on multiple proteins. Though contentious, it might also catalyze methyl-arginine demethylation. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Note that ERα and HSP70 are proposed demethylation susbtrates for JMJD6. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; ERα, estrogen receptor alpha; HSP70, heat-shock protein 70 kDa; SR, splicing regulatory.

(**Figure 6**) (85). JMJD5 is structurally related to FIH and JMJD6 and is another likely human JmjC hydroxylase (9, 86). Though some of these hydroxylases have been designated as KDMs (see the section titled 2OGXs as Protein Demethylases), most structural and bioinformatic analyses suggest it is more likely they produce stable alcohol products (9, 81). However, given the promiscuity of some 2OGXs, both activities are possible.

2OGXs AS PROTEIN DEMETHYLASES

Basic protein residues are especially prone to 2OGX-catalyzed PTMs. JmjC 2OGXs comprise the largest set of histone KDMs (14, 87). Of all residues involved in histone PTMs, lysines are modified most often, as their N^{ε}-methylation status is important in transcriptional regulation in eukaryotes (6, 14). Lysine N^{ε}-acetylation is normally transcriptionally activating. By contrast,

the sequence position of the histone and chromatin context determine whether histone N^{ε}-methylation either promotes or suppresses transcription.

Pioneering research leading up to the 1970s provided evidence of KDMs (14). Yet, many still thought histone methylation was irreversible until the KDM1 (or lysine-specific demethylase) family was discovered. KDM1 enzymes employ a flavin cofactor to catalyze demethylation of di- and mono-N^{ε}-methyl-lysine histone residues and likely act on the neutral form of their substrates. By contrast, the larger 2OGX JmjC KDM family catalyzes demethylation of all three lysine N^{ε}-methylation states, though not all members act on the positively charged N^{ε}-trimethylated state (**Figure 8**) (17, 28, 87). JmjC KDM catalysis starts with *N*-methyl group hydroxylation;

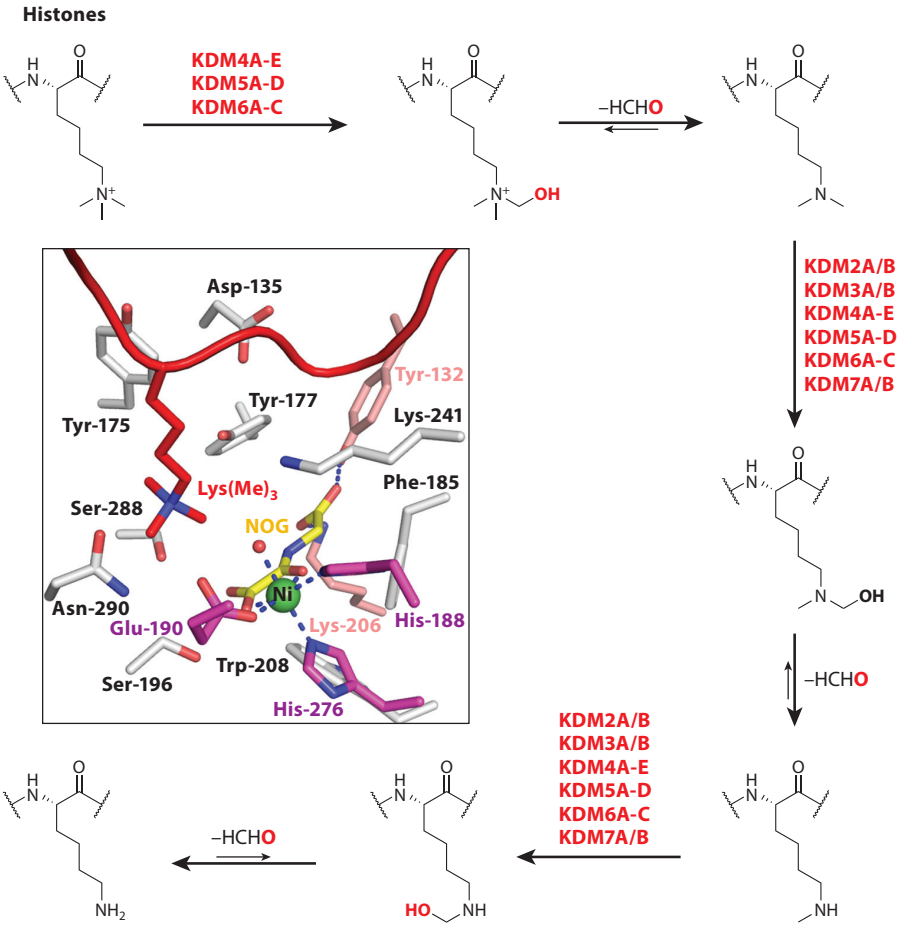


Figure 8

Summary of human JmjC KDM reactions. JmjC KDMs catalyze demethylation of N^{ε}-methylated lysine residues (multiple sites) on histones H1.4, H3, and H4. Demethylation proceeds via hydroxylation to form a hemiaminal that fragments, giving the demethylated product and formaldehyde. (*Boxed inset*) KDM4A structure complexed with Ni(II) [substituting for Fe(II)], NOG (2OG analog), and an H3-fragment peptide containing trimethylated lysine at position 9 (PDB ID: 2OQ6) (28), showing metal-binding residues (*magenta*) and NOG/2OG-binding residues (*pink*). Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; KDM, N^{ε}-methyl-lysine demethylase; NOG, *N*-oxalylglycine; PDB, Protein Data Bank.

the resultant unstable hemiaminal intermediate (as yet not directly observed) is then fragmented to give formaldehyde. Substrate analog studies support a mechanism that starts with N^{ε}-methyl group hydroxylation and, at least in some cases, requires a positively charged substrate.

Over the past decade, intensive research has investigated the roles of JmjC KDMs and of histone methylation in epigenetics and development, e.g., in sex determination. Mutations to genes encoding for JmjC KDMs are linked to genetic and mental disorders, midline defects, and cancer (88). Human JmjC KDMs are classified into approximately six subfamilies (KDM2/7, KDM3, KDM4, KDM5, and KDM6) with different sequence and methylation-state selectivities (**Figure 8**) (14, 89). Recent biochemical studies reveal some JmjC KDMs exhibit *N*-methyl-arginine residue demethylation activity (**Figure 9**), but the cellular relevance of this observation has not been validated (90). JMJD6 might play a role in histone residue demethylation (82), though such activity is controversial. It also functions as a lysyl 5-hydroxylase acting on splicing regulatory proteins (see above) and on histones. Several apparent JmjC KDM pseudoenzymes are predicted, e.g., human PHF2 (91) and JARID2 (24, 92), though they may have unassigned catalytic activities.

