CHAPTER 1

Biochemical Diversity of 2-Oxoglutarate-Dependent Oxygenases

ROBERT P. HAUSINGER*[a,b]

[a]Department of Microbiology and Molecular Genetics, [b]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA
*E-mail: hausinge@msu.edu

1.1 Introduction

This chapter summarizes the diverse array of biochemical transformations that are catalysed by Fe(II)- and 2-oxoglutarate (2OG, also known as α-ketoglutarate)-dependent oxygenases.[1–5] As described more comprehensively in Chapter 2, most of these enzymes coordinate their active site metal at one end of a double-stranded β-helix fold (Figure 1.1A)[6–8] using a 2-His-1-carboxylate motif, bind 2OG within the core, and interact with their primary substrates using less conserved regions of the core and additional loops. Most representatives of this enzyme family catalyse hydroxylation reactions (Figure 1.1B), but desaturation, ring formation, ring expansion, halogenation and other types of chemistry are known, as described for several examples in later sections of this chapter. The mechanisms of these enzymes are detailed in Chapter 3, supported by chemical model investigations summarized in Chapter 4. A highly simplified mechanistic scheme for hydroxylases

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based on investigations of TauD (taurine hydroxylase), a well-characterized taurine-degrading enzyme (see Section 1.6.4),[9] is depicted in Figure 1.1C. As illustrated, the binding of 2OG displaces two waters from an octahedral Fe(II) site (converting state a to state b); a third water is lost as the primary substrate binds (generating state c); the newly vacant coordination site is used to react with O_2 to form an Fe(III)–superoxo species (state d); oxidative decarboxylation of 2OG yields an Fe(IV)–oxo species (state e, also known as the ferryl intermediate); this powerful oxidant abstracts a hydrogen atom from the substrate to generate a radical and Fe(III)–hydroxide (state f); and hydroxyl radical rebound (or more complex chemistry)[10] completes

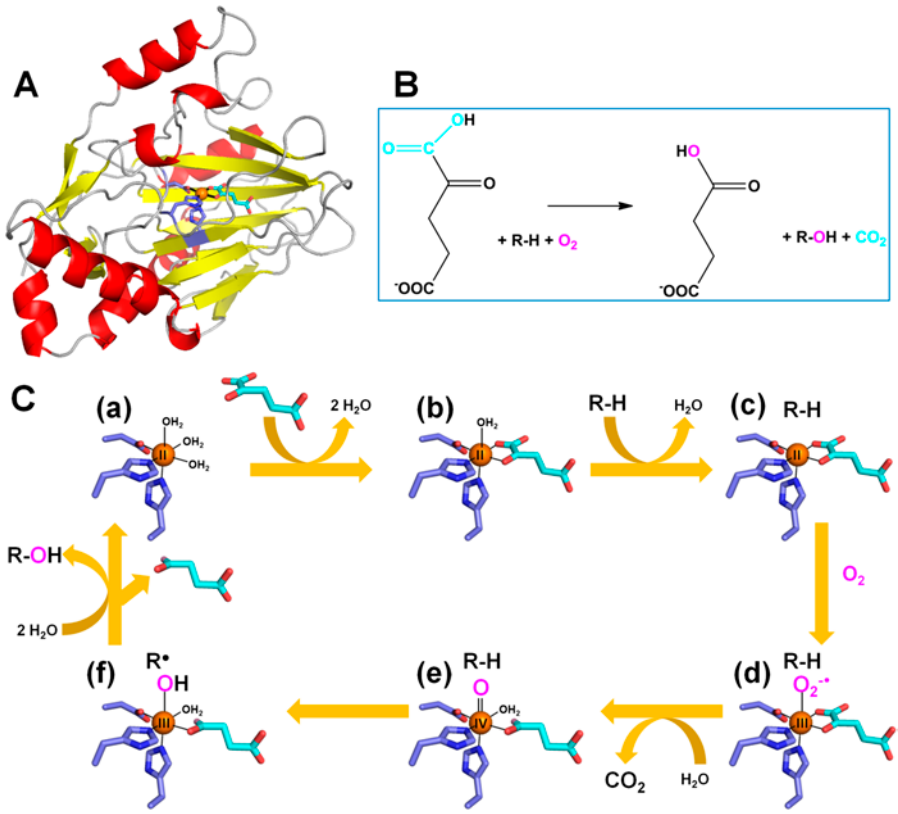

Figure 1.1 Structure and mechanism of a representative 2OG-dependent oxygenase. (A) Cartoon structure of TauD (PDB ID: 1GY9) with red α helices, yellow β strands, grey unstructured regions, Fe (orange sphere), metal ligands (sticks with blue carbons) and 2OG (sticks with cyan carbons). The major grouping of β strands forms a double-stranded β helix core. (B) Overall hydroxylation reaction in which one atom of O_2 is incorporated into substrate (R–H) in a step that is driven by the oxidative decarboxylation of 2OG; CO_2 is derived from the C-1 position of 2OG and succinate incorporates the second atom of O_2. (C) Simplified hydroxylation mechanism.

substrate hydroxylation and recycles the enzyme to the initial Fe(II) state. Such enzymes are dioxygenases because both atoms of oxygen are incorporated into substrates, with one atom ending up in succinate and the second in the hydroxylated product (which decomposes spontaneously in some reactions).[11] These enzymes are correctly referred to as primary substrate:2OG dioxygenases or primary substrate hydroxylases; it is incorrect to cite these enzymes as primary substrate dioxygenases or 2OG dioxygenases because these names imply both atoms of oxygen become incorporated into a single substrate. The other types of chemistries associated with this family of enzymes are also likely to utilize the Fe(IV)-oxo intermediate.

The total number of 2OG-dependent oxygenases found in biology is immense. For example, humans and other animals are thought to possess about 80 such enzymes.[7] Even more are predicted to be present in plants such as the model organism *Arabidopsis thaliana*.[12] Some 2OG-dependent oxygenases are widely distributed throughout aerobic life forms, whereas highly specialized enzymatic catalysts have evolved in various microorganisms or plants for synthesis or degradation of compounds found in their unique niches. In the following sections, the vast array of reactions catalysed by 2OG-dependent oxygenases are divided into seven categories. Section 1.2 describes the 2OG-dependent enzymes acting on proteins. These reactions may lead to structural consequences, have oxygen-sensing functions, alter histone properties, or possess other roles. Section 1.3 summarizes the metabolism of 2OG-dependent oxygenases acting on polynucleotides. The functions of these enzymes include DNA or RNA repair of alkylation damage, roles in transcriptional regulation, biosynthesis of base J in DNA or of specific tRNAs, and demethylation of 5-methylcytosine. Enzymes involved in lipid-related metabolism are discussed in Section 1.4. Examples include reactions related to carnitine biosynthesis, the degradation of phytanic acids, and the decoration of ornithine lipids or lipid A. Plant-specific representatives and close relatives are highlighted in Section 1.5. Of interest are reactions related to synthesis of flavonoids and anthocyanins, gibberellins, alkaloids, and other metabolites found predominantly in plants. Section 1.6 covers enzymes that act on a variety of small molecules including free amino acids, nucleobases or nucleosides, herbicides, sulfonates/sulfates and phosphonates. In some cases, the products resulting from these transformations are incorporated into antibiotics whereas other reactions are involved in metabolite recycling or alternative biochemical pathways. Additional 2OG-dependent oxygenases utilized for antibiotic biosynthesis are provided in Section 1.7. Examples include several halogenating enzymes and other representatives derived from bacterial and fungal sources. Finally, Section 1.8 covers enzymes that are related in structure or mechanism to the 2OG-dependent oxygenases. These include isopenicillin *N* synthase and the plant-specific ethylene-forming enzyme, which contain the 2OG oxygenase fold yet fail to utilize 2OG, and two enzymes that share a distinct fold and use the alternative oxo-acid 4-hydroxyphenylpyruvate.

1.2 2OG-Dependent Dioxygenases that Act on Proteins

This section describes 2OG-dependent dioxygenases that act directly on protein side chains or at modified sites in proteins and catalyse the reactions summarized in Figure 1.2.

1.2.1 Protein Substrates with Structural Roles

The earliest investigations of the 2OG-dependent dioxygenases were focused on enzymes acting on protein substrates with structural roles. For example, seminal studies from the 1960s demonstrated that prolyl 4*R*-hydroxylase (Figure 1.2A) utilizes 2OG as a cosubstrate for its function in collagen synthesis.[13,14] Similar findings were observed for two other enzymes needed to functionalize collagen, prolyl 3*S*-hydroxylase (Figure 1.2B)[15] and procollagen lysyl 5*R*-hydroxylase (PLOD, Figure 1.2C).[16] By 1982, a remarkably prescient mechanism was proposed for prolyl 4*R*-hydroxylase and contained many of the features shown in Figure 1.2C, including coordination of Fe(II) by a facial triad, bidentate coordination of 2OG, and the generation of a reactive metal–oxo intermediate.[17] Collagen, the most abundant protein in mammals, is first synthesized as procollagen, which undergoes extensive modifications including the formation of 4*R*-hydroxyproline (4Hyp, accounting for 10% of its residues), 3*S*-hydroxyproline (3Hyp, ~1% of its residues), and 5*R*-hydroxylysine (Hyl, 0.5–7% of its residues) prior to assembly into its triple helical structure and export to the extracellular matrix.[18] 4Hyp is also found in elastin and other human structural proteins, plant cell wall components, and various other proteins or peptides of algae, selected bacteria, and even a virus.[19] Similarly, 3Hyp and Hyl have been detected in non-collagen proteins. The collagen-specific prolyl 4*R*-hydroxylases form heterodimers with protein disulfide isomerase, but only the homomeric enzymes from the bacterium *Bacillus anthracis* and the alga *Chlamydomonas reinhardtii* have been crystallized.[20–22] These structures reveal the basis for stereospecificity and substrate specificity. The procollagen-specific 2OG-dependent dioxygenases are described in greater detail in Chapter 5.

1.2.2 Protein Substrates with Oxygen-Sensing Roles

An O_2-sensing role for 2OG-dependent dioxygenases was uncovered in 2001.[23,24] A key component of this signalling pathway is the hypoxia-inducible factor (HIF-1) which directs the transcription of several genes under low O_2 conditions. The two subunits of this heterodimer (HIF-α and HIF-β in humans) are constitutively synthesized, but human HIF-α is modified in cells under normoxic conditions by three HIF-α-specific prolyl 4*R*-hydroxylases (PHDs, Figure 1.2A). These enzymes act in a tissue-specific manner to oxidize Pro-402 and Pro-564 of HIF-1α, which results in enhanced affinity of the

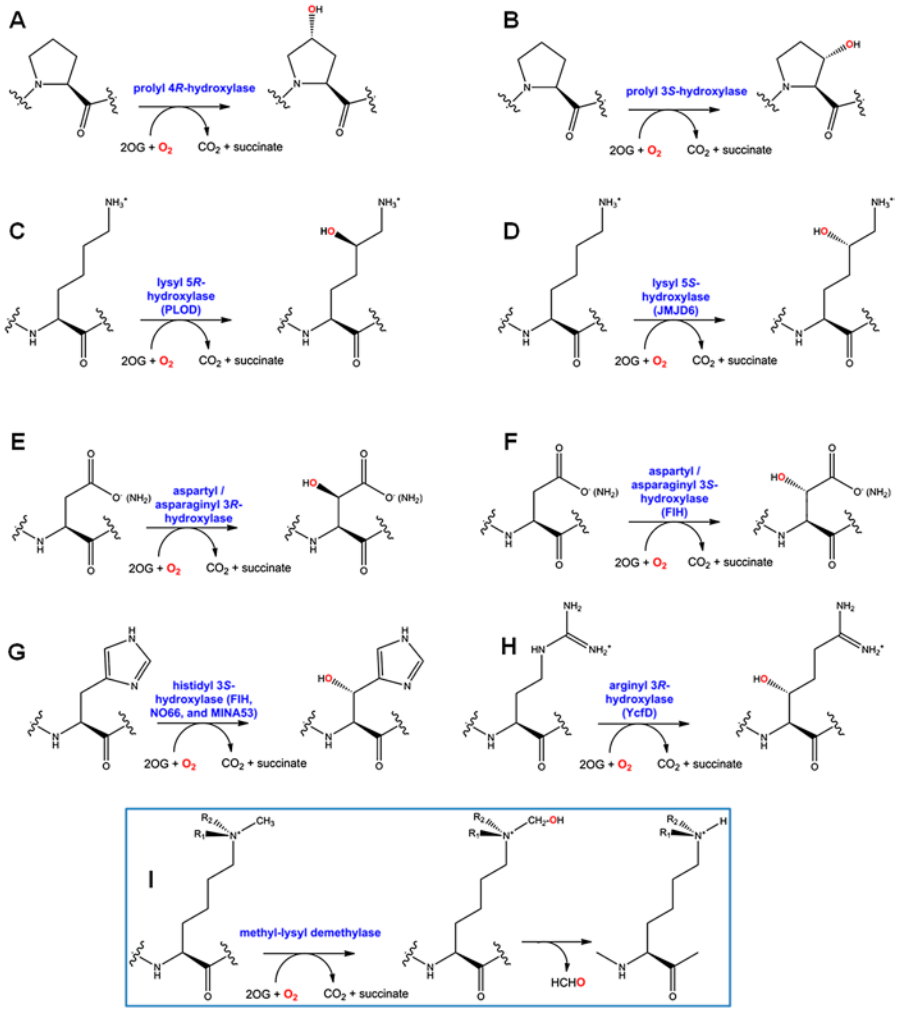

Figure 1.2 2OG-dependent dioxygenase reactions involving protein substrates. (A and B) Prolyl hydroxylases acting at the 4*R* and 3*S* positions. (C and D) Lysyl hydroxylases specific to the 5*R* and 5*S* positions. (E and F) Aspartyl or asparaginyl hydroxylases that insert oxygen at the 3*R* and 3*S* positions. (G) Histidyl 3*S*-hydroxylase. (H) Arginyl 3*R*-hydroxylase. (I) Methyl lysyl demethylases, where R_1 and R_2 are H or CH_3. A lysyl 4-hydroxylase is also known, but this reaction is not depicted because the enantiospecificity has not been reported.

protein towards the von Hipple–Lindau (VHL) tumour suppressor protein, elongin B and elongin C,[25,26] leading to polyubiquitinylation and proteosomal destruction of the transcription factor. The structure has been described for PHD2[27] and for PHD2 in complex with HIF-1α,[28] providing great insight into the geometric aspects of catalysis.

Independent of the PHD-specific process, a separate hydroxylation event involving Asn-803 of HIF-1α occurs in the presence of oxygen.[29] This modification is carried out by another 2OG oxygenase: factor-inhibiting HIF (FIH), an asparaginyl 3*S*-hydroxylase (Figure 1.2F).[30] The addition of a single atom of oxygen to HIF-1α results in loss of interaction with the p300/CBP transcription coactivators and abrogation of hypoxic signalling.[31,32] Several structures have been reported for FIH, including its interaction with a peptide derived from the coactivator.[33–35]

The O_2-sensing functions of the PHD and FIH 2OG-dependent dioxygenases have been summarized in several reviews,[36–38] and their structures and biological activities are described further in Chapters 2 and 6.