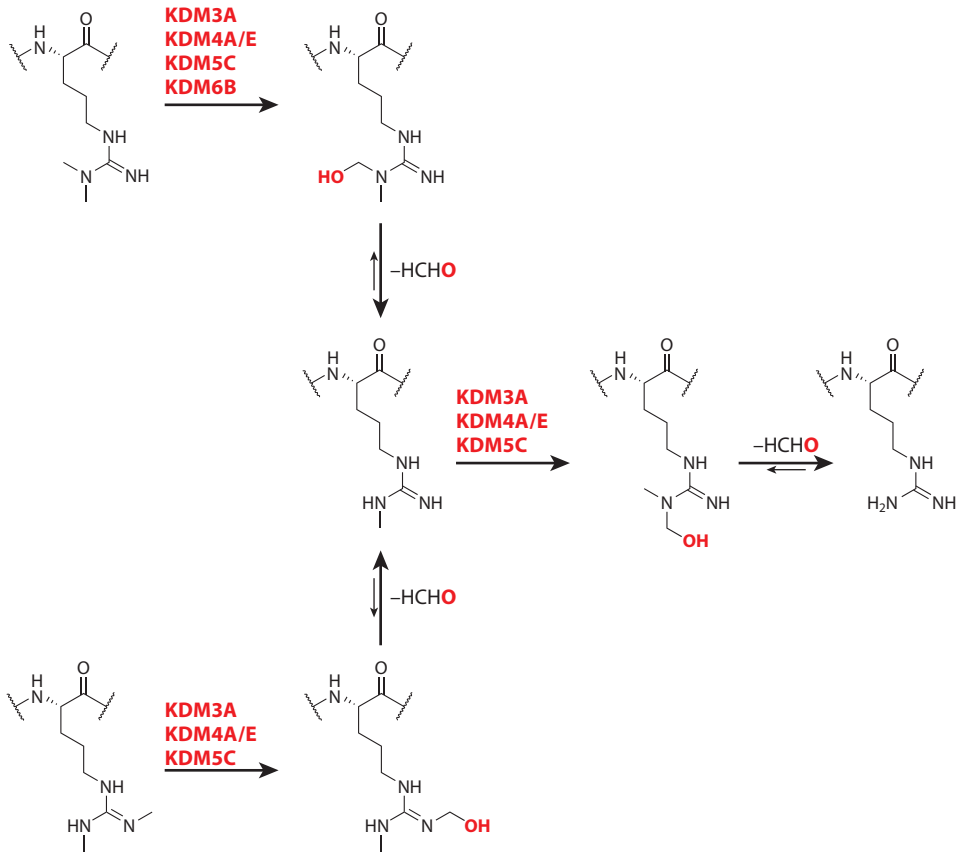


Figure 9

Demethylation reactions reported on methylated arginine residues catalyzed by JmjC KDMs. Demethylation proceeds via hydroxylation, in a similar manner to lysyl demethylation. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; KDM, N^{ε}-methyl-lysine demethylase.

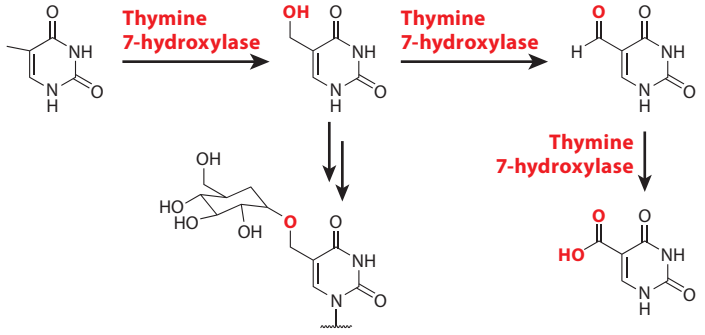


Figure 10

Oxidative reactions on thymine catalyzed by thymine 7-hydroxylase. Each 2-oxoglutarate (2OG)-dependent oxygenase reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2.

Nearly all JmjC KDMs have noncatalytic domains, many of which are likely important for biological function. These domains guide or regulate (promote or inhibit) catalysis; KDM7B (PHF8) exemplifies this function, and its mutations are linked to midline defects (93). It is important to note that interactions between JmjC KDMs and their nucleosome substrates involve substantial protein-protein and, in some cases, protein–nucleic acid interactions. Thus, KDM activity might best be considered as part of a dynamic process, rather than as having switch-like signaling effects.

2OGXs ACTING ON NUCLEOBASES AND NUCLEIC ACIDS

Nucleobases/nucleic acids are a major class of oligomeric 2OGX substrates (40, 94–97). Thymine 7-hydroxylase catalyzes formation of 7-hydroxy-, 7-formyl-, and then 7-carboxythymine (**Figure 10**) (98). Reactions of other 2OGX-catalyzed nucleobase/nucleoside modifications occur on both base and sugar.

AlkB is a 2OGX that catalyzes repair of methylation-induced DNA and RNA damage. This finding opened new functional possibilities for oxidative modifications to nucleic acids (40). Like KDM catalysis, AlkB-enabled demethylation repair proceeds via methyl-group oxidation to give a hemiaminal intermediate that fragments to give formaldehyde. In some cases, hemiaminal adducts formed on nucleobases through this process are more stable than those derived from N^{ε}-methyllysine demethylation (99). AlkB is also promiscuous and catalyzes multiple dealkylations (40).

AlkB homologs are widely distributed in prokaryotes and eukaryotes. Most of the human AlkB homologs (AlkBH1–9) (95) are *N*-methyl DNA/RNA demethylases, and some likely play more than one role, e.g., AlkBH2/3 in DNA repair of both 1meA and 3meC (100, 101). AlkBH5 and FTO (fat-, mass-, and obesity-associated protein) function principally as *N*-methyl RNA demethylases regulating mRNA stability/levels (94), but they are also linked to roles in metabolism and obesity (FTO) as well as roles in circadian clock regulation (AlkBH5) (**Figure 11**) (94).

Two 2OGXs catalyze tRNA oxidation (40): TYW5 (tRNA-wybutosine synthesizing enzyme 5; or wybutosine hydroxylase) and AlkBH8. TYW5 is a member of the JmjC 2OGX subfamily, members of which normally act on proteins (see above) (**Figure 12**). TYW5 catalyzes yW-72 hydroxylation to give undermodified hydroxywybutosine during wybutosine biosynthesis; the role of wybutosine hydroxylation is uncertain but may promote translation fidelity (as does wybutosine) (102, 103). AlkBH8 catalyzes both methyltransferase and hydroxylase reactions. In conjunction with TRM112, AlkBH8 catalyzes methylation to give 5-methylcarboxymethyl uridine at the wobble position in tRNA. The 2OGX domain of AlkBH8 then catalyzes hydroxylation of this

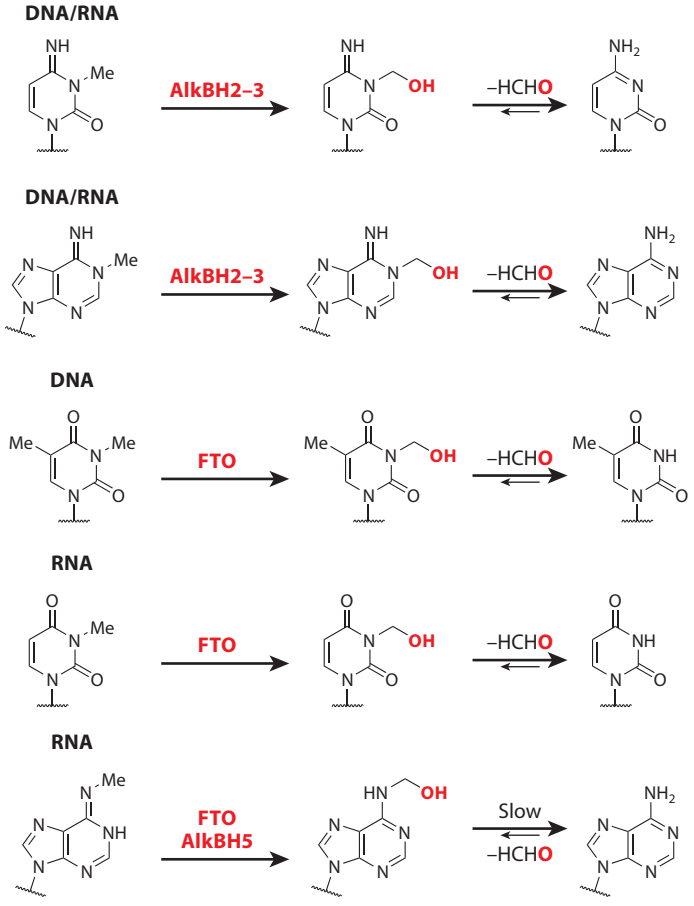


Figure 11

Reactions catalyzed by nucleic acid 2OGXs. Demethylation of *N*-methylated nucleic acid bases in DNA and RNA occurs via initial hydroxylation, forming *N*-hydroxymethylated products that fragment to release formaldehyde (HCHO). Stability of *N*-hydroxymethylated species depends on the position of the hydroxymethyl group; in some cases, they may be sufficiently stable to have functional roles (notably 6-hydroxymethyladenine). Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; AlkBH, AlkB homolog; FTO, fat-, mass-, and obesity-associated protein.

nucleoside to give (*S*)-methoxycarbonylhydroxymethyluridine (**Figure 12**) (104, 105); its (*R*)-epimer is also observed but may be produced via an unknown epimerization mechanism. Like TYW5, AlkBH8-catalyzed nucleobase modifications enhance translation accuracy/efficiency.

The ten-eleven translocation 2OGXs (TET1–3 in humans) catalyze sequential oxidations of 5-methylcytosine, to give 5-hydroxymethylcytosine, 5-formylcytosine, and then 5-carboxycytosine in DNA and RNA (**Figure 13**). In DNA, TET functions are linked to epigenetic transfer of information and development; the nucleic acid modifications they catalyze may also affect DNA structure/stability (106–109). TET catalysis is linked to excision of 5-methylcytosine, though the precise mechanism(s) by which this occurs is not fully established. TETs are evolutionarily related to thymidine hydroxylase that catalyzes the first step in biosynthesis of Base J (β-D-glycosyl-hydroxymethyluracil) that occurs in telomeric sequences in kinetoplastids, a group of

TET: ten-eleven translocation

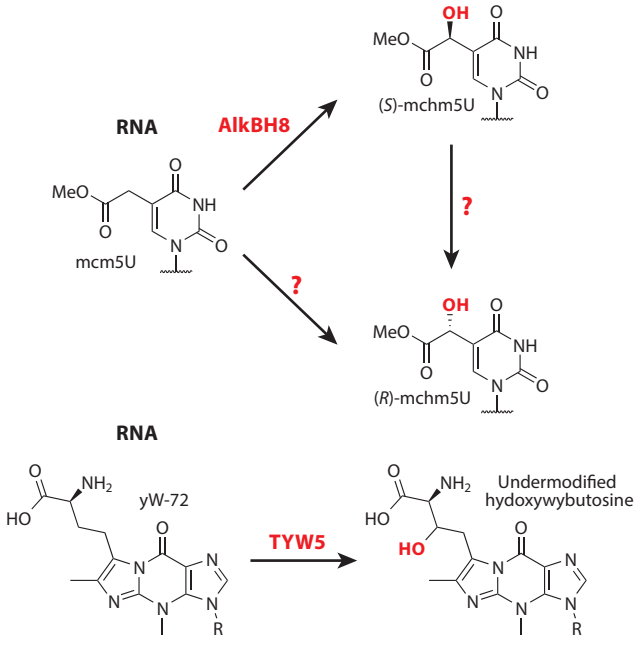


Figure 12

Hydroxylation reactions on nucleic acids catalyzed by AlkBH8 and TYW5. Whereas AlkBH8-catalyzed hydroxylation of mcm5U leads to formation of (*S*)-mchm5U, the epimer (*R*)-mchm5U is also produced, possibly either via oxygenase-catalyzed epimerization of (*S*)-mchm5U or via oxygenase-catalyzed hydroxylation of mcm5U. The enzyme is currently unknown. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; AlkBH8, AlkB homolog 8; -mchm5U, -methoxycarbonylhydroxymethyluridine; mcm5U, 5-methylcarboxymethyluridine; TYW5, tRNA-wybutosine synthesizing enzyme 5.

early eukaryotes (97). Base J has broad roles in genome regulation and is biosynthesized in two steps: JBP1 and JBP2 catalyze thymidine hydroxylation in DNA, thereby enabling glycosylation (110). JBP1, but not JBP2, also has a Base J double-strand DNA-containing binding domain (110).

2OGX-CATALYZED SMALL-MOLECULE OXIDATIONS

Although hydroxylation is likely the dominant 2OGX reaction on both small and large molecules, the potential catalytic diversity of 2OGXs is more fully realized in oxidation of the former. Some of the small-molecule reactions in which they are involved are biologically and societally important. In plants and animals, 2OGXs catalyze reactions during biosynthesis of most major classes of secondary metabolites, including peptides, alkaloids, terpenoids, carbohydrates, and mixed-origin molecules (3). They introduce diversity by modifying molecules prior to (e.g., amino acid hydroxylation) and during (e.g., by catalyzing tethered peptide halogenation) oligomerization. Postassembly modifications of, e.g., gibberellins and peptides, introduce further diversity. In microbes, 2OGXs also have roles in sulfur and phosphate metabolism: For example, taurine dioxygenase catalyzes hydroxylation of 2-aminoethane sulfonate (taurine) to give an alcohol that nonenzymatically fragments to give an aldehyde and sulfite, which is recycled in sulfur metabolism (3).

Proteinogenic amino acids that undergo 2OGX hydroxylations include proline to give C-4/C-3 hydroxylated products (112, 113), asparagine (114), aspartate (115), arginine to give C-3