1.2.3 Ribosomal Protein Hydroxylases

Hydroxylation of ribosomal proteins has been demonstrated for 2OG-dependent enzymes, as first identified for cells from humans and *Escherichia coli*.[39] The human proteins NO66 and MINA53 catalyse 3*S*-hydroxylation of histidyl residues (Figure 1.2G) in Rpl8 and Prl27a, respectively, while the homologous enzyme YcfD of *E. coli* catalyses arginyl 3*R*-hydroxylation of Rpl16 (Figure 1.2H). The precise biological functions of these modifications are unclear, but when the *ycfD* strain was provided with low nutrient concentrations its growth rate was reduced compared to a wild-type strain. The crystal structure was first determined for the *E. coli* version of YcfD,[40] but additional structures quickly became available for truncated versions of NO66 and MINA53 along with the YcfD homologue of the thermophile *Rhodothermus marinus* and the same YcfD with bound substrate.[41] The structure of the yeast enzyme, known as Tpa1, had also been reported prior to identifying its role in ribosome hydroxylation.[42] A 2OG and Fe(II)-dependent oxygenase domain protein (OGFOD1) is widely found in eukaryotes, where it hydroxylates a particular prolyl residue in small ribosomal protein S23 (RPS23).[43] In mammals, loss of the enzyme leads to the formation of stress granules, stoppage of translation and growth inhibition. Whereas the human enzyme catalyses prolyl 3*S*-hydroxylation (Figure 1.2B), the corresponding enzymes from *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* (Tpa1, see above), and *Ostreococcus tauri* catalyse dihydroxylation of the cognate residues.[44] Furthermore, a *Drosophila* version of 2OGFOD1, known as Sudestada1, was studied; RNAi-mediated reduction in levels in tissue culture cells leads to smaller cell size, fewer cells and decreased translation efficiency.[45]

1.2.4 Other Protein Substrates

Some 2OG-dependent dioxygenases utilize protein substrates beyond those described above, often without a clear role being identified for the modification. Two Jumonji domain (JMJD)-containing proteins and several other examples are illustrated here.

The nuclear protein JMJD6 hydroxylates the C5 position of a lysyl residue in a protein associated with RNA splicing.[46] Of special interest, the stereospecificity of the enzyme is opposite to that of PLOD, yielding lysyl 5*S*-hydroxylysine (Figure 1.2D).[47] Alternatively, JMJD6 was proposed to catalyse demethylation of methylated argininyl residues,[48] and recent work extends this theme to support such a role in regulation of transcriptional pause release.[49] The crystal structure of the catalytic domain of JMJD6 is known.[50,51] Another Jumonji domain protein, JMJD4, was shown to catalyse C4 hydroxylation of a lysyl residue (not depicted in Figure 1.2 because the enantiospecificity is not reported) in the NIKS motif of eukaryotic release factor 1.[52] Hydroxylation at this site is needed for efficient translation termination.

The hydroxylation of side chains of aspartic acid and asparagine residues (Figure 1.2E) in epidermal growth factor-like domains of various vitamin K-dependent proteins, coagulation factors and complement proteins is also carried out by 2OG-dependent dioxygenases.[53,54] The resulting 3*R*-hydroxyaspartyl (Hya) and 3*R*-hydroxyasparaginyl (Hyn) groups are thought to be important because mice lacking aspartyl (asparaginyl) β-hydroxylase activity exhibit developmental defects and greater susceptibility to intestinal neoplasia.[55] In addition, overexpression of the gene encoding the enzyme is linked to cellular transformation, at least in the case of biliary epithelial cells.[56] Of related interest, a bacterial enzyme, CinX from *Streptomyces cinnamoneus*, catalyses the same type of reaction to modify an aspartyl group in a 15-residue peptide during the synthesis of cinnamycin, an antimicrobial peptide known as a lantibiotic.[57]

Aside from its O_2-sensing function related to hydroxylation of HIF-α Asn-803, FIH hydroxylates ankyrin repeat domains (ARDs) of endogenous Notch receptors.[58] The ARD modifications appear to stabilize the domains and probably affect protein–protein interactions.[59] In these roles, FIH catalyses aspartyl 3*S*-hydroxylase (Figure 1.2F)[60] and histidyl 3*S*-hydroxylase (Figure 1.2G)[61] activities using Asp and His residues in ARD domains. FIH also hydroxylates members of the apoptosis-stimulating p53-binding protein (ASPP) family, such as modification of ASPP2 at Asn-986.[62] An alternative function of type I collagen prolyl 4-hydroxylase involves oxygen addition to Pro-700 of Argonaute 2, an essential component of the RNA-induced silencing complex for RNA interference.[63] This modification is also thought to promote stabilization of the protein.

In addition to catalysing the hydroxylation of target proteins as described above, many 2OG-dependent oxygenases catalyse self-hydroxylation reactions. This process is exemplified by TfdA and TauD, described in Sections 1.6.3 and 1.6.4, which generate hydroxytryptophan and dihydroxyphenylalanine from Trp and Tyr, respectively.[64–68] It is unclear whether such modifications have any beneficial/regulatory role, but the abundance of aromatic residues near the active sites of many 2OG-dependent enzymes led to the proposal of a sacrificial function for these side chains, thus sparing the enzymes from more deleterious chemistry.[69] Other examples of

auto-catalysed oxidative modifications are found in procollagen 4*R*-hydroxylase that adds oxygen atoms to unknown sites of four of its own peptides;[70] JMJD6 that catalyses lysyl 5*S*-hydroxylation of one of its own Lys residues in the absence of its RNA splicing factor, perhaps as a regulatory mechanism;[71] FIH that catalyses the formation of hydroxytryptophan when HIF-1α is absent;[72] AlkB and ALKBH3 (both described in Section 1.3) that hydroxylate Trp and Leu residues, respectively, at their active sites.[73,74] Two reviews of autocatalysed oxidative modifications in this enzyme family have been published.[75,76]

1.2.5 Histone Demethylases

Another group of 2OG-dependent dioxygenases using protein substrates are those acting on N^{ε}-methylated lysine residues of histones. Eukaryotic DNA wraps around the histone core (composed of two each of histone H2A, H2B, H3 and H4) leaving the histone amino terminal regions accessible; these regions undergo several types of post-translational modification including the addition of methyl groups on specific lysyl or arginyl residues. The positions and extents of these modification have long been known to play a critical role in gene regulation.[77] In 2006, several research groups demonstrated that 2OG-dependent enzymes containing JmjC (jumonji domain C) domains catalyse demethylation reactions by hydroxylating the target methyl groups, with the hemiaminal intermediates subsequently decomposing with release of formaldehyde (Figure 1.2I).[78–82] The enzymes act on particular residues of specific histones with explicit levels of methylation. Unlike the flavin-dependent N^{ε}-methyl lysyl demethylase, the 2OG oxygenases can act on all three N^{ε}-methylation states of the modified residue. The first crystal structure for one of these enzymes was published that same year,[83] and follow-up studies have clarified the basis of the exquisite substrate specificities.[84–88] A molecular threading mechanism for the peptide substrate was proposed in the case of methylated H3K36 histone-specific KDM2A protein,[89] and the degree of methylation was suggested to influence whether the ferryl group was aligned according to the previously described 'in line' or 'off line' modes.[1] Additional discussion of this important family of enzymes is provided in Chapter 7 and selected reviews.[90–92]

1.2.6 Other Protein Demethylases

Several proteins other than histones are methylated in cells, so corresponding demethylases are likely to be identified. As one example, ALKBH4 was shown to mediate the removal of the methyl group from Lys84 in cytoplasmic actin, a reaction that regulates actin dynamics.[93] Of additional interest, ALKBH4 was also found to associate with several proteins associated with chromatin or involved in transcription, although the functions of these interactions remain unknown.[94]

1.3 2OG-Dependent Dioxygenases that Act on DNA or RNA

1.3.1 Demethylation of Alkylated DNA or RNA Substrates

In 2002, the *E. coli* enzyme AlkB was shown to directly repair alkylation damage to DNA by 2OG-dependent oxidation of 1-methyladenine (1meA) and 3-methylcytosine (3meC) lesions, resulting in the spontaneous loss of formaldehyde and restoration of the native base (Figure 1.3A and B).[95,96] Soon thereafter, AlkB-specific chemistry was expanded to include oxidative repair of alkylated RNA[97] and the range of lesions corrected was extended to 1-methylguanine (1meG, Figure 1.3C),[98] 3-methylthymine (3meT, Figure 1.3D),[98,99] N^6-methyladenine (N^6meA, Figure 1.3E),[100] N^2-methylguanine and N^4-methylcytosine (not shown),[101] as well as bases with slightly larger alkyl groups and exocyclic adducts (not shown). Several structures of AlkB have provided keen insights into the mechanism of substrate binding and substrate specificity.[102–105] Furthermore, the hemiaminal intermediate has been trapped in the protein by binding 3meT and exposing to oxygen.[106]

AlkB-like proteins are widely distributed in other bacteria,[107] viruses[108] and many types of eukaryotes. Of special interest, mammals have nine homologues of AlkB that are referred to as ALKBH (or ABH) followed by numbers 1–8 along with FTO (sometimes referred to as ALKBH9).[109–111] No direct evidence of polynucleotide demethylation has been identified for several of these proteins, including ALKBH4 mentioned earlier in Section 1.2.6. In contrast, ALKBH2 and ALKBH3 were shown to repair 1meA and 3meC in DNA,[112] with ALKBH3 also active with alkylated RNA.[97] Consistent with this differential specificity, knockout mouse studies demonstrated that ALKBH2 is the primary demethylase for repairing alkylated DNA.[113] The structures of ALKBH2 and ALKBH3 reveal the basis of their distinct specificities.[74,103,114–116] ALKBH1 has a more restricted specificity, acting only on 3meC in DNA and RNA,[117] but it also was proposed to be a methylated-histone demethylase[118] and is associated with lyase activity at abasic sites.[119,120] ALKBH5 exhibits N^6meA demethylase activity (Figure 1.3E) for alkylated mRNA.[121] This modification on RNA can affect its processing and has regulatory implications, such as controlling the length of the circadian clock.[122] Human and zebrafish ALKBH5 structures reveal the basis for nucleic acid recognition and catalysis.[123–125] The reaction catalysed by ALKBH8 is described in the next section. Meanwhile, the chemistries catalysed by ALKBH6 and ALKBH7 remain unknown, but the structure of ALKBH7 has been determined and shown to lack a nucleotide recognition lid consistent with a potential protein hydroxylation role.[126]

FTO is named for the fat mass and obesity-associated gene, whose inactivation protects mice from obesity.[127] Early studies reported the enzyme acts on 3meT in DNA,[128] or 3meT and 3-methyluracil (3meU, Figure 1.3F) in DNA and RNA;[129] however, more recent studies have shown that N^6meA in RNA is a major substrate.[130] The RNA-associated N^6-hydroxymethyladenine resulting

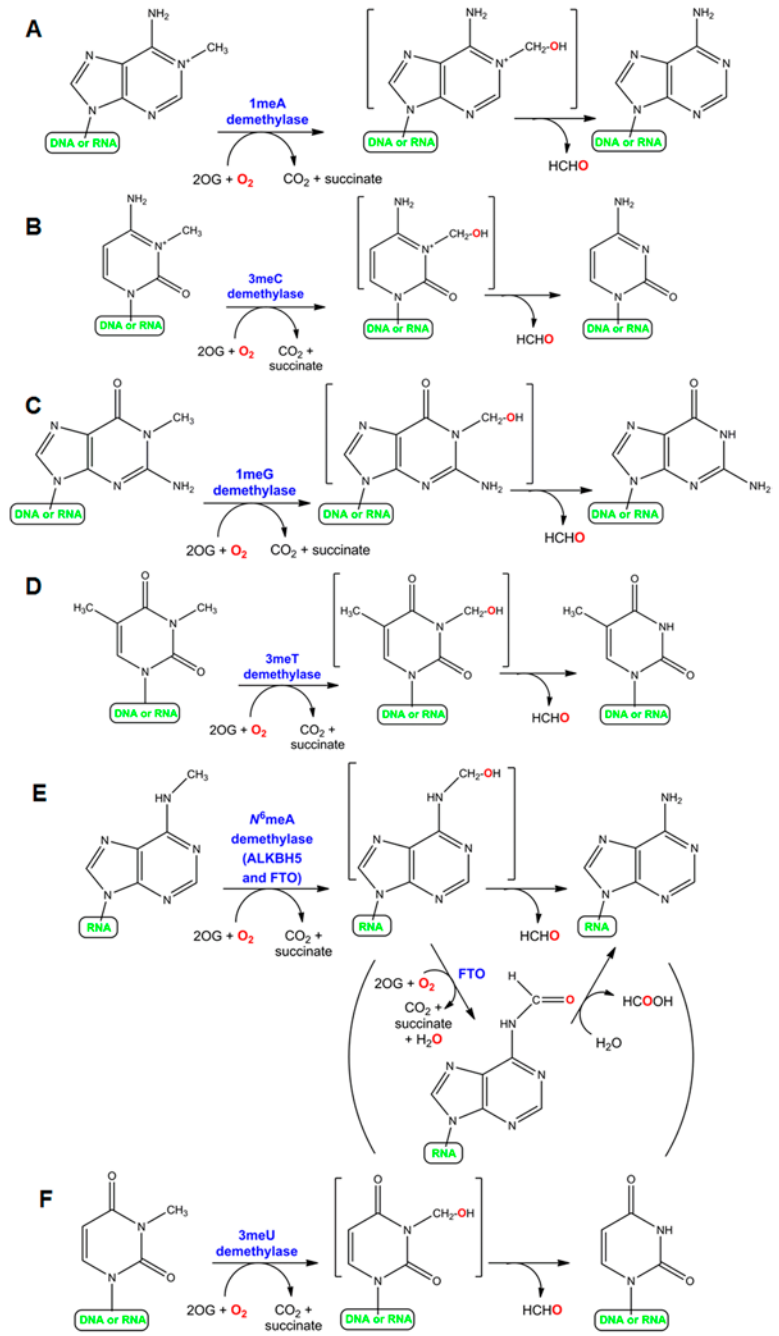

Figure 1.3 2OG-dependent dioxygenase reactions involving demethylations of alkylated DNA and RNA substrates. The reactions reverse lesions related to (A) 1meA, (B) 3meC, (C) 1meG, (D) 3meT, (E) N^6meA and (F) 3meU.

from the latter activity is sufficiently stable that it can be further hydroxylated by FTO to form N^6-formyladenine (the reaction shown in parentheses in Figure 1.3E).[131] The structure of FTO has been determined in the presence of 3meT mononucleotide and with several inhibitors.[132,133]

Further details on polynucleotide repair by AlkB and ALKBH species are found in Chapter 8, while ALKBH5 and FTO are described more fully in Chapter 9.

1.3.2 Other Oxidative Modifications of DNA or RNA

In addition to the oxidative demethylations of alkylated bases in DNA and RNA discussed above, 2OG-dependent dioxygenases catalyse several additional types of reaction using these substrates (Figure 1.4).