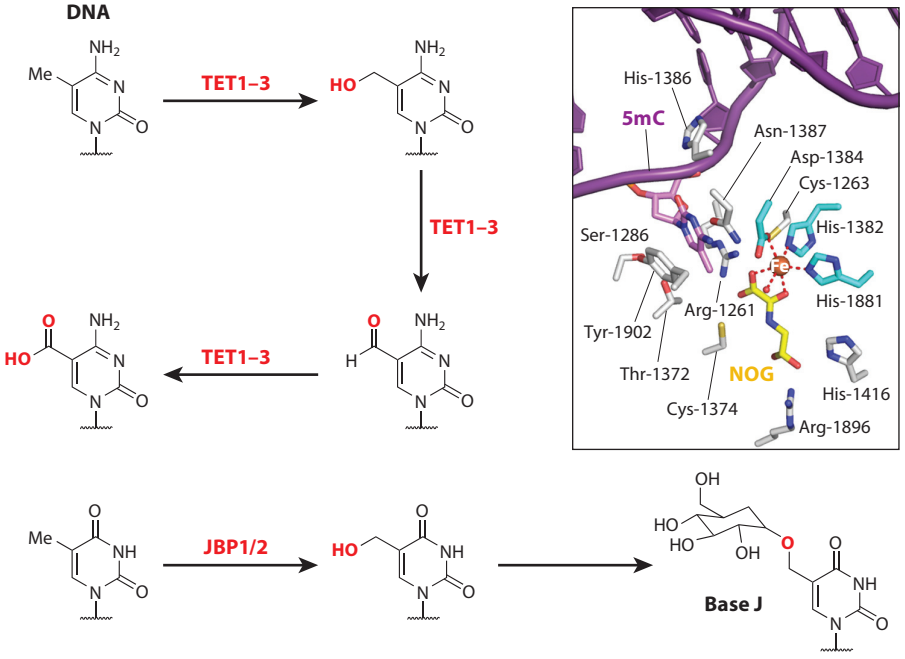


Figure 13

Oxidative reactions on DNA bases catalyzed by TETs and related 2OGXs. Sequential oxidations of 5-methylcytosine (5mC) bases are catalyzed by TET1–3 in humans. (*Boxed inset*) Human TET2 complexed with iron (*orange*), NOG (2OG analog) (*yellow*), and double-stranded DNA containing the substrate 5-methylcytosine (*purple*) (PDB ID: 4NM6) (111). Residues involved in binding iron (*blue*), NOG, and substrate (*gray*) are also shown. In kinetoplastids, hydroxylation of thymine on the 5-methyl group by JBP1/2 forms 5-hydroxymethyluracil, which is then glycosylated, forming Base J. The glycosyltransferase(s) catalyzing the glycosylation step is unidentified. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; JBP, Base J binding protein; NOG, *N*-oxalylglycine; PDB, Protein Data Bank; TET, ten-eleven translocation.

hydroxylated products (116), leucine to give C-4/C-5 hydroxylated products (117), and isoleucine to give C-4 hydroxy and ketone products (118–120). Multiple modified proteinogenic amino acids and peptides (121), including N^{ε}-trimethyl-lysine, γ-butyrobetaine (47), enduracididine (122), and ectoine (123), also undergo 2OGX oxidation.

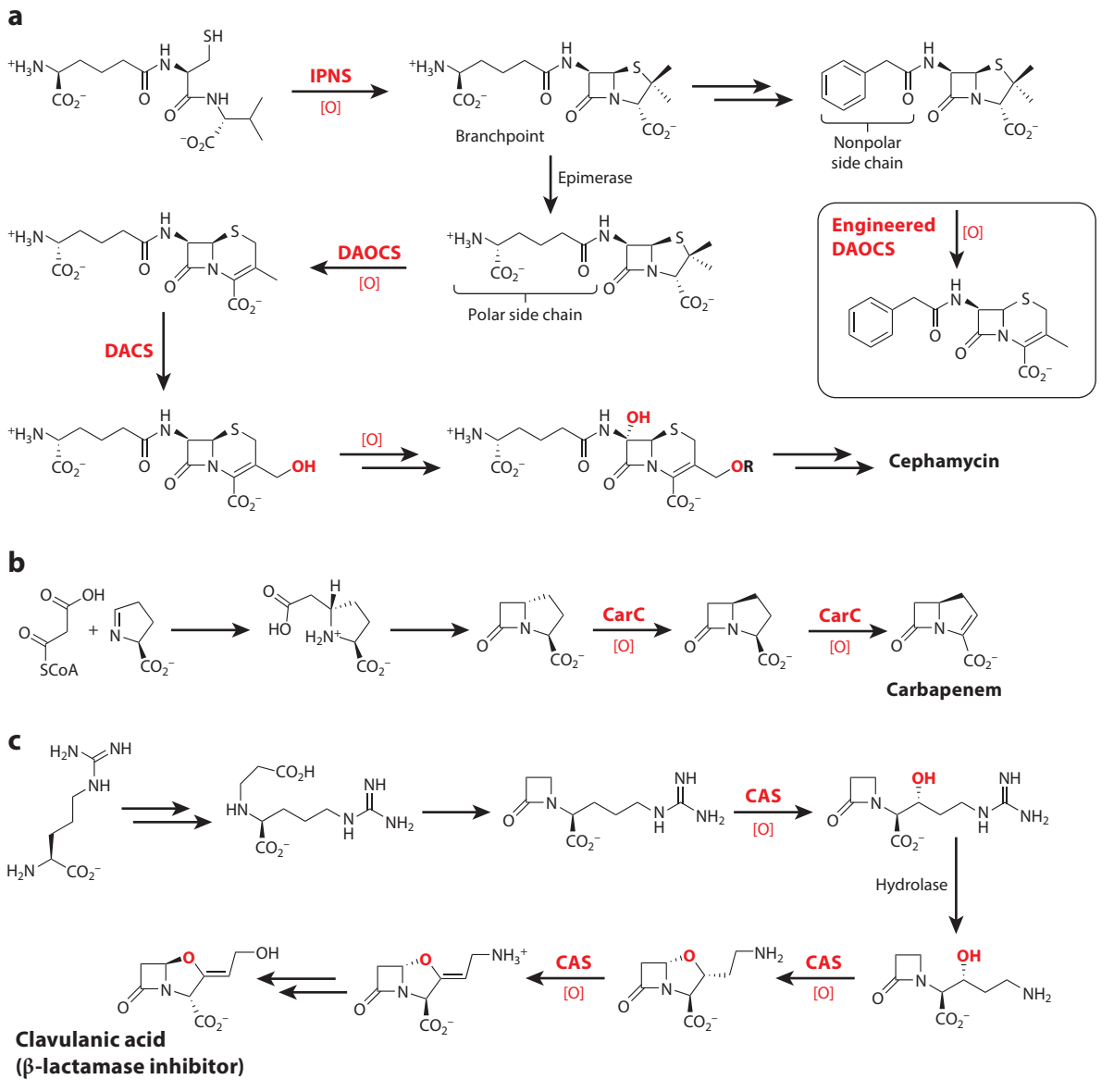
An ethylene-forming enzyme from *Pseudomonas* spp. catalyzes C-5 arginine hydroxylation, uncoupled 2OG turnover, and, unusually, 2OG fragmentation (**Supplemental Figure 3**) (124, 125). In one mechanistic branch, arginine promotes uncoupled turnover of 2OG to succinate and CO_2; C-5 arginine hydroxylation leading to pyrroline-5-carboxylate can also occur. Alternatively, Grob-type fragmentation of a 2OG-derived succinate linked to a ferryl intermediate (or succinyl peroxide) is proposed to produce ethylene and CO_2. Crystallographic and quantum chemical studies reveal fragmentation that produces ethylene is promoted by binding of arginine in a nonoxidized conformation and of 2OG in an unusually hydrophobic pocket and with an unprecedented high-energy conformation that favors ethylene rather than succinate formation (**Supplemental Figure 3**).

An additional role of 2OGXs is ring formation in β-lactam biosynthesis (**Figure 14**) (32). Penicillin formation proceeds via IPNS-catalyzed oxidation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine to give isopenicillin N (**Supplemental Figure 3**). IPNS does not use 2OG but instead

Supplemental Material

catalyzes four-electron oxidation, which proceeds via a monocyclic β-lactam linked via its cysteinyl thiol to an Fe(IV)=O intermediate that undergoes thiazolidine ring formation to give bicyclic penicillin (36). IPNS accepts several substrate analogs, which may relate to direct ligation of its substrate thiol to Fe(II); this process does not occur during normal 2OGX catalysis (36).