Two types of tRNA modifications have been shown to require 2OG-dependent hydroxylases. Working with an accessory protein, ALKBH8 first utilizes its RNA recognition and methyltransferase motifs to convert 5-carboxymethyluridine (cm^5U) to 5-methoxycarbonylmethyluridine (mcm^5U) at position 34 in the anticodon loop of human tRNA.[134] This modified tRNA then serves as the substrate for the hydroxylase domain of ALKBH8 which forms 5*S*-methoxycarbonylhydroxymethyluridine ($mchm^5U$) (Figure 1.4A).[135,136] The structure of the RNA recognition and hydroxylase domains of ALKBH8 has been reported.[137] Curiously, protozoan ALKBH8 catalyses both tRNA modification and DNA repair reactions.[138] Additional discussion of the biosynthesis of this unique base modification can be found in Chapter 10. A second tRNA-related hydroxylase acts on 7-(α-amino-α-carboxypropyl)wyosine known as wybutosine and abbreviated yW (Figure 1.4B), a tricyclic base that abuts the anticodon loop of $tRNA^{Phe}$ in eukaryotes and archaea.[139] Among the numerous derivatives formed from this base are hydroxylated species generated by TYW5 (tRNA-yW-synthesizing enzyme 5), a member of the JmjC domain family (see Section 1.2.4), whose structure has been reported.[140]

A family of ten-eleven translocation (TET) proteins contributes to the epigenetic regulation of a wide range of genes in many eukaryotes by controlling the methylation status of cytosine in CpG islands of DNA. For example, TET1 was shown to convert 5-methylcytosine (5meC) to 5-hydroxymethylcytosine (5hmC) in 2009 (Figure 1.4C).[141] Soon thereafter this result was confirmed and extended to TET2 and TET3.[142] More recently, TET enzymes were shown to catalyse subsequent reactions to create 5-formylcytosine (5fC)[143] and 5-carboxylcytosine (5caC),[144,145] including in a fungus.[146] In addition, the TET-catalysed oxidation of thymine to 5-hydroxymethyluracil in DNA of embryonic stem cells has been reported.[147] The various oxidized derivatives may have regulatory functions,[148] such as the hypoxic gene induction noted in neuroblastoma.[149] Moreover, the series of reactions from 5mC is suggested to play a role in active demethylation in DNA, either *via* (i) excision of 5fC or 5caC by thymine-DNA glycosylase, (ii) deamination of 5hmC followed by glycosylase removal, (iii) retro-aldol chemistry of 5hmC or 5fC with release of formaldehyde or formate, or (iv) decarboxylation of 5caC.[150] The structures of human TET2 in complex

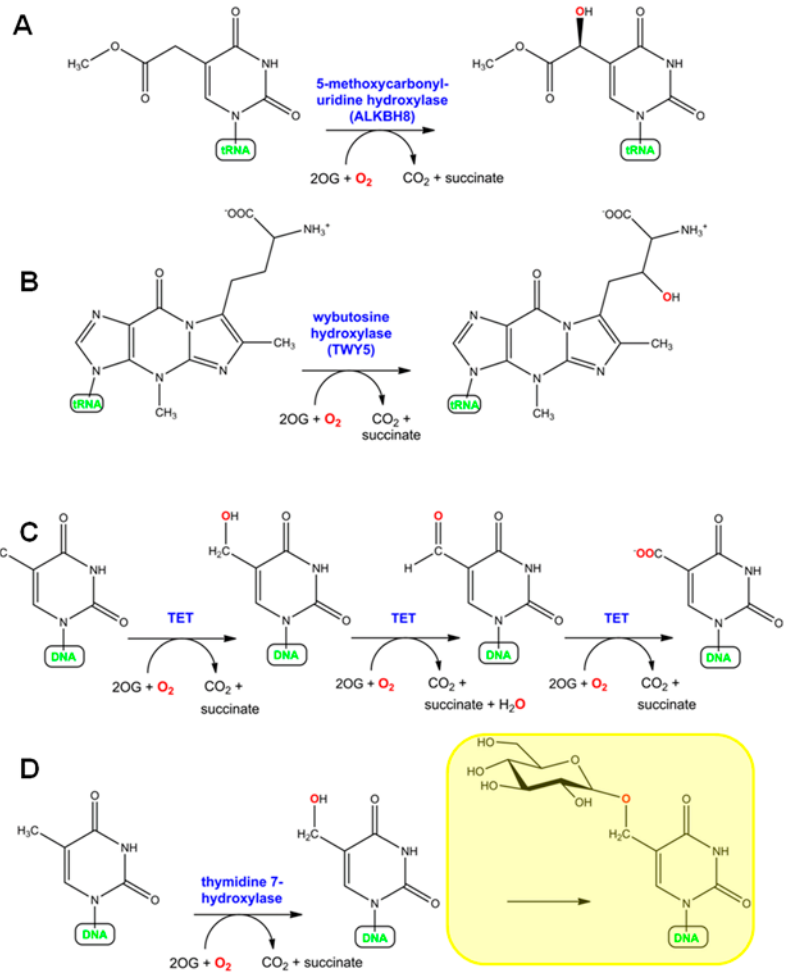

Figure 1.4 Non-demethylase 2OG-dependent dioxygenase reactions utilizing RNA or DNA substrates. (A) Action of ALKBH8 in the synthesis of a modified tRNA. (B) Wybutosine hydroxylase catalysis by TWY5. (C) The sequential reactions of TET proteins acting on 5meC to form 5hmC, 5fC and 5caC. (D) Thymidine 7-hydroxylase activity catalyses the first step in formation of base J (a subsequent glucosyl transfer reaction is highlighted in yellow).

with DNA and a TET family protein from *Naegleria gruberi* in complex with 5meC-containing DNA are reported.[151,152] Additional information on the biology of the TET proteins is provided in Chapter 11 and in reviews.[153,154]

Nuclear genomes of kinetoplastid flagellates and some unicellular flagellates contain β-D-glucopyranosyloxymethyluracil in their telomeric repeats.[155] This modified base, first identified in 1993, is also called β-D-glucosyl-hydroxymethyluracil or base J.[156] Synthesis of base J involves

two steps, hydroxylation of thymidine to form hydroxymethyluracil followed by glucosylation (Figure 1.4D).[157,158] JBP1, a protein that binds to and enhances the levels of base J,[159,160] was shown to belong to the 2OG-dependent dioxygenase family and is related to the TET proteins.[161,162] JBP1 along with the related JBP2, which does not bind to base J in DNA,[163] were directly demonstrated to catalyse thymidine hydroxylation.[164] Further information about the role of 2OG-dependent dioxygenases in base J biosynthesis is provided in Chapter 12.

1.4 Lipid-Related Metabolism Involving 2OG-Dependent Oxygenases

Several 2OG-dependent hydroxylase reactions are used in lipid biosynthesis (Figure 1.5).

Carnitine or γ-trimethyl-hydroxybutyrobetaine is a small molecule that becomes linked to fatty acids, allowing for their transport across the inner mitochondrial membrane and degradation in the matrix by the β-oxidation pathway.[165] The synthesis of carnitine initiates with protein-derived ε-*N*-trimethyllysine that undergoes β-hydroxylation, aldolase cleavage to glycine plus trimethylaminobutyraldehyde, dehydrogenation to γ-butyrobetaine, and another hydroxylation (Figure 1.5A). The ε-*N*-trimethyllysine hydroxylase activity was shown to require 2OG in 1978,[166] and the enzyme was purified and characterized in 2001.[167] The hydroxylation of γ-butyrobetaine was found to require 2OG in 1968,[168] and the enzyme was purified from *Pseudomonas* spp. AK1 in 1977 and from calf liver in 1981.[169,170] A detailed comparison of substrate specificities for the human and bacterial enzymes revealed surprisingly large differences in reactivity for several γ-butyrobetaine analogues.[171] The structure of human γ-butyrobetaine hydroxylase has been reported.[172,173] Further discussion of the enzymes involved in carnitine metabolism is provided in Chapter 13.

Phytanic acid (2,6,10,14-tetramethylhexadecanoic acid), a polyisoprenoid derived from the phytol moiety of chlorophyll, cannot be directly degraded *via* the β-oxidation pathway due to its 3-methyl group. To overcome this hurdle, the C-1 carbon is removed by action of a series of four enzymes: a ligase converts the molecule to phytanoyl-coenzyme A (CoA),[174] hydroxylation occurs at the C-2 position,[175] a lyase releases formyl-CoA and methylpentadecanal (pristanal),[176,177] and a dehydrogenase forms pristanic acid (2,6,10,14-tetramethylpentadecanoic acid)[178] which is a substrate for β-oxidation. Interruption of this pathway results in Refsum disease, which is associated with dysfunctions including retinitis pigmentosa, polyneuropathy and ataxia.[179] Phytanoyl-CoA hydroxylase (Figure 1.5B), shown to be a 2OG-dependent enzyme,[175] was purified from rat liver and as the recombinant human protein.[180,181] The human enzyme was structurally characterized, providing an understanding of mutations associated with Refsum disease.[182] Further description of phytanic acid metabolism is found in Chapter 14.

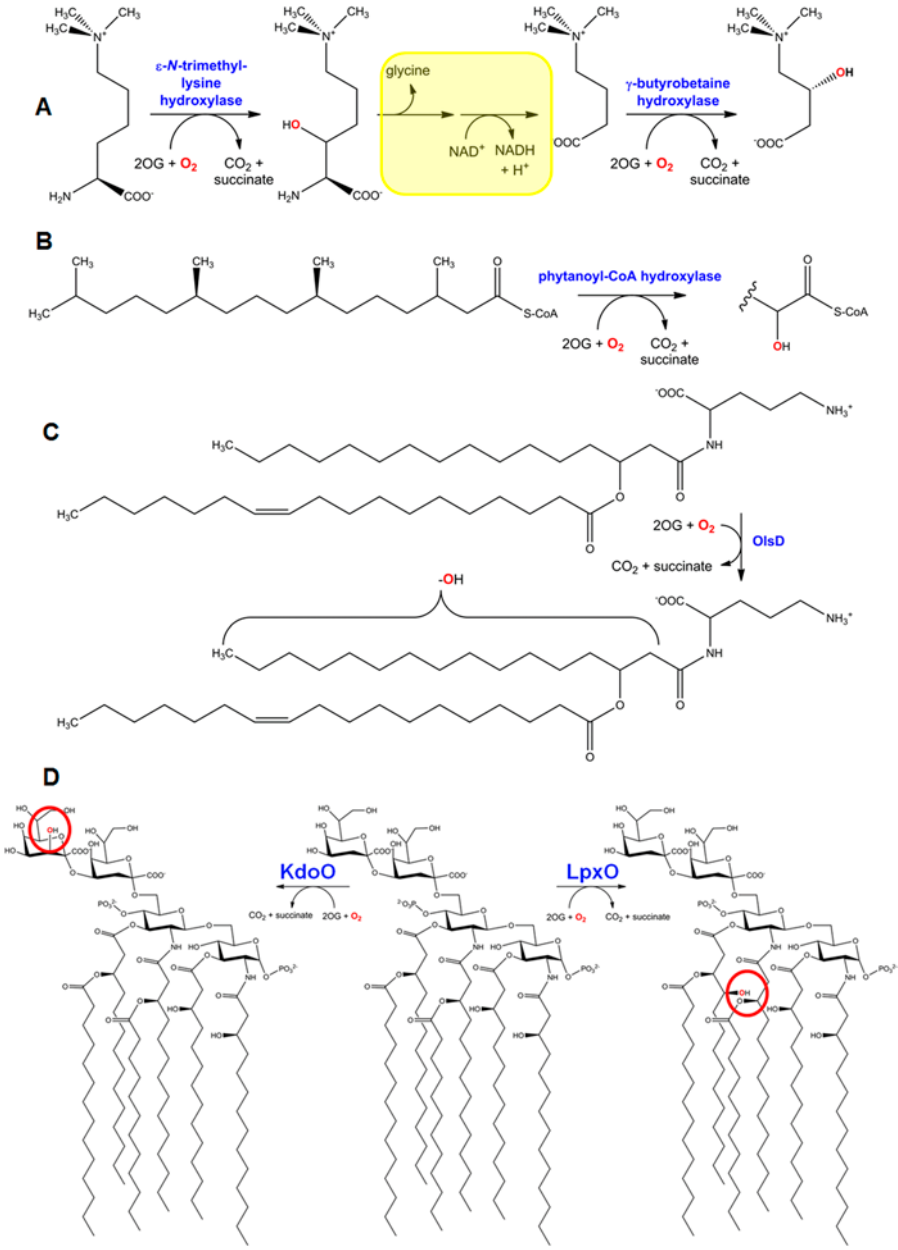

Figure 1.5 2OG-dependent dioxygenases involved in lipid metabolism. (A) Biosynthesis of the acyl group carrier molecule carnitine includes two 2OG-dependent hydroxylases that act on ε-*N*-trimethyllysine and γ-butyrobetaine (with intervening aldolase and dehydrogenase reactions highlighted in yellow). (B) Phytanic acid metabolism includes the activity of phytanoyl-CoA hydroxylase. (C) Hydroxylation of the acyl chain of ornithine lipids is catalysed by OlsD. (D) KdoO and LpxO hydroxylase reactions involving lipid A.

Much like phytanoyl-CoA hydroxylase, OlsD from *Burkholderia cenocepacia* (and probably from other *Burkholderia* and *Serratia* species) catalyses the hydroxylation of an acyl chain (Figure 1.5C) as part of a lipid metabolism pathway.[183] In this case, however, the pathway is biosynthetic rather than degradative. The first step of the pathway involves OlsB, an *N*-acyl transferase that transfers a 3-hydroxy fatty acyl group from the acyl carrier protein to the α-amino group of ornithine. The resulting lyso-ornithine lipid is the substrate of OlsA, an *O*-acyltransferase that transfers a fatty acyl group from the acyl carrier protein to the C-3 hydroxyl group. This ornithine-containing lipid, or its C-2 hydroxylated derivative (not shown), is the substrate of OlsD that hydroxylates somewhere on the amide-linked fatty acyl chain.

Two 2OG-dependent enzymes have been shown to modify lipid A (Figure 1.5D), with one targeting a specific acyl side chain and the other acting on the 3-deoxy-D-*manno*-oct-2-ulosonic (Kdo) moiety. *Salmonella typhimurium*, previously known to synthesize lipid A containing secondary *S*-2-hydroxyacyl chains, was demonstrated to use the O_2- and 2OG-dependent enzyme LpxO to achieve this modification.[184] LpxO was the first integral membrane protein representative of this enzyme family.[185] A Kdo 3-hydroxylase (KdoO) that adds an oxygen atom to the deoxysugar was identified in *Burkholderia ambifaria* and *Yersinia pestis*.[186] Homologues to the genes encoding these proteins are found in other Gram-negative bacteria.

1.5 Plant Metabolite Biosynthesis Using 2OG-Dependent Oxygenases

Plants possess many of the 2OG-dependent oxygenases already mentioned, but in addition they utilize members of this enzyme family for some of their unique biosynthetic needs.[187] Here we illustrate how plants (and, in a few cases, other organisms) use these enzymes for generating flavonoids, gibberellins, alkaloids and other predominantly plant-specific products.

1.5.1 2OG-Dependent Oxygenases in Flavonoid Biosynthesis

The flavonoids are polyphenolic compounds that include flavanones, flavones, isoflavones, flavonols and anthocyanins with more than 9000 such compounds known (see examples in Figure 1.6). These substances are used by plants for defence against pathogens, as signalling molecules in plant–microbe interactions, to minimize photodamage, in flower colour, and other roles.[188] When consumed by humans they function as antioxidants, antimalarials and potential anticancer agents.[189] The entrance to the main pathway shown in Figure 1.6 (indicated by the green arrow) is the flavanone naringenin, the substrate for two distinct 2OG-dependent oxygenases. Flavone synthase I (FNS) catalyses a desaturation reaction at the C-2/C-3 position forming *trans*-dihydroflavonol,[190] while flavanone 3β-hydroxylase (FHT) adds a hydroxyl group to C-3.[191] The cytochrome P450 enzyme flavanone

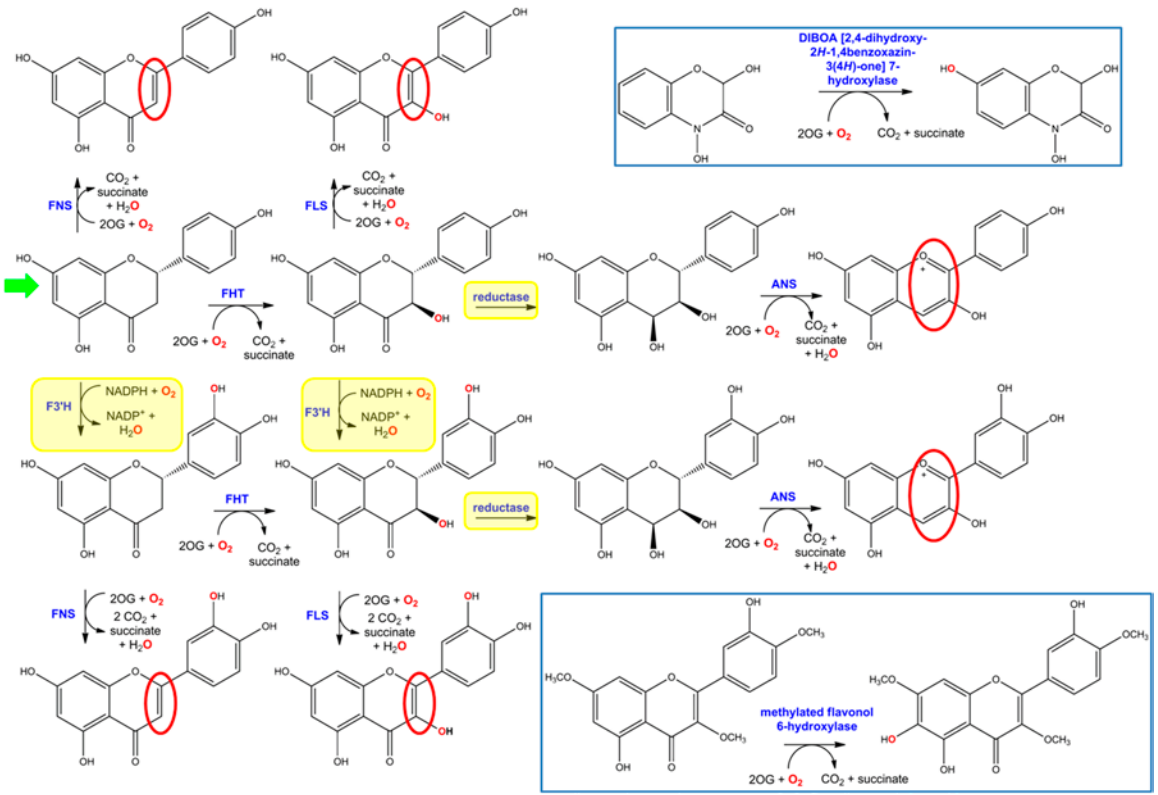

Figure 1.6 Representative 2OG-dependent oxygenases of flavonoid metabolism. Flavanone (indicated by the green arrow) can undergo desaturation by flavone synthase I (FNS), C-3 hydroxylation by flavanone 3β-hydroxylase (FHT), or C-3′ hydroxylation by flavanone 3′-hydroxylase (F3′H, not a 2OG-dependent enzyme as indicated by yellow highlighting), with the product of the latter reaction also serving as substrate for both FNS and FHT. The FHT-derived products can undergo desaturation by flavonol synthase (FLS) or can be reduced by a non-2OG enzyme (also highlighted in yellow). The reduced products are converted to anthocyanins by anthocyanidin synthase (ANS). The boxes to the upper and lower right depict the reactions of related enzymes that hydroxylate at the 7 and 6 positions of flavonoid compounds.

3′-hydroxylase (F3′H) uses naringenin (or its 3β-hydroxy derivative) and converts the appended phenol to a catechol, with this species also being a substrate for FNS and FHT. The FHT-derived *trans*-dihydroflavonol products are substrates for another 2OG-dependent desaturase called flavonol synthase (FLS).[192,193] Alternatively, the *trans*-dihydroflavonols can be reduced *via* non-2OG-dependent enzymes to provide leucoanthocyanidin substrates for anthocyanidin synthase (ANS).[194] The structure of ANS from *Arabidopsis thaliana* has been elucidated with the bound substrate analogue dihydroquercetin and the unnatural substrate naringenin.[192,195] Two other flavonoid-related reactions catalysed by 2OG-dependent hydroxylases are 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) 7-hydroxylase and methylated flavonol 6-hydroxylase (see boxes in Figure 1.6).[196–200] Flavonoid biosynthesis is discussed in much greater detail in Chapter 15.[201]

1.5.2 2OG-Dependent Oxygenases of Gibberellin Biosynthesis

Tetracyclic diterpenoids known as gibberellins (GAs) are widely distributed in higher plants and are also found in some lower plants, bacteria and fungi.[202] At least 136 distinct GA structures are reported (commonly referred to as GA_1-GA_{136}; see http://www.plant-hormones.info/gibberellins.htm). A small sampling of such structures is shown in Figure 1.7, which depicts selected 2OG-dependent transformations of these molecules. Many GAs possess a carboxylate at C-7, introduced by oxidation of the GA_{12}-aldehyde to form GA_{12}, with this product undergoing partial oxidization at C-7 by a hydroxylase to form GA_{53}. Depending on their biological sources these reactions can be catalysed by either cytochrome P450 or 2OG-dependent oxygenases (Figure 1.7A).[203] The GA C20 oxidase can catalyse sequential reactions that convert the C-20 methyl group (*e.g.* GA_{12}/GA_{53}) to the alcohol (GA_{15}/GA_{44}), aldehyde (GA_{24}/GA_{19}), and in some cases the carboxylate (GA_{25}/GA_{17}) (Figure 1.7B).[204,205] Alternatively, the same enzyme can catalyse an oxidative transformation that eliminates the carboxylate as CO_2 while forming a γ-lactone (GA_9/GA_{20}).[206] GA 3β oxidase converts these products to the corresponding hydroxylated species (GA_4/GA_1).[207] GA 2β oxidase acts on the same substrates (producing GA_{51}/GA_{29}) or on the products of the prior reaction (producing GA_{34}/GA_8).[208] In addition, other 2OG-dependent enzymes can catalyse several types of desaturation reactions (not shown),[202,209] such as a recently characterized fungal GA_4 desaturase that introduces a double bond between C-1 and C-2.[210] Further information on this remarkable family of enzymes is available in Chapter 16.

1.5.3 2OG-Dependent Oxygenases in Alkaloid Synthesis

Four examples are depicted to illustrate how plants use 2OG-dependent oxygenases for alkaloid biosynthesis. Scopolamine is a hallucinogenic tropane alkaloid produced by *Hyoscyamus niger* (henbane). The last two steps in its synthesis are catalysed by hyoscyamine 6β-hydroxylase that carries out both

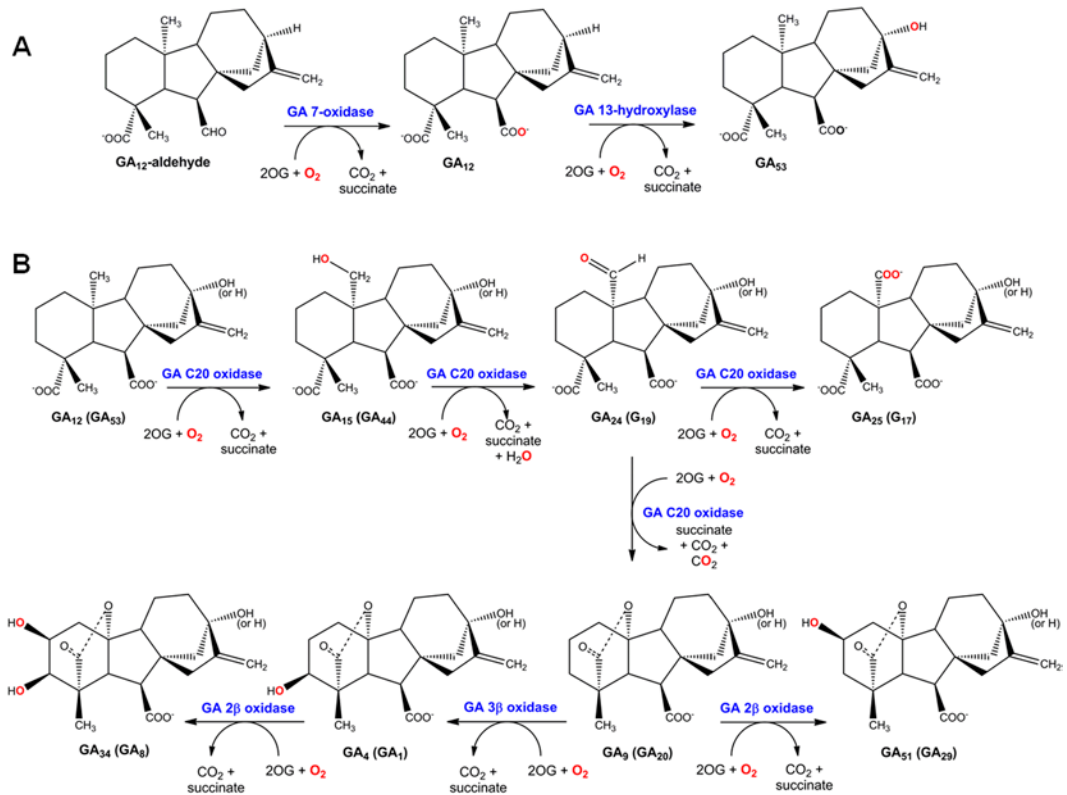

Figure 1.7 Selected 2OG-dependent oxygenases of gibberellin (GA) biosynthesis. (A) Reactions of GA 7-oxidase and GA 13-hydroxylase. (B) Three consecutive reactions of GA C20 oxidase that convert a methyl group to an alcohol, aldehyde and carboxylic acid; a distinct reaction catalysed by GA C20 oxidase to eliminate CO_2 and form a γ-lactone; GA 2β and GA 3β oxidase activities.

hydroxylation and epoxidation steps (Figure 1.8A).[211,212] Vinblastine and vincristine are alkaloids produced by *Caranthus roseus* (periwinkle), which have been used for treatment of Hodgkin's lymphoma and acute leukaemia. These compounds are synthesized by a complex biosynthetic pathway that utilizes a 2OG-dependent hydroxylase to generate deacetylvindoline (Figure 1.8B), which is then further modified.[213,214] Codeine and morphine are important pharmaceuticals obtained from *Papaver somniferum* (opium poppy). Their complex biosynthetic pathway includes an intermediate named thebaine, which can be demethylated at one site by thebaine 6-*O*-demethylase to produce codeinone (Figure 1.8C) or demethylated at a second site by codeine *O*-demethylase to form oripavine (Figure 1.8D).[215] These two reactions plus that catalysed by codeinone reductase yields morphine. A series of additional reactions (not shown) involving alkaloid metabolism in opium poppy are catalysed by these enzymes and the 2OG-dependent dioxygenase protopine *O*-dealkylase, PODA.[216] In the fungus *Claviceps purpurea*, a cyclization reaction (not shown) in the synthesis of D-lysergic acid alkaloid peptides is catalysed by a member of this enzyme family, and the 2OG-bound holoprotein, EasH, was structurally characterized.[217] Another fungal example is found in the synthesis of loline alkaloids by *Epichloë* species, where the 2OG-dependent oxygenase LolO introduces an ether bridge into a pyrrolizidine ring system (Figure 1.8E).[378]

1.5.4 Other Plant-Specific 2OG-Dependent Oxygenases

Additional representatives of the 2OG-dependent oxygenases that are primarily restricted to plants include enzymes involved in the biosynthesis of phytosiderophores, coumarins and glucosinolates, or the degradation of hormonal compounds.

Under iron-deficient conditions, some grasses, cereals and rice produce and secrete iron-binding compounds such as the mugineic acid-related species made by *Hordeum vulgare* (barley). This plant contains two 2OG-dependent dioxygenases, IDS2 and IDS3, that act on 2′-deoxymugineic acid to form 3-epihydroxy-2′-deoxymugineic acid and mugineic acid, respectively (Figure 1.8F).[218] 2′-Deoxymugineic acid is converted to 3-epihydroxymugineic by combined actions of the two enzymes.

Coumarins (1,2-benzopyrones) such as scopoletin and umbelliferone are synthesized by many higher plants where they are used for defence against phytopathogens. A key enzyme in the pathway used by *Arabidopsis thaliana* for generating scopoletin is a 2OG-dependent enzyme that hydroxylates the *ortho* position of feruloyl-CoA,[219] with subsequent isomerization, hydrolysis and lactonization steps providing the product (Figure 1.8G). The same activity was detected using two recombinant proteins from *Ipomoea batatas* (sweet potato), one of which also used *p*-coumaroyl-CoA to form umbelliferone (where H is present at the 3′ position in Figure 1.8G).[220] An enzyme with the latter dual activity has also been characterized from *Ruta graveolens* (the common rue).[221]

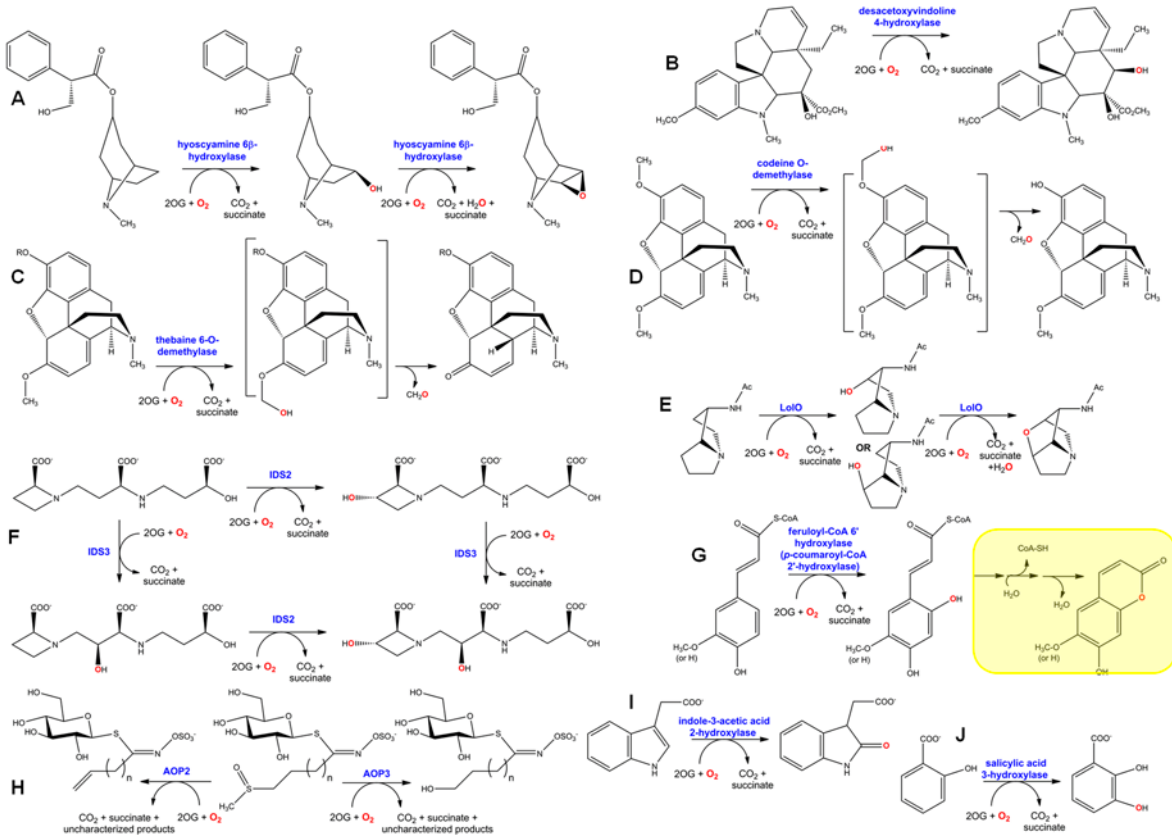

Figure 1.8 Steps in the synthesis of selected alkaloids and other primarily plant-specific products catalysed by 2OG-dependent oxygenases. (A) Hydroxylase and epoxidase activities of hyoscyamine hydroxylase. (B) Desacetoxyvindoline 4-hydroxylase. (C) Thebaine 6-*O*-demethylase. (D) Codeine *O*-demethylase. (E) LolO-catalysed ether bridge formation in loline alkaloid biosynthesis. (F) Mugineic acid phytosiderophore synthesis. (G) Feruloyl-CoA 6′-hydroxylase and *p*-coumaroyl-CoA 2′-hydroxylase in the pathways for synthesis of scopoletin and umbelliferone (yellow highlight). (H) Action of AOP2 and AOP3 on methylsulfinylalkyl glucosinolates. (I) Indole-3-acetic acid 2-hydroxylase. (J) Salicylic acid 3-hydroxylase.