During cephalosporin biosynthesis, (*D*)- to (*L*)-α-aminoadipoyl side-chain epimerization is followed by ring expansion of the penicillin N amide thiazolidine ring, as catalyzed by DAOCS, to give the dihydrothiazine cephem ring (likely via a methylene radical generated by reaction between a ferryl intermediate and the penicillin N β-methyl group) (**Figure 14*a***). Further 2OGX-catalyzed



(*Caption appears on following page*)

Figure 14 (*Figure appears on preceding page*)

Roles of 2OGXs in biosynthesis of bicyclic β-lactam antibiotics and β-lactamase inhibitors illustrate the ability of the Fe(IV)=O intermediate to enable formation of functionalized rings of medicinal importance. (*a*) IPNS does not use 2OG but is structurally homologous with 2OGXs and catalyzes four-electron oxidation of a dipeptide to produce the penicillin nucleus. DAOCS and DACS catalyze oxidative ring expansion and hydroxylation, respectively, during cephem biosynthesis. IPN is a branch point between biosynthesis of penicillins with hydrophobic side chains and cephalosporins with hydrophilic side chains (in different microorganisms). Engineering of DAOCS to accept hydrophobic penicillins is a biocatalytic objective. (*b*) CarC catalyzes a redox-neutral, but 2OG- and O_2-dependent, epimerization as well as more typical desaturation reactions during carbapenem biosynthesis. (*c*) CAS catalyzes three types of two-electron oxidation during clavam biosynthesis (CAS I, CAS II, CAS III); the latter cyclization (II) and desaturation (III) steps are separated from the initial hydroxylation (I) step catalyzed by the guanidine hydrolase PAH, which effectively mutates the product of the first CAS-catalyzed step to enable the second and third CAS-catalyzed reactions. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; CarC, carbapenem synthase; CAS, clavaminic acid synthase; DACS, deacetylcephalosporin C synthase; DAOCS, deacetoxycephalosporin C synthase; IPN, isopenicillin N; IPNS, IPN synthase; PAH, proclavaminate amidino hydrolase.

oxidations, including at the sterically demanding C-7 position, are involved in DAOC modification to give the cephamycins (32). 2OGXs also catalyze steps in the biosynthesis of other β-lactam antibiotics, including carbapenems and clavams (**Figure 14*b*,*c***) (32, 126). During carbapenem biosynthesis, the 2OGX carbapenem synthase catalyzes a chemically challenging epimerization likely involving a tyrosinyl-radical intermediate; it also catalyzes a desaturation reaction (**Figure 14*b***) (127). During clavam biosynthesis, clavulanic acid synthase catalyzes three oxidative reactions: hydroxylation, oxidative ring closure, and desaturation (**Figure 14*c***) (126).

Such reactions, as well as those in terpene and alkaloid biosynthesis, exemplify the capability of 2OGXs to catalyze sequential substrate oxidations (which also occur on other substrate classes, e.g., during thymine hydroxylase and TET catalysis) and myriad small-molecule oxidative reactions. Halogenations occur on synthetase-tethered and free small-molecule substrates (4, 128, 129). Halogenase catalysis may proceed via initial hydrogen abstraction by the Cl/Br-Fe(IV)=O species to give a substrate radical, which in turn reacts with the metal-bound halide ion to give the halogenated product and restore the Fe(II) oxidation state (4, 128, 129).

Important small-molecule oxidations in plant biology and agrochemistry include 2OGX-catalyzed biosynthesis of plant signaling molecules such as gibberellins (130) (**Figure 15*a***) and flavonoids (131) (**Figure 15*b***). S-glucosinolate metabolism is also significant. In crops, biosynthesis of gibberellins, which are also present in microbes (**Figure 15*a***), has been targeted to introduce beneficial trials, e.g., to hinder plant lodging in rice (130). Catalytic flexibility in this family is further illustrated by their roles in hydroxylations, desaturations, and methyl group oxidation; the latter involves sequential oxidation of the C-20 methyl group to an alcohol, then aldehyde, and finally oxidative decarboxylation to give a lactone linking C-4 to C-10 (**Figure 15*a***). In hydroxylations and desaturations, plants use flavonoids for flower coloring and to protect against microbes and photodamage (131). Depending on the organism, the same step in flavonoid biosynthesis is catalyzed by either P_{450} oxygenase or 2OGX (131).

Ethylene is an important plant signaling molecule involved in development and regulation of its biosynthesis as well as fruit ripening. Ethylene is produced from methionine in the Yang cycle (132). The two-electron oxidation of 1-amino-cyclopropane-1-carboxylate (ACC) is catalyzed by ACC oxidase to give ethylene, hydrogen cyanide, and CO_2. This 2OG-independent reaction is strongly stimulated by CO_2 and ascorbate (**Figure 16**) (132). Crystallographic analyses, however, reveal a clear relation between the folds of ACC oxidase and 2OGX, especially those involved in flavonoid biosynthesis and IPNS (133).

By contrast, 2OGXs appear to be rarely involved in agrochemical/drug resistance. However, 2,4-dichlorophenoxyacetic acid herbicides undergo hydroxylation followed by fragmentation

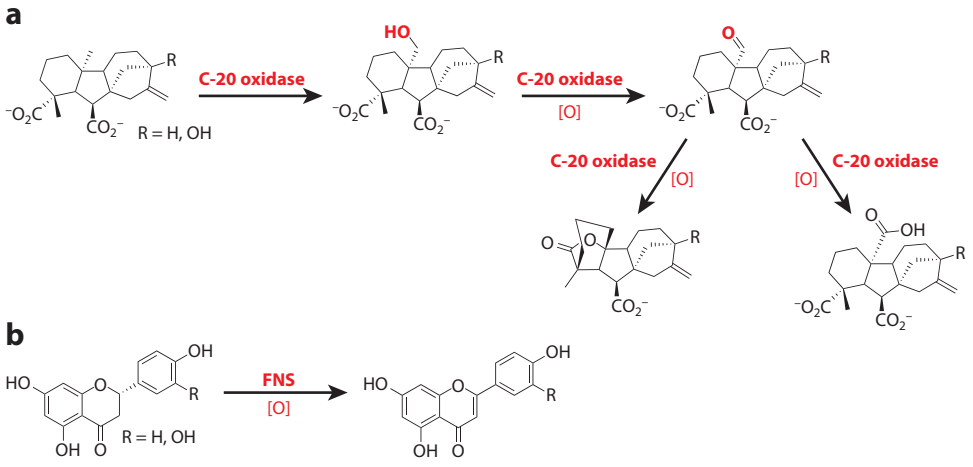


Figure 15

Examples of oxidative reactions catalyzed by 2OGXs in biosynthesis of (*a*) gibberellins and (*b*) flavonoids. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; FNS, flavone synthase 1.

(134). Additional examples of important 2OGX-catalyzed reactions continue to emerge; for example, EasH catalyzes oxidative cyclopropanation during ergot alkaloid biosynthesis (135) (**Supplemental Figure 4**).