Glucosinolates (over 130 are known) are predominantly associated with the Brassicaceae family of plants where, following tissue disruption, they are decomposed to form compounds with diverse protective roles against herbivores and pathogens.[222] Two sequence-related 2OG-dependent enzymes in *A. thaliana*, named AOP2 and AOP3, have been shown to participate in glucosinolate biosynthesis by converting methylsulfinylalkyl glucosinolate to the alkenyl or hydroxyalkyl species (Figure 1.8H).[223] The chemistry of these reactions has not been well defined.

Indole-3-acetic acid and salicylic acid are plant hormones that play important roles in growth, development, disease resistance or other functions, but until recently their degradation pathways within plants were unclear. Using rice, a dioxygenase for auxin oxidation (DAO) has now been identified and shown to transform this substrate into 2-oxoindole-3-acetic acid (Figure 1.8I).[224] Mutants affecting the corresponding gene display male sterility and produce infertile seeds. Similarly, a salicylic acid 3-hydroxylase (Figure 1.8J) of *Arabidopsis* was identified and mutants in the corresponding *s3h* gene were found to accumulate salicylate species and exhibit early senescence.[225] When assayed *in vitro*, both 2,3- and 2,5-dihydroxybenzoate are formed; however, only the former appears to be made *in vivo*.

1.6 2OG-Dependent Oxygenases Catalysing Reactions with Free Amino Acids, Nucleobases, Herbicides and Sulfur- or Phosphorous-Containing Compounds

The reactions described in this section are diverse, but generally involve rather small-sized molecules. Several products from these reactions are precursors that become incorporated into antibiotics, whereas 2OG-dependent tailoring enzymes that act directly during antibiotic synthesis are described in Section 1.7.

1.6.1 Amino Acid Hydroxylases

2OG-Dependent hydroxylases acting on free amino acids (*i.e.* not as a side chain of a protein) are known. In the case of L-Pro, different enzymes exhibit each of four distinct specificities. Pro 4*R*-hydroxylase (Figure 1.9A, left) from *Streptomyces griseoviridus* P8648 produces the *trans*-isomer of 4-hydroxyproline that is subsequently utilized for etamycin synthesis.[226,227] This enzyme activity is also found in other bacterial strains of *Streptomyces*, *Dactylosporangium* and *Amycolatopsis*,[228] and in the pneumocandin-producing fungus *Glarea lozoyensis*.[229] L-Pro 4*S*-hydroxylase (Figure 1.9A, right), producing the *cis* isomer, has been identified in *Mesorhizobium loti* and *Sinorhizobium meliloti* where it was shown to require 2OG.[230] L-Pro 3*S*-hydroxylase (Figure 1.9B, left) has been reported in the *G. lozoyensis* fungus mentioned

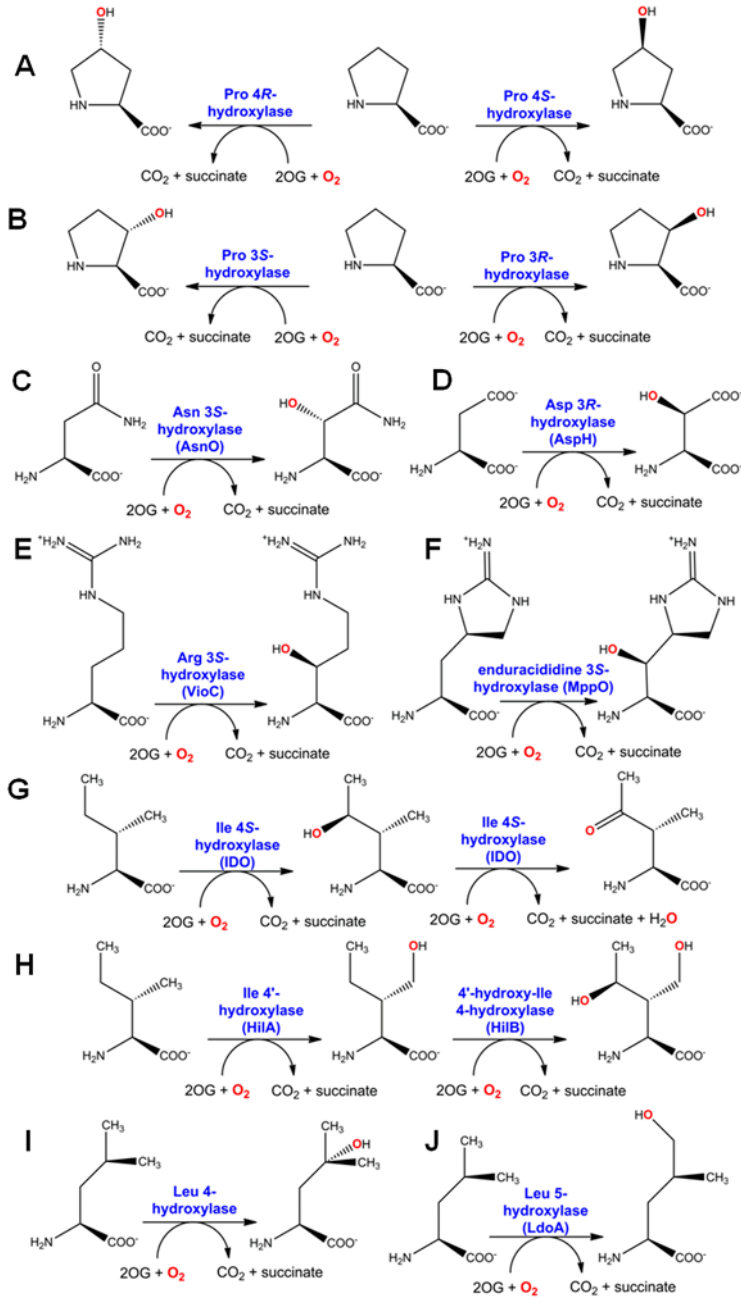

Figure 1.9 2OG-dependent hydroxylases acting on free L-amino acids. (A) Pro 4*R*- and 4*S*-hydroxylases. (B) Pro 3*S*- and 3*R*-hydroxylases. (C) Asn 3*S*-hydroxylase. (D) Asp 3*R*-hydroxylase. (E) Arg 3*S*-hydroxylase. (F) Enduracididine 3-hydroxylase. (G) Ile 4*S*-hydroxylase. (H) Ile 4′- and 4-hydroxylase. (I) Leu 4-hydroxylase. (J) Leu 5-hydroxylase.

above.[229] Enzymes from two actinomycetes were shown to exhibit both 3*S*- and 4*S*-hydroxylase activities using L-Pro.[231] The best studied enzyme using this substrate is L-Pro 3*R*-hydroxylase (Figure 1.9B, right), an activity identified in strains of *Streptomyces* and *Bacillus*.[232] The enzyme was purified from *Streptomyces* sp. strain TH1[233] and the structure of the apoprotein was elucidated.[234]

Several 2OG-dependent hydroxylases act on free L-amino acids with polar side chains. A 3*S*-hydroxylase of L-Asn (creating the *threo* isomer, Figure 1.9C) was characterized from recombinant cells containing *asnO* from *Streptomyces coelicolor*.[235] High-resolution crystal structures of AsnO reveal the basis of this substrate specificity. The product of the reaction is subsequently incorporated (at position nine) into a daptomycin-type lipopeptide called the calcium-dependent antibiotic. A single amino acid substitution (D241N) of AsnO led to use of L-Asp as substrate, forming the *threo* isomer of hydroxyaspartic acid (not shown).[236] By contrast, AspH from *Pseudomonas syringae* catalyses 3*R*-hydroxylation of L-Asp (and L-Asp thioesters, not shown) to form the *erythro* isomers (Figure 1.9D).[237] VioC, a 2OG-dependent Arg 3*S*-hydroxylase (Figure 1.9E), was shown to be used by *Streptomyces vinaceus* to provide a precursor for viomycin biosynthesis.[238] The VioC structure was elucidated and explains the hydroxylation specificity.[239] Enduracididine is an Arg-derived amino acid that undergoes 3*S*-hydroxylation by MppO (Figure 1.9F) to provide β-hydroxyenduracididine, which is incorporated into mannopeptimycins, glycopeptide antibiotics produced by *Streptomyces hydrogroscopicus* NRRL 30439.[240]

Hydroxylases of L-Ile and L-Leu have been widely studied since the first report of 2OG-dependent 4-hydroxyisoleucine synthesis in *Trigonella foenum-graecum* (fenugreek).[241] The recombinant enzyme (IDO) from *Bacillus thuringiensis* was shown to exhibit L-Ile 4*S*-hydroxylase activity,[242] and the enzyme was later shown to also form 2-amino-3-methyl-4-ketopentanoate, probably arising from dihydroxylation of the same carbon position to produce a gem diol that loses water (Figure 1.9G).[243] The products of two adjacent genes in *Pantoea ananatis*, HilA and HilB, catalyse sequential hydroxylation reactions at the 4′ and 4 positions, respectively, of L-Ile (Figure 1.9H).[244] Related family members were identified in a range of other bacteria, in several cases using L-Leu as the preferred substrate and yielding 4-hydroxyleucine (Figure 1.9I).[245] Alternatively, L-Leu 5-hydroxylase (LdoA, Figure 1.9J) was characterized from *Nostoc punctiforme*.[246] All of these enzymes also act on other substrates with reduced catalytic efficiencies (including Leu/Ile substitution), in various cases forming Met sulfoxide, 4-hydroxyvaline and 4-hydroxythreonine (not shown).[243–245]

Four other enzymes are mentioned in this section because they modify amino acid-like substrates or combine another reaction with amino acid hydroxylation. SadA from *Burkholderia ambifaria* acts on several *N*-substituted amino acids with hydrophobic side chains, notably catalysing 3*R* hydroxylation of *N*-succinyl-L-Leu (Figure 1.10A).[246] The structure of SadA has been reported,[247] and variants with altered specificity have been studied.[248]

PvcB of *Pseudomonas aeruginosa* functions in the biosynthesis of the siderophore pyoverdine by participating in the formation of 2-isocyano-6,7-dihydroxycoumarin (reminiscent of the coumarin product shown in Figure 1.8G). The *P. aeruginosa* enzyme catalyses a cyclization reaction (Figure 1.10B),[249] although the mechanistic details remain obscure, and the structure of the protein has been characterized in the absence of ligands.[249] Ectoine is an acid with a secondary amine, so the associated enzyme is described here although this tetrahydropyrimidine could alternatively be included in the next section with nucleobases and nucleosides. Bacteria synthesize ectoine and a variety of other compatible solutes to prevent excessive loss of water when grown in high salinity conditions, and some species convert ectoine

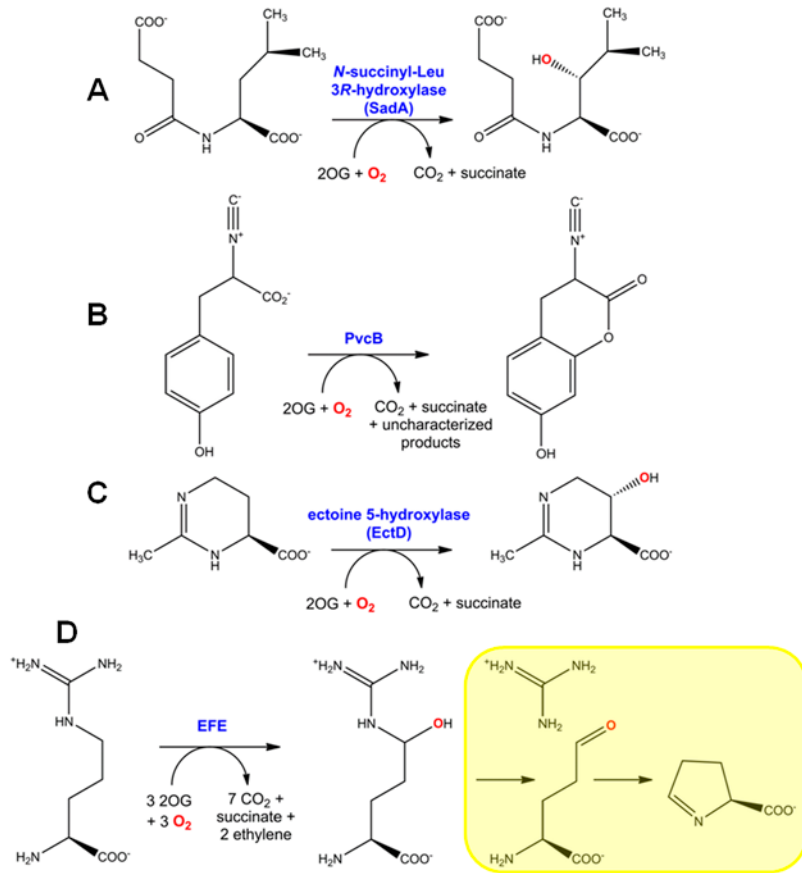

Figure 1.10 2OG oxygenases using substrates resembling amino acids. (A) *N*-succinyl-L-Leu 3*R*-hydroxylase. (B) PvcB reaction in pyoverdine biosynthesis. (C) Ectoine 5-hydroxylase. (D) A poorly characterized bacterial enzyme reported to convert 2OG to ethylene while simultaneously hydroxylating L-Arg, with subsequent spontaneous reactions shown in yellow highlight.

to 5-hydroxyectoine during stationary growth. EctD is a 2OG-dependent enzyme responsible for this ectoine 5-hydroxylase activity (Figure 1.10C).[250] The properties of this protein have been examined from a wide distribution of microorganisms, including extremophiles.[251] Of particular interest, the structure of EctD from *Virgibacillus* (formerly *Salibacillus*) *salexigens* in the absence of substrate has been characterized,[252] and a model of the holoprotein with ectoine and 2OG has been simulated.[253] Finally, *Pseudomonas syringae* pv. *phaseolicola* contains the ethylene-forming enzyme (EFE) which is reported to convert 2OG to ethylene and three molecules of CO_2 while simultaneously converting Arg into guanidine and Δ^1-pyrroline-5-carboxylate.[254] The presumed Arg 5-hydroxylase activity (Figure 1.10D) and formation of the resulting degradation products have not been clearly documented. 2OG-dependent ethylene formation has been observed in other microorganisms, including a mushroom,[255] and the gene encoding EFE is functional when inserted into other hosts,[256,257] including cyanobacteria,[257,258] with the latter microorganisms offering the potential for generating a biofuel from CO_2. The mechanism of 2OG conversion to ethylene by EFE is not understood.