Supplemental Material

2OGX-CATALYZED LIPID OXIDATIONS

In animals and some earlier organisms, 2OGXs catalyze two hydroxylation steps during biosynthesis of carnitine, which is required for fatty acid transport into mitochondria; in humans, carnitine is obtained through both endogenous biosynthesis and diet (**Figure 17**) (46). N^{ε}-Trimethyl-lysine hydroxylase catalyzes the first step, whereas γ-butyrobetaine hydroxylase (BBOX) catalyzes the

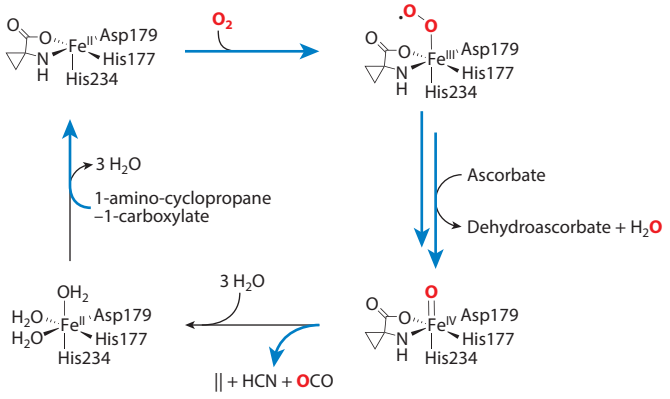


Figure 16

Ethylene formation catalyzed by ACC oxidase. ACC is converted to ethylene, hydrogen cyanide, and CO_2 in an O_2-dependent reaction that likely proceeds via an Fe(IV)-oxo intermediate. 2OG is not required for catalysis, but CO_2/bicarbonate stimulates the reaction via an unknown mechanism. Abbreviations: 2OG, 2-oxoglutarate; ACC, 1-amino-cyclopropane-1-carboxylate.

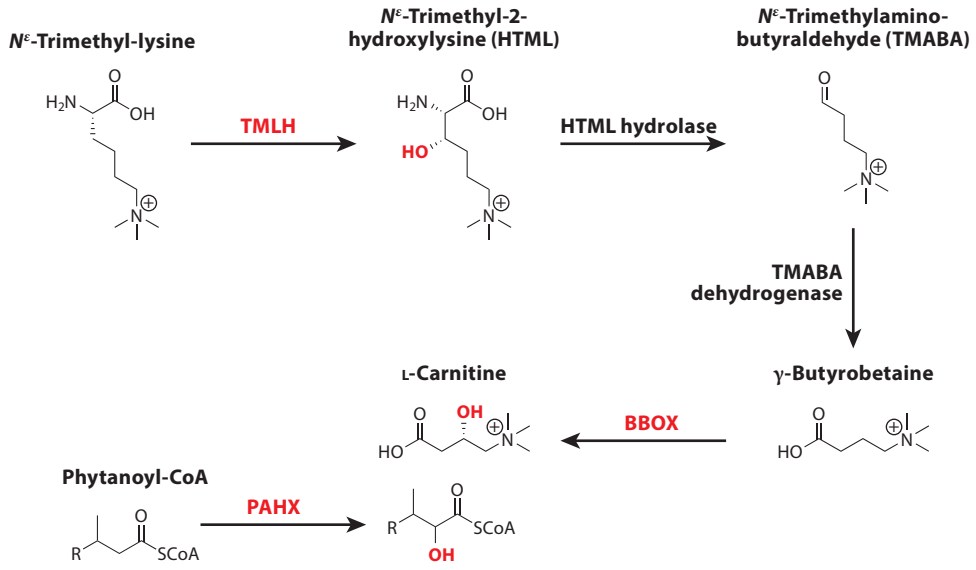


Figure 17

Hydroxylations catalyzed by 2OGXs during L-carnitine biosynthesis and metabolism of phytanic acid. Two 2OGXs are involved in L-carnitine biosynthesis; TMLH catalyzes C-3 hydroxylation of N^{ε}-trimethyl-lysine (first step), whereas BBOX (or GBBH) catalyzes C-3 hydroxylation of γ-butyrobetaine (last step). Meldonium (Mildronate) is a cardioprotectant in clinical use; this BBOX substrate reacts to form multiple products (including one rearrangement product). PAHX catalyzes α-hydroxylation of phytanoyl-CoA during phytanic metabolism. Each 2OGX reaction (enzyme denoted in *red*) is coupled to conversion of 2OG and O_2 to succinate and CO_2. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; BBOX, γ-butyrobetaine hydroxylase; GBBH, γ-butyrobetaine hydroxylase; PAHX, phytanoyl-CoA hydroxylase; TMLH, N^{ε}-trimethyl-lysine hydroxylase.

final step (**Figure 17**) (136). In carnitine biosynthesis, N^{ε}-trimethyl-lysine availability may be rate limiting. Thus, its hydroxylation may provide a direct link between proteins, including histone methylation status, and fatty acid metabolism (47). BBOX is one of the targets of Meldonium (or Mildronate), a clinically used cardioprotectant that athletes have also used for performance enhancement (137, 139–141) (see **Figure 17**).

Chlorophyll-derived phytanic acid has an epimeric C-3 (β) methyl group that prevents its degradation via the normal β-oxidation pathway (136). Phytanoyl-CoA hydroxylase (PAHX) catalyzes C-2 (α) hydroxylation of phytanoyl coenzyme A, enabling subsequent fragmentation to give pristanal, which is converted to pristanoic acid. Mutations to the PAHX gene cause Refsum disease, whose symptoms result from elevated phytanic acid (136). PAHX catalyzes stereoselective hydroxylation of the naturally occurring phytanoyl CoA C-3 epimers to give diastereomeric products (**Figure 17**). 2OGXs also catalyze lipid oxidation in bacteria. In particular, the bacterial cell-wall component Lipid A undergoes hydroxylation of its sugar and lipid moieties, though the biological relevance of these modifications remains undetermined (3).

BBOX: γ-butyrobetaine hydroxylase

Meldonium: drug that inhibits γ-butyrobetaine hydroxylase

INHIBITION OF 2OGXs

Interest in human 2OGX inhibition has increased owing to their roles in regulating protein biosynthesis. Inhibitors of HIF PHDs are in trials for treatment of anemia, and orally active inhibitors of the JmjC KDM5 subfamily have been reported (34, 138). Researchers have developed efficient

assays for 2OGX-inhibitor screening (34), including strategies based on cosubstrate depletion or coproduct formation. Formaldehyde detection assays employ coupling with formaldehyde dehydrogenase and have been developed for JmjC KDMs. However, owing to the possibility of uncoupling 2OG and substrate oxidation, challenges in analyzing gases, and deficiencies with coupled assays, methods that directly monitor substrate consumption or product formation, e.g., by mass spectrometry, NMR (in lower-throughput mode), or antibody-based methods, are preferred (34). Binding assays have been developed using ligand-observe NMR and more general methods (e.g., fluorescence) that observe 2OG or substrate displacement (36, 37, 142, 143).

The combination of crystallography and solution-based analyses can be particularly useful in understanding inhibitor binding modes, though protein-observe NMR is often not possible owing to the size of many 2OGXs. Overall, the available assay methods are tractable for most 2OGXs if sufficient quantities of active enzyme can be prepared. More biologically representative assays including for multidomain 2OGXs with protein or nucleic acid substrates are important because the efficiency and nature of some 2OG-dependent hydroxylations can vary with substrate length and conformation. Thus, assays with full-length JmjC KDMs and intact nucleosomes as well as the ability to target non-catalytic domains of 2OGXs are needed.

The quality and biological relevance of cellular assays for 2OGXs vary. Direct quantitative assays are available for some small-molecule oxygenases, e.g., BBOX (34, 142, 143). However, cellular assays often employ antibody-based methods for protein and nucleic acid substrates. These methods do not necessarily give a direct readout of enzyme activity, as is likely the case for analysis of JmjC KDM inhibition using global histone-methylation status. Though less common, mass spectrometry–based methods have been used and are likely to be of increasing importance for analyzing the effects of inhibitors of biomacromolecule modifications. In upregulation of erythropoietin/erythropoiesis by HIF PHD inhibitors, physiologically relevant assays on intact organisms are possible (37). Recent work on JmjC KDMs, in particular KDM5, suggests selective inhibition of cancer cells, including drug-resistant types, may be possible (138). Cell-based work should also consider the different kinetic properties of 2OGXs, including any variations in 2OG binding strength and variations in 2OG concentrations among different cell types.

Most 2OGX inhibitors can be classified into (*a*) those that compete with 2OG and/or substrate and (*b*) those that do or do not bind to Fe(II). In the latter, inhibitors may bind to Fe(II) either at the active site and/or in solution. Some 2OGXs, e.g., JmjC KDMs, contain non-catalytic domains involved in substrate targeting, which are potential inhibition targets.

Small-molecule inhibition of 2OGXs was pioneered in the agrochemical and pharmaceutical fields prior to modern molecular and structural biology. This work has identified potent inhibitors, most of which likely complex to active site Fe(II) and compete with 2OG (**Figure 18**) (34); *N*-oxalylglycine (**Figure 18**) and pyridine-dicarboxylate derivatives, which are 2OG analogs, are used extensively in cell biology (34). There are variations in potencies of these broad-spectrum inhibitors against particular types of 2OGX (34, 144). Although multiple crystallographic analyses have been reported, binding modes observed for these and related compounds likely do not provide full details of the precise inhibition mode in solution; there is evidence that coordination mode and movement of the active site metal may occur (144).

Cellular analyses are complicated by variations in binding affinities of 2OGXs for 2OG and by variations in intracellular 2OG concentrations (see above). Despite these issues, inhibitors complexing active site Fe(II) and competing with 2OG have been developed for clinical use (25, 145, 146). Some of these inhibitors block substrate binding (at least at the active site), whereas others stabilize it (147). *N*-oxalylglycine is a catalytically inactive analog of 2OG that stabilizes enzyme-substrate complexes and has been used in crystallographic and proteomic substrate-trapping studies.

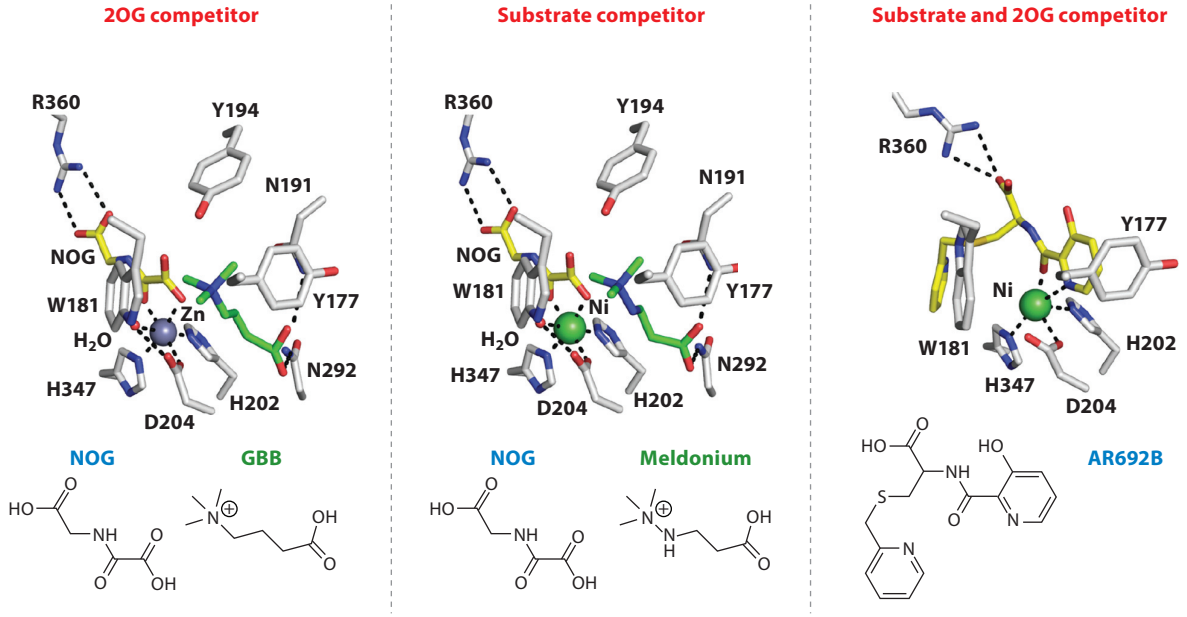


Figure 18

Views from BBOX1-inhibitor complex structures illustrating different 2OGX inhibitor types. Inhibitors competing with 2OG are exemplified in a crystal structure of BBOX1 complexed with 2OG analog NOG and its substrate GBB (PDB ID: 3O2G), which reveals very similar binding modes for NOG and 2OG. Substrate-competing inhibitors are exemplified in the BBOX1 structure in complex with Meldonium (Mildronate) and NOG (PDB ID: 3MS5). Note that Meldonium is a BBOX1 substrate. Inhibitors competing with both 2OG and substrate are exemplified in a BBOX structure complexed with AR692B that occupies both 2OG and GBB binding sites. Abbreviations: 2OG, 2-oxoglutarate; 2OGX, 2OG-dependent oxygenase; BBOX, γ-butyrobetaine hydroxylase; GBB, γ-butyrobetaine; NOG, *N*-oxalylglycine; PDB, Protein Data Bank.

Although displacement of active site Fe(II) from 2OGX active sites might be difficult to achieve in a selective manner, the erythropoetic effect of Co(II) ions (which have been used to treat anemia) is likely due to inhibition of HIF PHDs via competition of Co(II) and Fe(II) for active site binding (148, 149). If this mechanism is correctly assigned, toxicity due to inhibition of other 2OGXs might result. Relatively low levels of toxicity might reflect an alternative mechanism, or Co(II) ions may have an unusually high effect on PHD activity in vivo for reasons not understood. Crystallographic evidence suggests organic small molecules may also displace active site Fe(II) (144).

Work in agriculture to develop plant growth retardants has led to important applications of 2OGX inhibitors (130, 150). These inhibitors target enzymes involved in biosynthesis of the gibberellin plant growth hormones. The most important of such inhibitors are tricarbonyl compounds, e.g., prohexadione and trinexapac-ethyl (150), which likely are 2OG competitors and Fe(II) chelators. Other plant oxygenase inhibitors include daminozide [4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid]. It was used to control fruit ripening until concern regarding potential carcinogenicity precluded its use (151), but it is still used as a growth retardant of ornamental plants (150). Daminozide inhibits some JmjC KDMs through 2OG competition and Fe(II) chelation via carbonyl O_2 and dimethylated nitrogen of its dimethylhydrazinyl group (152). Tricarbonyl compounds are also commercially important inhibitors of the herbicide target *p*-hydroxyphenylpyruvate dioxygenase (HPPD). This enzyme is not a 2OGX (153) but a 2-oxo acid oxygenase with similar active site chemistry, albeit with a different protein fold. The HPPD

inhibitor Nitisinone {2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione} is used to inhibit human HPPD in the treatment of type-I tyrosinemia (153, 154).