1.6.2 Hydroxylases of Nucleobases and Nucleosides

Two distinct 2OG-dependent oxygenases are known to act on nucleobases. Thymine 7-hydroxylase from the fungus *Neurospora crassa* was among the first 2OG-dependent oxygenases to be characterized.[259,260] The enzyme catalyses sequential oxygen additions to the methyl group of the free base, forming 5-hydroxymethyluracil, 5-formyluracil and 5-carboxyuracil (Figure 1.11A).[261,262] Additional studies have focused on the purified fungal enzyme from *Rhodotorula glutinis*,[263] including analysis of its broad substrate specificity.[264,265] Xanthine hydroxylase, XanA, is a fungal enzyme that catalyses the reaction of Figure 1.11B.[266] This enzyme was discovered by studies involving a mutant strain of *Aspergillus nidulans* that was defective in xanthine dehydrogenase, a widely distributed molybdopterin-containing enzyme, yet was able to grow on xanthine as a nitrogen source. XanA was purified both from the fungal host and as a recombinant protein from *E. coli*; although differing in various types of post-translational modifications, both forms exhibited the activity shown.[267] Although no crystal structure is available for XanA, the likely active site residues were identified from mutagenesis studies and shown to support results of a homology model.[268]

Three types of 2OG-dependent oxygenases have been reported to catalyse reactions with the sugar components of nucleosides. Several fungi contain pyrimidine deoxyribonucleoside 2′-hydroxylases that form the corresponding ribonucleosides (Figure 1.11C).[269,270] Furthermore, *R. glutinis* contains a deoxyuridine or uridine 1′-hydroxylase that forms an unstable intermediate which decomposes with release of the nucleobase and formation of a lactone (Figure 1.11D).[271] Finally, a uridine-5′-monophosphate 5′-hydroxylase (Figure 1.11E) named LipL has been purified from a *Streptomyces* species where it provides a precursor for incorporation into antibiotic A-90289.[272]

Although the enzyme has not been characterized, it appears that a thymine 7-hydroxylase-like activity might be used for nucleoside modification during polyoxin biosynthesis in *Streptomyces avermitilis*.[273] The cells produce 14 distinct forms of polyoxins that possess a common nucleoside core containing 1-(5′-amino-5′-deoxy-β-D-allofurauronosyl)pyrimidine. The SAV_4805 open reading frame of this microorganism was shown to be associated with enhancement of structural diversity at the C-5 position of the pyrimidine ring, consistent with hydroxylation of this site by the encoded protein (Figure 1.11F).

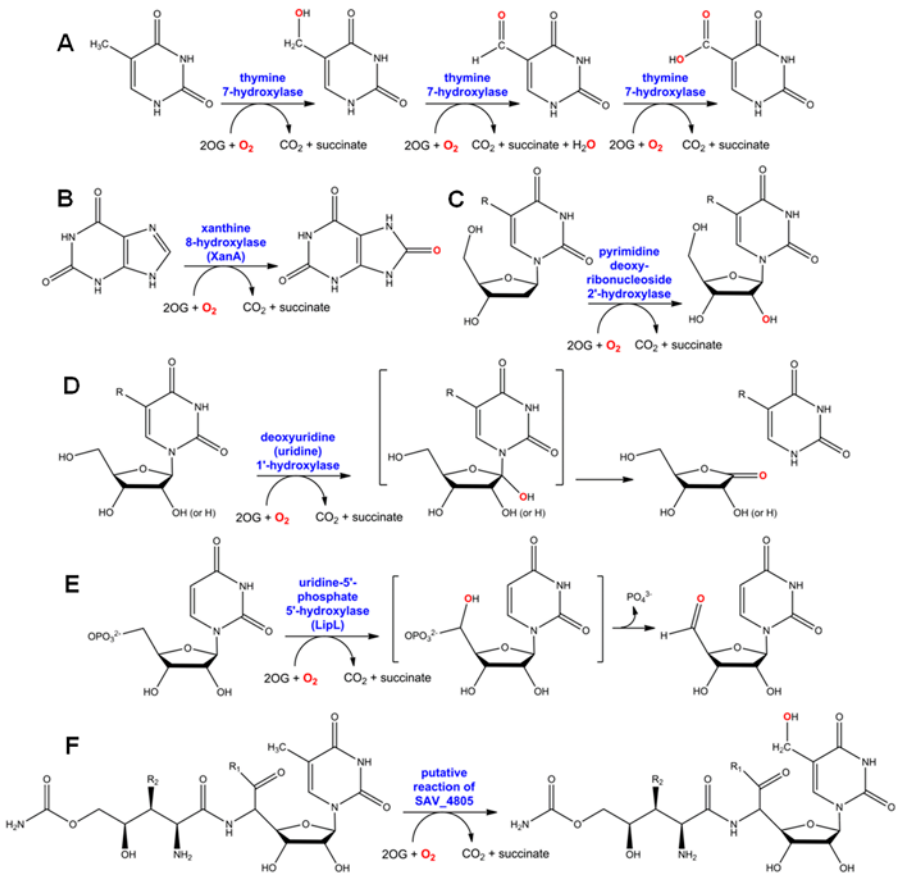

Figure 1.11 2OG-dependent oxygenase reactions involving nucleobases and nucleosides. (A) Thymine 7-hydroxylase. (B) Xanthine hydroxylase. (C) Pyrimidine deoxyribonucleoside 2′-hydroxylase. (D) Deoxyuridine (uridine) 1′-hydroxylase. (E) Uridine-5′-monophosphate 5′-hydroxylase. (F) Possible reaction catalysed by the enzyme associated with the SAV_4805 open reading frame.

1.6.3 Herbicide Degradation by 2OG-Dependent Oxygenases

Phenoxyalkanoic acids are widely used as herbicides for selective control of broad-leaf weeds. An important example is 2,4-dichlorophenoxyacetic acid (2,4-D), for which the biodegradative process has been extensively studied.[274] Bacteria that are capable of growth on 2,4-D as sole carbon source often possess a series of enzymes: TfdA (initially referred to incorrectly as 2,4-D monooxygenase), 2,4-chlorophenol hydroxylase (TfdB), 3,5-dichloro–catechol dioxygenase (TfdC), dichloromuconate cycloisomerase (TfdD), dienelactone hydrolase (TfdE) and maleylacetate reductase (TfdF) yielding 3-oxoadipate which enters intermediary metabolism. Using the enzyme from *Cupriavidus necator* (formerly *Ralstonia eutropha*), the reaction of TfdA was revised to be that of a 2OG-dependent hydroxylase (Figure 1.12A).[275] The properties of TfdA have been extensively characterized, including the specificity towards a diversity of phenoxyalkanoic acids, several spectroscopic features of the protein, and its ability to catalyse self-hydroxylation.[64,276,277] This was the first example of a 2OG-dependent dioxygenase being used for biodegradation of a xenobiotic compound. By incorporating *tfdA*-like genes into transgenic plants, enhanced herbicide tolerance has been obtained.[278]

2-Phenoxypropionic acids differ from phenoxyacetic acids by a single methyl group, resulting in two enantiomeric forms of these compounds. Only the (*R*) enantiomer of 2-(2,4-dichlorophenoxy)propionic acid (dichlorprop) or 2-(4-chloro-2-methyl-phenoxy)propionic acid (mecoprop) are active as herbicides. *Sphingomonas herbicidovorans* MG was shown to possess two proteins, RdpA and SdpA, specific for hydroxylating the (*R*) and (*S*) enantiomers, respectively (Figure 1.12B).[279] The structural basis of the distinct enantiospecificities for these proteins has been assessed by homology modelling, substrate docking and mutagenesis.[280]

The mechanism of herbicidal action of the above aryloxyalkanoic acids depends on the interaction of the compounds with the auxin receptor of plants. It is thus of some interest that rice possesses DAO (see Section 1.5.4), which converts indole-3-acetic acid into 2-oxoindole-3-acetic acid (Figure 1.8I).[224] The distribution of this activity in other plants remains to be defined.

1.6.4 Sulfonate and Sulfate Metabolism by 2OG-Dependent Dioxygenases

TauD is the best characterized 2OG-dependent oxygenase in terms of understanding its catalytic mechanism.[9,281] This *E. coli* enzyme hydroxylates 2-aminoethanesulfonate (taurine), with the hydroxylated sulfonate intermediate spontaneously decomposing to aminoacetaldehyde and sulfite (Figure 1.12C), which is used as a sulfur source by the cells.[282] Crystal structures are available for the *E. coli* protein as well as that from *Pseudomonas putida* KT2440.[283–285] Several of the intermediate states of catalysis

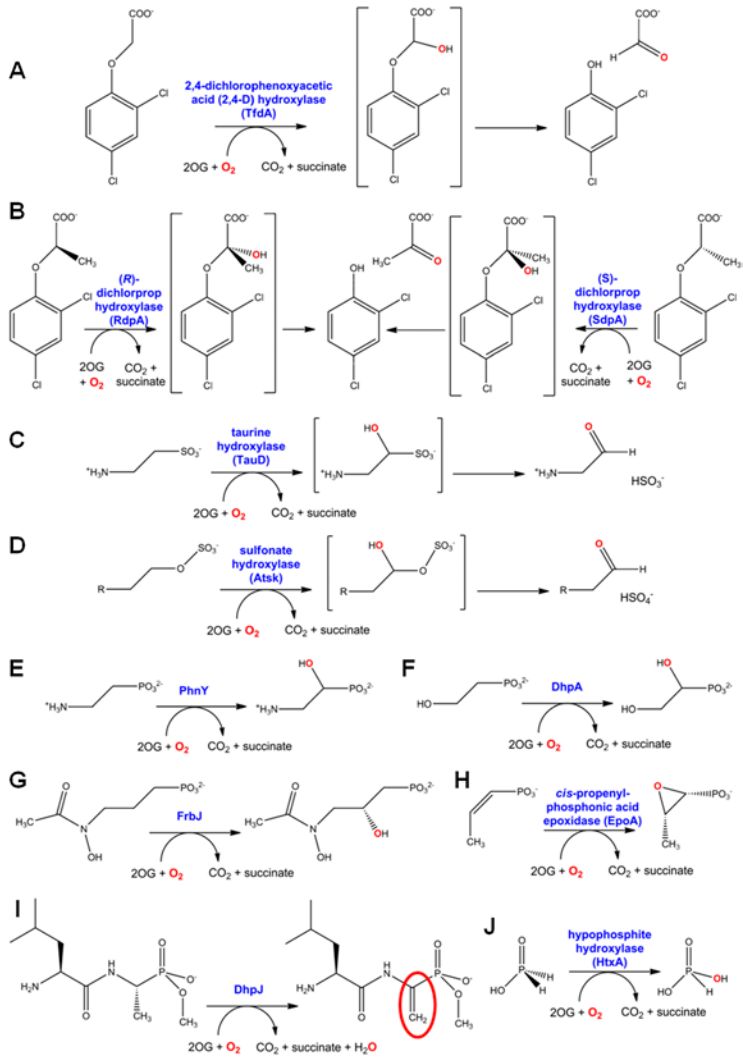

Figure 1.12 2OG-dependent oxygenases involved in metabolism of herbicides, sulfur-containing species and phosphonate compounds. (A) 2,4-D metabolism by TfdA. (B) RdpA and SdpA reactions with enantiomers of dichlorprop. (C) Taurine metabolism by TauD. (D) Sulfate metabolism by AtsK. (E) PhnY catalysed hydroxylation of 2-aminoethylphosphonate. (F) DhpA reaction with hydroxyethylphosphonate. (G) FrbJ catalysed hydroxylation. (H) Epoxidation reaction of EpoA. (I) Desaturation reaction of DhpJ. (J) HtxA conversion of hypophosphite to phosphite.

have been examined spectroscopically,[286,287] including the Fe(IV)-oxo[288–291] and later species.[10] In addition, at least two distinct types of self-hydroxylation chemistries have been studied with TauD, with one case involving a transient tyrosyl radical.[66–68] The enzyme decomposes a variety of other sulfonates (not depicted), including the widely used buffer 3-(*N*-morpholino)

propanesulfonic acid, MOPS.[282] A yeast homologue is most active with MOPS among the potential substrates tested, but physiologically it functions to degrade taurocholate – the amide formed between taurine and cholic acid.[292]

Similar to the sulfonate-degrading enzymes discussed above, 2OG-dependent hydroxylases are also used to decompose alkylsulfates (Figure 1.12D). The first such enzyme discovered was AtsK from *P. putida* S-313; this protein is 38% identical to TauD, but it exhibits no activity with taurine.[293] A related enzyme is associated with the Rv3406 locus of *Mycobacterium tuberculosis*.[294] Crystal structures have been obtained with AtsK proteins from both sources.[294–296]

1.6.5 2OG-Dependent Oxygenases in Phosphonate Metabolism

Several members of the 2OG oxygenase enzyme family have been shown to act on various types of phosphonate compounds. The gene encoding PhnY was identified from a screen of ocean-derived metagenomic DNA that allowed *E. coli* cells deficient in C–P lyase (*Δphn*) to grow on 2-aminoethylphosphonic acid as the sole source of phosphorus. The substrate, a close analogue of taurine, is hydroxylated by PhnY (Figure 1.12E)[297] much like the reaction of TauD; however, the hydroxylated phosphonate is stable – unlike the hydroxylated sulfonate. DhpA catalyses a very similar reaction, oxygen addition to hydroxyethylphosphonate (Figure 1.12F) or its O-phosphomonomethyl ester (not shown), in the pathway for biosynthesis of dehydrophos, a vinyl phosphonate tripeptide antibiotic of *Streptomyces luridus*.[298] A hydroxylation reaction is also catalysed by FrbJ of *Streptomyces rubellomurinus*, in this case using the antibiotic FR-900098 as substrate (Figure 1.12G).[299] Rather than hydroxylation, EpoA of *Penicillium decumbens* catalyses epoxidation using *cis*-propenylphosphonic acid (Figure 1.12H) during synthesis of the antibiotic fosfomycin.[300] Another enzyme participating in the synthesis of dehydrophos by *S. luridus* is DhpJ, which catalyses a desaturation reaction of the monomethyl diester of L-Leu-L-1-aminoethylphosphonic acid (Figure 1.12I).[301] While the substrate is not a phosphonate, it is also appropriate to mention here the phosphite-producing hypophosphite hydroxylase activity (Figure 1.12J) of HtxA from *Pseudomonas stutzeri* WM88.[302]

1.7 2OG-Dependent Oxygenases Involved in Antibiotic Biosynthesis

Several 2OG-dependent oxygenases described earlier function in the synthesis of antibiotics, but their actions typically involve provision of small precursor molecules that become incorporated into the final compounds. This section focuses on other family members that function in antibiotic biosynthesis by forming bicyclic β-lactams, tailoring terpenoids, modifying protein-bound *S*-pantetheinyl thioesters compounds, and other roles.

1.7.1 Bicyclic β-Lactam Antibiotic Biosynthesis

This section covers several 2OG-dependent oxygenases that participate in the formation of clinically important bicyclic β-lactam antibiotics.[303] Also of interest for this topic, Section 1.8.1 describes isopenicillin *N* synthase, a structurally-related enzyme that does not use 2OG.