In an overlapping timeframe in agrochemistry, inhibitors of human collagen prolyl hydroxylases were developed for testing as antifibrotics (34). Potent heteroatomic inhibitors of these enzymes (all likely 2OG competitors) (**Figure 18**) were developed, but their toxicity curtailed further efforts. Functional work on HIF PHDs reawakened interest in prolyl hydroxylase inhibition; inhibitors for these domains are now in late-stage clinical trials for anemia treatment (25, 145). Some PHD inhibitors are related to those developed for inhibition of plant gibberellin biosynthesis enzymes and the collagen prolyl hydroxylases, and at least at the biochemical level, most reported HIF PHD inhibitors are likely not completely selective. However, next-generation compounds, including those that replace close 2OG analogs with more complex heterocyclic frameworks, are emerging. Work on HIF hydroxylases reveals inhibitor selectivity may be obtained for PHDs or FIH (25). Structural and kinetic studies (26) imply the development of isoform-selective inhibitors should be possible. Such inhibitors not only are of interest from a pharmaceutical perspective but also will be useful for dissecting the context-dependent roles of the different HIF hydroxylases in the hypoxic response.

The roles of JmjC KDMs in epigenetic regulation stimulated substantial work on inhibition within academia and industry (34, 90). At present, JmjC KDM inhibitors are mostly aimed at treating cancer, and many are typical 2OGX inhibitors (34, 89, 155). However, new types are emerging, and the most advanced compounds are orally active KDM5 inhibitors. Crystallographic studies indicate a large surface area of interaction between JmjC oxygenases and their substrates; thus, other types of inhibition may be possible, e.g., JmjC KDM inhibitors that function via substrate competition (**Figure 18**) (87). Available evidence from recent work on cyclic peptide inhibitors of the JmjC KDM4A-C subfamily also indicates that substrate-competitive inhibition is more likely to enable highly selective inhibition, e.g., of specific isoforms (156). Nonetheless, the use of 2OG competition is a viable strategy, at least in the short term, as evidenced by the apparent lack of acute toxicity in trials for HIF PHD inhibitors.

The development of mechanism-based inhibitors of 2OGXs is of interest but has not been widely explored. Although the BBOX inhibitor Meldonium was not identified by enzyme assays, it undergoes oxidative fragmentation to give a mixture of products, including some produced by an oxidative Stevens-type rearrangement (**Supplemental Figure 5**) (139, 140).

Supplemental Material

Cell-based inhibition of 2OGXs by endogenous molecules may be important in tumor biology (45). Mutations to genes encoding for TCA cycle enzymes can cause large increases in the concentrations of succinate, fumarate, or 2-hydroxyglutarate. In the latter, this increase is due to gain-of-function mutations to the genes encoding for both cytosolic and mitochondrial forms of isocitrate dehydrogenase. Levels of 2OG were also decreased, and these metabolic changes may cause 2OG-competitive inhibition of 2OGXs, e.g., in the regulation of transcription/chromatin, with tumorigenic consequences. HIF hydroxylases, JmjC KDMs, and TETs may all be deregulated in this manner (157–159). Though evidence for the latter is uncertain, 2-hydroxyglutarate may function as both inhibitor and substrate of some 2OGXs (160, 161). Despite little consideration to date, 2OGX activity alters total 2OG/succinate concentrations, particularly when their levels are reduced. Candidates for this role include 2OGXs with multiple substrates (e.g., FIH in animals) or substrates that are efficient catalysts (e.g., antibiotic biosynthesis enzymes in highly evolved strains).

BIOCATALYTIC APPLICATIONS OF 2OGXs

The diversity in 2OGX reactions suggests they have substantial potential in industry. Use of isolated 2OGXs for large-scale biocatalysis may be challenging owing to issues associated with

generation of reactive oxidizing species leading to enzyme damage. However, work with proline hydroxylases has demonstrated the validity of using recombinant 2OGXs in cells for industrial-scale biocatalysis (162). Recombinant prolyl-4-hydroxylase in bacteria is used to produce *trans*-4-hydroxyproline, which functions as pharmaceutical starting material (162). The ability of proline hydroxylases to accept different ring sizes and catalyze different types of reactions (e.g., epoxidation) exemplifies how 2OGXs carry out amino acid oxidations. This implies additional possibilities for applying recombinant 2OGX, e.g., for production of 4-hydroxy-isoleucine for diabetes treatment (163).

To enable production of cephalosporins with hydrophobic side chains, which are easier to purify, engineering is targeting the cephalosporin biosynthesis pathway, which naturally produces cephalosporins with the hydrophilic α-D-α-aminoadipoyl side chain (**Supplemental Figure 3**) (164–166). One approach involves altering DAOCS selectivity so it efficiently accepts a penicillin with a hydrophobic side chain (e.g., penicillin G or V) rather than the polar natural D-δ-α-aminoadipoyl side chain (**Supplemental Figure 3**). This work exemplifies how a 2OGX catalyzing a synthetically difficult reaction can be used in an engineered pathway.

Supplemental Material

Although more challenging, the ability of 2OGXs to catalyze protein modifications also has potential in biocatalysis. The relaxed substrate selectivity of the amino acid hydroxylases is mirrored at least in FIH, one of the analogous JmjC protein hydroxylases. With peptide substrates, albeit under forcing conditions, FIH catalyzes hydroxylation of hydrophobic residues (167). These results suggest that 2OGXs could help introduce functional groups into proteins, e.g., halogens or reactive rings, that could be employed for further protein modification, e.g., to enable cross-linking of antibody-drug conjugates.

Work on 2OGXs and related nonheme oxygenases has also inspired development of biomimetic catalysts that add alcohols and other functional groups to small molecules. Following initial breakthroughs (168, 169), advances in analytical methodologies, especially liquid chromatography–mass spectrometry, have enabled work to progress. Although still at an early stage, recent work has identified reagents that catalyze hydroxylation, oxidation, azidations, and halogenations, sometimes at unactivated positions. Owing to the potential for late-stage functionalization of drug-like molecules, interest in this field will likely increase to enable efficient structural diversification.

CONCLUSIONS AND FUTURE PROSPECTS

Work over the past half century has established the major biological and societal importance of the 2OGX superfamily. Both genetic and small-molecule approaches have enabled targeting of plant 2OGXs to be significant contributions to the Green Revolution. Pharmaceutical targeting of 2OGXs has been less successful to date. However, compounds targeting their roles in transcriptional regulation are in clinical trials to treat diseases such as cancer and anemia. If one or more of these treatments are successful, research on 2OGXs will likely grow substantially. Biologically, the identification of roles for 2OGXs in modifying the core genetic and epigenetic machinery in eukaryotes has massively stimulated scientific work on them. Understanding how their biophysical and kinetic properties connect with their physiology, as likely occurs in the hypoxic response, is of particular biochemical interest. To date, the diversity of reactions catalyzed by 2OGXs involving small molecules has been only partially reflected in their macromolecular substrates. Hence, the full extent of protein and nucleic acid oxidation by 2OGXs has likely not yet been defined.

FUTURE ISSUES

1. A deeper understanding of dynamics during 2OGX catalysis in solution is needed.
2. It remains unclear how multidomain 2OGXs involved in regulation of protein biosynthesis interact with their macromolecular substrates. Cryo-EM may be used to investigate JmjC KDM binding to nucleosomes.
3. Highly potent and isoform-selective human 2OGX inhibitors are needed for use in functional assignments and medicinal chemistry target validation.
4. How ascorbate and other redox-active small molecules affect 2OGX activity in vivo requires mechanistic understanding.
5. An understanding of how Fe(II) is delivered to and maintained in 2OGXs in cells is needed.
6. High-resolution structural information on the catalytic domains of human procollagen prolyl and lysyl hydroxylases is of interest.
7. Knowledge of 2OGXs could be applied to develop useful small-molecule catalysts for synthesis reactions.
8. The early evolution of 2OGXs and related oxygenases remains unknown.

DISCLOSURE STATEMENT

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Errata

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