Clavaminate synthase (CAS) is a remarkable trifunctional 2OG-dependent enzyme in the pathway for synthesis of clavulanic acid in *Streptomyces clavuligerus*.[304] Starting with L-Arg and glyceraldehyde-3-phosphate, the cells utilize a thiamine diphosphate-dependent enzyme to form N^2-(2-carboxyethyl)arginine, which cyclizes *via* an ATP-dependent ligase reaction to generate deoxyguanidinoproclavaminate. This compound is the substrate for CAS, which catalyses a 2OG-dependent hydroxylation reaction (Figure 1.13A, left reaction). The guanidine group is removed from the resulting guanidinoproclavaminic acid by a separate hydrolase to yield proclavaminate. This substrate is used in a 2OG-dependent cyclization reaction catalysed by CAS (Figure 1.13A, middle reaction) to form dihydroclavaminic acid, which undergoes desaturation to clavaminic acid in the third 2OG-dependent reaction of CAS (Figure 1.13A, right reaction). Subsequent amino transferase and reductase reactions provide the final clavulanic acid (with an alcohol replacing the amine in the last structure shown). Purified CAS in its various states was studied by several spectroscopic methods, which provided evidence that the binding of both substrates (2OG and the antibiotic precursors) led to the loss of all metal-bound water, thus creating an oxygen binding site on the metal.[305,306] Structures of CAS have been solved in the presence of Fe(II), 2OG and either *N*-α-acetyl-L-Arg, proclavaminic acid, or deoxyguanidinoproclavaminate (with the oxygen analogue NO bound to the metal).[307,308]

Cephalosporin biosynthesis starts with the formation of isopenicillin (see Section 1.8.1), which is epimerized to penicillin, modified by two 2OG-dependent enzymes in prokaryotes or a single dual-function enzyme in eukaryotes, and additionally tailored by other reactions, sometimes including another 2OG family member. The first two 2OG-dependent oxygenases in *S. clavuligerus* are deacetoxycephalosporin C synthase (DAOCS), which catalyses a ring expansion reaction (Figure 1.13B, left), and deacetylcephalosporin C synthase (DACS; Figure 1.13B, right), a hydroxylase.[309–311] In contrast, both reactions are catalysed by the same DAOCS/DACS enzyme from *Cephalosporium acremonium*.[312,313] Several structural studies of DAOCS included the first structure for any 2OG-dependent oxygenase[314] and reveal the modes of substrate and product binding – with the surprising finding of overlapping binding sites of 2OG and penicillin substrates.[315–319] Recent pre-steady state kinetics and binding studies have called into question the interpretations from earlier results and conclude that a ternary complex does form in the protein.[320] Modelling combined with mutagenesis studies suggest that a single residue controls whether the enzyme catalyses ring expansion or hydroxylation, and offers insight into how the *C. acremonium* DAOCS/DACS enzyme catalyses both reactions.[321] The deacetylcephalosporin C resulting

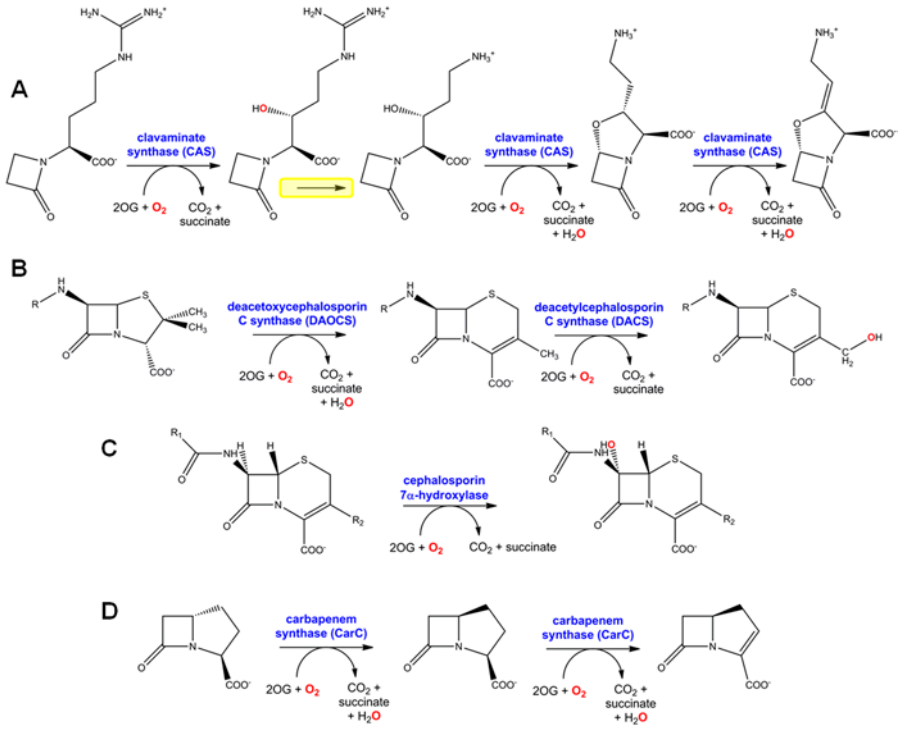

Figure 1.13 2OG-dependent oxygenases for synthesis of bicyclic β-lactam antibiotics. (A) Three reactions of clavaminic acid synthase and a separate non-CAS reaction (yellow highlight). (B) Ring expansion and hydroxylation reactions of cephalosporin biosynthesis. (C) Cephalosporin 7α-hydroxylase. (D) Carbapenem synthase epimerase (an external source of electrons is also needed for this reaction) and desaturation reactions.

from these reactions undergoes further transformations including acylation of the newly introduced hydroxyl group, 2OG-dependent hydroxylation at the 7α position (Figure 1.13C),[322] and methylation of the latter site. These enzymes are described in greater detail in Chapter 17.

Carbapenems are a third grouping of the bicyclic β-lactams that utilize a 2OG-dependent oxygenase in their synthetic pathway. *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) contains the enzyme (2*S*,5*S*)-carboxymethylproline synthase (CarB) that uses glutamate semi-aldehyde and malonyl-CoA to produce the carboxymethylproline derivative. This substrate is subjected to a ligase reaction by (3*S*,5*S*)-carbapenam synthetase (CarA), forming carbapenam-3-carboxylate with its β-lactam ring. Carbapenem synthase (CarC) is a 2OG-dependent oxygenase proposed to catalyse two sequential non-hydroxylase reactions: epimerization to (3*S*,5*R*)-carbapenam-3-carboxylate and desaturation to yield (5*R*)-carbapenem-3-carboxylate (Figure 1.13D).[323] The crystal structure of CarC with bound Fe(II) and 2OG

has been elucidated.[324] The stereoinversion reaction appears to involve ferryl abstraction of a substrate hydrogen atom followed by hydrogen atom donation from a tyrosyl side chain, limiting the enzyme to a single turnover unless external electrons are provided.[325]

1.7.2 Synthesis of Terpenoid Antibiotics

The rich biochemistry of terpenoid metabolism includes several examples of reactions catalysed by 2OG-dependent oxygenases. The synthesis of pentalenolactone, a sesquiterpenoid produced by dozens of strains of *Streptomyces*, nicely illustrates this point. A hydroxylase reaction, the conversion of 1-deoxypentalenic acid to 11β-hydroxy-1-deoxypentalenic acid (Figure 1.14A), is catalysed by *S. avermitilis* PltH.[326] The structure of this protein in complex with Fe(II), 2OG and *ent*-1-deoxypentalenic acid (a non-reactive enantiomer), has been defined.[327] Analogous proteins appear to be present in *S. exfolatus* UC5319 and *S. arenae* TU469, where they are named PenH and PntH, respectively.[328] These three strains each possess a second 2OG-dependent oxygenase (PtlD, PenD and PntD, respectively) capable of desaturating pentalenolactone D to pentalenolactone E, with PenD and PntD subsequently forming the epoxide pentalenolactone F (Figure 1.14B). In addition, these same three enzymes can transform neopentalenolactone D to an unstable enollactone desaturation product which is hydrolysed (Figure 1.14C).[328]

Two 2OG-dependent oxygenases utilize the same substrate, fusicocca-2,10(14)-diene-8β,16-diol, to produce distinct diterpene phytohormone-like compounds in fungi.[329] An enzyme from *Alternaria brassicicola* first abstracts a hydrogen atom from the eight-membered ring, the carbon-centered radical migrates, and hydroxyl radical rebound yields the distal hydroxylated product (Figure 1.14D, left) during a step in the synthesis of cotylenin A or brassicicene C. In contrast, an enzyme from *Phomopsis amygdali* catalyses oxidation at C-16 to yield the aldehyde 8β-hydroxyfusicocca-1,10(14)-diene-16-al (Figure 1.14D, right) during fusicoccin A biosynthesis.[329]

A final example of a 2OG-dependent oxygenase involved in synthesis of a terpene glycoside antibiotic is PlaO1 from *Streptomyces* sp. Tü6071.[330] PlaO1 catalyses the critical formation of a γ-butyrolactone during synthesis of phenalinolactone (Figure 1.14E). The chemical mechanism associated with this remarkable reaction has not been detailed, but a cogent hypothesis has been proposed.[331]

1.7.3 2OG-Dependent Oxygenases Acting on Tethered Substrates in Non-Ribosomal Peptide Synthesis

Several 2OG-dependent oxygenases act on protein-tethered *S*-pantetheinyl thioesters of amino acids which undergo non-ribosomal incorporation into peptide-related antibiotics. For example, *Pseudomonas syringae* pv. *syringae* B301D produces the non-ribosomal peptide phytotoxin called syringomycin

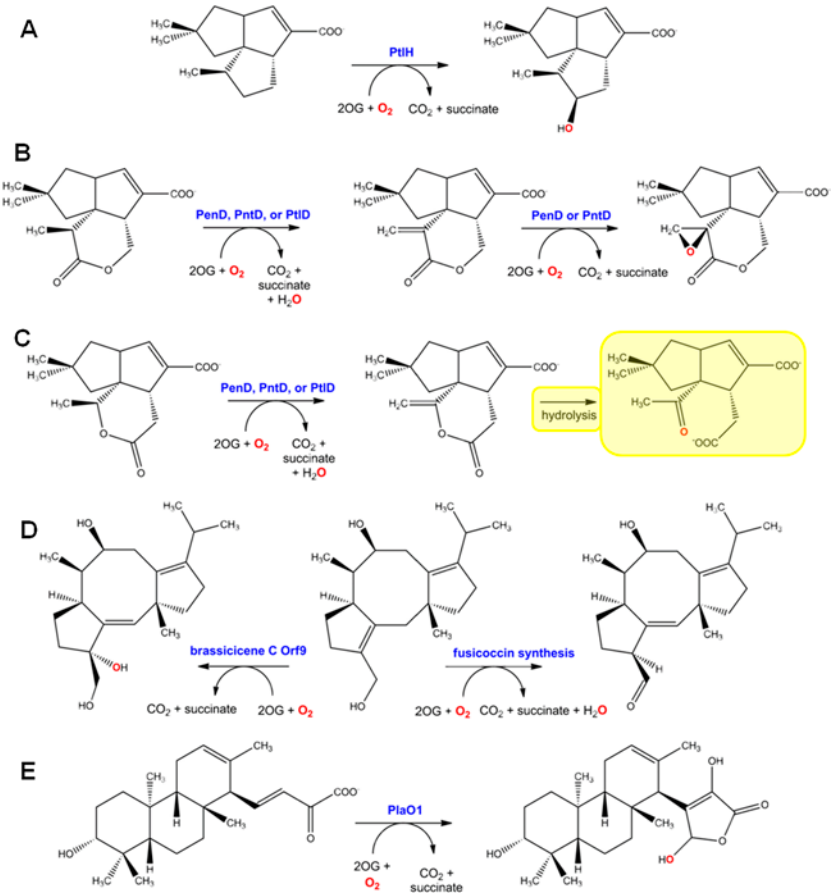

Figure 1.14 Representative 2OG-dependent oxygenases in terpenoid biosynthesis. (A) 1-deoxypentalenic acid 11β-hydroxylase, PtlH. (B) Desaturase and epoxidase reactions of PenD, PntD and PtlD using pentalenolactone D. (C) Neopentalenolactone desaturase reaction by the same enzymes, followed by spontaneous hydrolysis (yellow highlight). (D) Two reactions using fusicocca-2,10(14)-diene-8β,16-diol. (E) PlaO1-catalysed γ-butyrolactone formation in phenalinolactone synthesis.

E by assembling components on a multimodular megasynthetase, SyrE. SyrP is a 2OG-dependent hydroxylase that acts on L-Asp bound to the eighth module of SyrE to yield the *threo* isomer of 3-hydroxyaspartic acid which is inserted into the eighth position of the final phytotoxin (Figure 1.15A).[237]

Two other proteins involved in syringomycin E biosynthesis, SyrB1 and SyrB2, are required for synthesis of the 4-chloro-L-Thr located at position nine of the phytotoxin. Remarkably, SyrB2 was shown to be a 2OG-dependent oxygenase that catalyses a halogenation reaction using L-Thr tethered to SyrB1 (Figure 1.15B).[332] The enzyme also catalyses bromination, dichlorination, nitration and azidation reactions (not depicted).[333,334] The structure

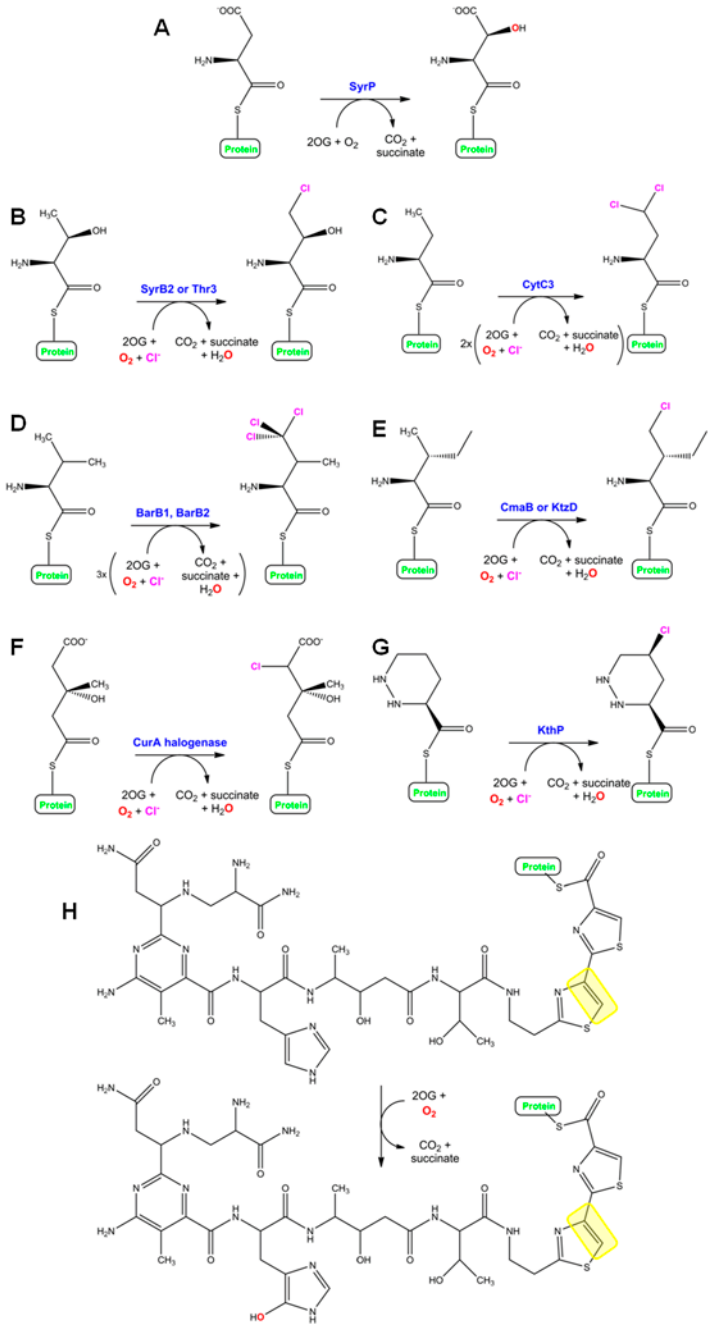

Figure 1.15 Use of protein-tethered substrates by 2OG-dependent oxygenases in antibiotic biosynthesis. (A) SyrP-catalysed formation of a 3-hydroxy-aspartyl group and (B) SyrB2-dependent synthesis of a 3-chlorothreonyl group in syringomycin E synthesis. (C) CytC3 generation of the

of SyrB2 reveals a metallocentre in which Fe(II) is bound only by two histidyl residues (with the typical carboxylate ligand replaced by an alanyl side chain), and coordinated to 2OG and chloride ion.[335] Spectroscopic studies have revealed evidence for a chloroferryl intermediate in catalysis,[336] and studies with substrate analogues suggest that substrate positioning determines whether halogenation or hydroxylation take place.[337] *Streptomyces* sp. OH-5093 produces free 4-chlorothreonine by a similar pathway, in which Thr3 catalyses the halogenation of the tethered amino acid, followed by thioester hydrolysis.[338]

Much like the SyrB1/SyrB2 system just discussed, a cytotrienin-producing *Streptomyces* sp. uses CytC3 to catalyse halogenation of L-2-aminobutyric acid (or L-Val) tethered to CytC2 (Figure 1.15C).[339] Tandem chlorinations followed by thioesterase activity lead to the γ,γ-dichloraminobutyrate antibiotic that is released into the soil. Two interconverting Fe(IV) intermediates were detected in that study, and later work provided evidence for the formation of a bromoferryl intermediate when bromide was used to replace chloride.[340] The structure of CytC3 exhibits the same general architecture as seen for SyrB2, including an Ala residue replacing the typical aspartyl metal ligand.[341]

Five other examples illustrate additional interesting features of 2OG-dependent halogenation enzymes. The marine cyanobacterium *Lyngbya majuscula* produces barbamide, an antibiotic containing trichlorinated leucine that is active against molluscs. BarB2 converts BarA-tethered L-Leu or 4-chloro-L-Leu to the dichlorinated species and BarB1 halogenates the mono- or dichlorinated species to the trichlorinated species (Figure 1.15D).[342] *Pseudomonas syringae* pv. *tomato* DC3000 synthesizes the phytotoxin coronatine which contains 1-amino-1-carboxy-2-ethylcyclopropane (coronamic acid). Synthesis of coronamic acid derives from L-*allo*-isoleucine which is tethered to CmaD, chlorinated by CmaB (Figure 1.15E), and converted to the cyclopropane species by CmaC with elimination of chloride in this cryptic chlorination pathway.[343] An analogous system is present in the ascomycete *Kutzneria*, where KtzD chlorinates L-Ile bound to KtzC, with the product cyclized to the bound (1*S*,2*R*)-allocoronamic acid by KtzA.[344] Another example of cryptic chlorination occurs during curacin A synthesis by *L. majuscula*, the marine bacterium mentioned above. In this case, acyl carrier protein-bound (*S*)-3-hydroxy-3-methylglutarate is chlorinated by CurA (Figure 1.15F), followed by dehydration using CurC, decarboxylation *via* a CurF domain, and

mono- or dichloro L-2-aminobutyryl group in cytotrienin synthesis. (D) Trichlorination of leucine by BarB1 and BarB2 to make barbamide. (E) CmaB- or KtzD-dependent hydroxylation of isoleucyl groups for subsequent cyclization to coronamic acids. (F) CurA chlorination of a 3-hydroxy-3-methylglutaryl group as part of curacin A synthesis. (G) KthP halogenation of a piperazyl group during formation of kutznerides. (H) Hydroxylation of a histidyl group during synthesis of bleomycin, tallysomycin and zorbamycin (yellow highlight is a single bond in the case of zorbamycin).

reductive dechlorination by another CurF domain to yield the protein-bound (1*R*,2*S*)-2-methylcyclopropane-1-carboxylate which is incorporated into curacin A.[345] The structures of CurA in five ligand states have been elucidated.[346] *Kutzneria* sp. 744 chlorinates a protein-bound piperazate residue by using the KthP halogenase (Figure 1.15G) during biosynthesis of a family of antifungal kutznerides.[347] Finally, *Lyngbya majuscula* contains the three-domain protein HctB that catalyses dichlorination (along with hydroxylation and introduction of a vinyl chloride group) on a fatty acyl group attached to its acyl carrier domain region (not depicted).[348] Interestingly, the oxidative reactions of this enzyme are stimulated more than 200-fold by the presence of saturating concentrations of chloride, thus providing an explanation for why hydroxylation does not dominate in the absence of halide salts.[349] Further discussion of 2OG-dependent halogenases can be found in Chapter 18.

Final examples for this section relate to hydroxylation reactions during synthesis of four antitumour natural products made by non-ribosomal peptide synthesis pathways: bleomycin, tallysomycin, zorbamycin and spliceostatin made by *Streptomyces verticillus* ATCC15003, *Streptoalloteichus hindustanus* E465-94, *Streptomyces flavoviridis* ATCC21892 and *Burkholderia* spp., respectively. A 2OG-dependent hydroxylase, potentially associated with open reading frames 1, 10 and 30 of the gene clusters in the first three microbes, is thought to catalyse histidyl group hydroxylation of a large precursor of the final antibiotic species bound to carrier proteins (Figure 1.15H), rather than hydroxylating just the tethered amino acid.[350] The 3-hydroxyhistidyl group is the site of glycation and additional tailoring reactions are used to generate the final antibiotics. The *Burkholderia* enzyme catalyses the synthesis of a hemiketal group (not shown) using the 2OG-dependent mechanism.[351]

1.7.4 Other Roles for 2OG-Dependent Oxygenases in Antibiotic Synthesis

Other 2OG-dependent oxygenases serve important roles in antibiotic biosynthesis, but don't fit into the above categories. These examples are not meant to be comprehensive, but provide useful illustrations of the diversity of reactions catalysed by these enzymes. The just mentioned antibiotic tallysomycin requires TmlH for its synthesis. This enzyme catalyses two 2OG-dependent hydroxylations, at positions C-41 and C-42 (Figure 1.16A).[352] The carbanolamide produced by hydroxylation of C-41 is unstable, but appears to be immediately modified further by TlmK to produce a stable species. It remains unclear whether TlmH acts on the protein-free species as shown or if the modification takes place on the protein-tethered substrate.[350] The fumonisins are mycotoxins produced by *Fusarium verticillioides* and several other filamentous fungi *via* polyketide synthetic routes. Hydroxylation at the C-5 position (Figure 1.16B) is carried out by the 2OG-dependent enzyme Fum3p (formerly associated with the *FUM9* locus).[353,354] Another mycotoxin, verruculogen, is produced by *Aspergillus fumigatus*. The novel creation of an endoperoxide within fumitremorgin B is catalysed by the 2OG-dependent FtmOx1 protein

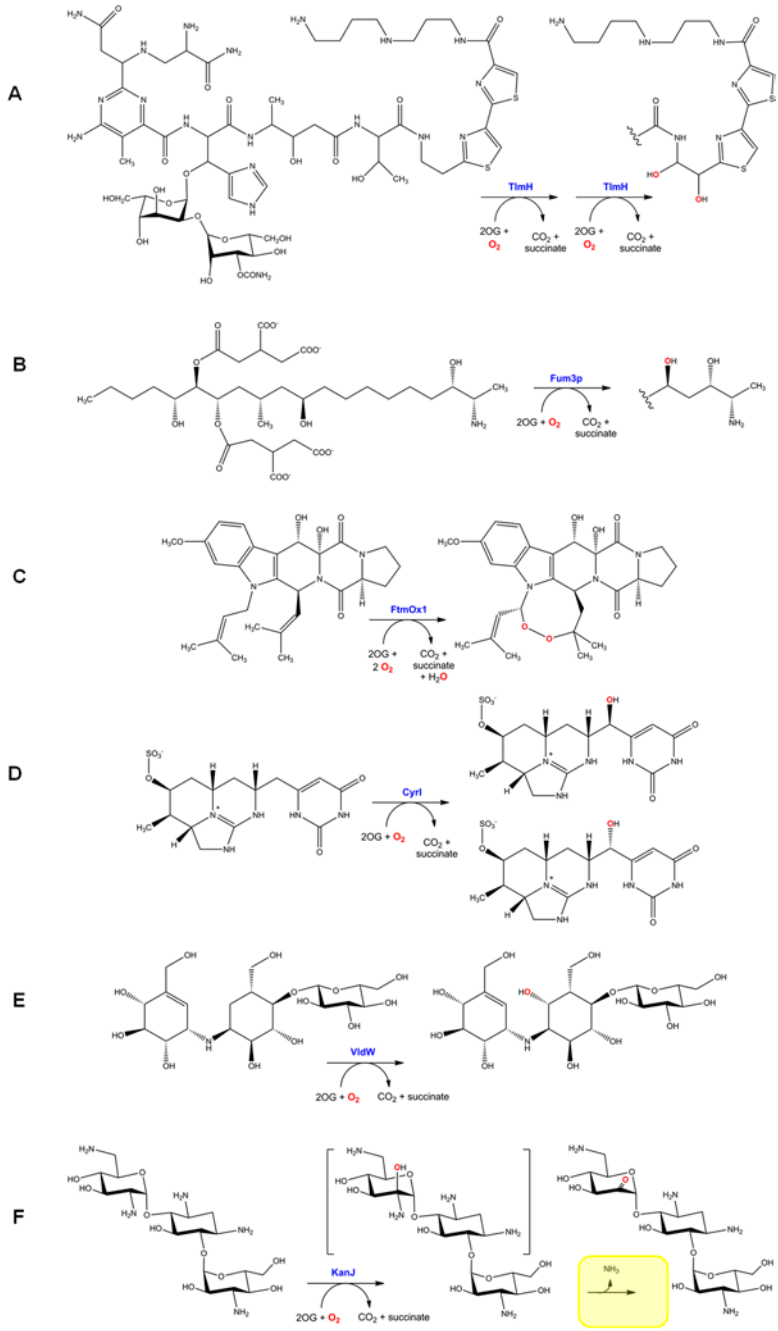

Figure 1.16 More 2OG-dependent oxygenases involved in the biosynthesis of antibiotics. (A) TlmH functioning in tallysomycin biosynthesis. (B) Fum3p modification of a fumiosin. (C) FtmOx1 creation of an endoperoxide in the verruculogen pathway. (D) CyrI production of two cylindrospermopsin products. (E) VldW role in decreasing the toxicity of a validamycin. (F) KanJ hydroxylation and a spontaneous follow-up reaction (yellow highlight) in the penultimate step of kanamycin synthesis.

(Figure 1.16C). Notably, this reaction requires two molecules of oxygen.[355] Several *Oscillatoria* and other cyanobacterial species produce the cyanotoxins cylindrospermopsin and 7-*epi*-cylindrospermopsin. The final step in the synthesis of these compounds is catalysed by the 2OG-dependent hydroxylase CyrI (Figure 1.16D).[356] *Streptomyces hydrogscopicus* subsp. *Limoneus* produces a family of validamycin compounds with antifungal activity. The main species, validamycin A, is converted to the less effective validamycin B by VldW, a 2OG-dependent hydroxylase (Figure 1.16E).[357] Finally, the penultimate step in kanamycin biosynthesis by *Streptomyces kanamyceticus* is carried out by KanJ. This protein catalyses the hydroxylation of an amino sugar (Figure 1.16F) to yield an unstable hemiaminal which decomposes with release of ammonia to yield 2′-oxokanamycin; the latter compound is the substrate for an NADPH-dependent reductase, KanK, to produce the final antibiotic.

1.8 Related Enzymes

1.8.1 Isopenicillin *N* Synthase

The enzyme isopenicillin *N* synthase (IPNS) catalyses the fascinating transformation of a linear tripeptide, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine, into a bicyclic structure, as shown in Figure 1.17A. The resulting product is subsequently metabolized into penicillins and cephalosporins as described earlier in Section 1.7.1.[303] Although IPNS does not utilize 2OG as a cosubstrate, it is related in sequence and structure to the 2OG-dependent oxygenases; indeed, it was the first family member to be structurally characterized.[358] Like the 2OG-dependent enzymes, IPNS coordinates Fe(II) *via* a 2-His-1-carboxylate motif and binds substrate within a double-stranded β helix fold.[359] The thiolate sulfur atom of the substrate forms a ligand to the metallocentre. Snapshots of the reaction were structurally visualized after brief exposure of anaerobic crystals to high pressures of oxygen.[360] This remarkable enzyme is described further in Chapter 19.

1.8.2 1-Aminocyclopropane-1-Carboxylate Oxidase

Another enzyme that does not utilize 2OG, yet is related by sequence and structure to the 2OG-dependent oxygenases, is 1-aminocyclopropane-1-carboxylate oxidase (ACCO).[361,362] This plant enzyme catalyses the synthesis of ethylene (Figure 1.17B) in a reaction that is distinct from that described in Section 1.6.1. The gaseous product is a phytohormone that functions in germination, fruit ripening and senescence. The cyclopropane-containing substrate of ACCO binds to the Fe(II) site *via* its α-amino and α-carboxylate groups according to spectroscopic analyses with isotopically-labelled substrates and substrate analogues;[363,364] such coordination is reminiscent of the bidentate binding of 2OG to the metallocentre of related family members. Ascorbate is required for multiple turnovers of ACCO, but the reductant is not needed for a single turnover.[365] Surprisingly, carbon dioxide is essential

for catalysis in addition to being a product, and this molecule is proposed to stabilize the enzyme from undergoing inactivation reactions.[366] Oxidative inaction of ACCO occurs rapidly and can result in cleavage of the peptide backbone.[367,368] Crystal structures have been resolved for the apoprotein and holoprotein forms of ACCO,[369] and modelling studies have led to proposals for the binding sites of substrate, bicarbonate and ascorbate.[370] This enzyme is described in further detail in Chapter 20.

1.8.3 4-Hydroxyphenylpyruvate Dioxygenase and Hydroxymandelate Synthase

Hydroxymandelate synthase (HMS) and 4-hydroxyphenylpyruvate dioxygenase (HPPD) are of special interest because they transform the same substrate into distinct products (Figure 1.17C) by reactions that are mechanistically related to those carried out by the 2OG-dependent oxygenases; however, HMS and HPPD are unrelated to the latter enzymes while being closely related to each other.[371] The substrate for both enzymes is 4-hydroxyphenylpyruvate, a 2-oxo acid, and like 2OG it undergoes oxidative decarboxylation with formation of carbon dioxide and 4-hydroxyphenylacetate. The enzyme intermediate resulting from the C–C cleavage reaction is used either to hydroxylate the

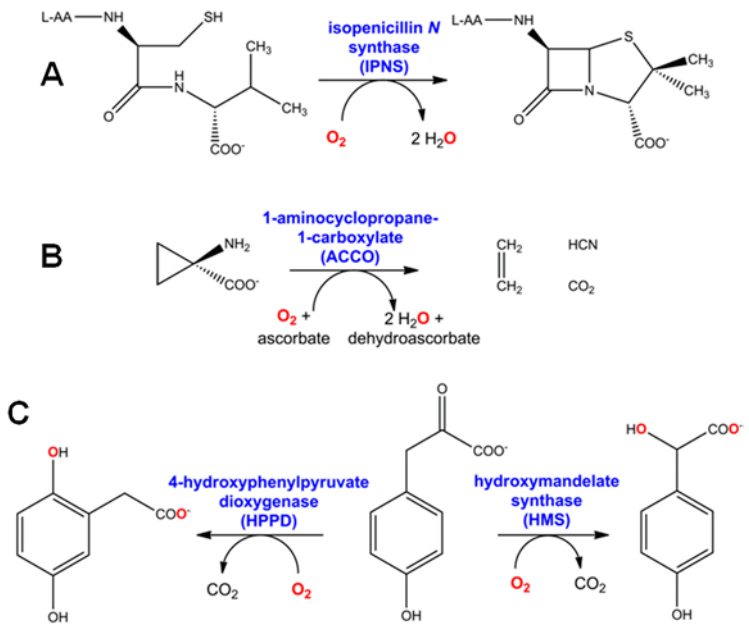

Figure 1.17 Enzymes that are structurally or functionally related to 2OG-dependent oxygenases, but do not utilize 2OG. (A) Isopenicillin *N* synthase. (B) 1-Aminocyclopropane-1-carboxylate oxidase. (C) 4-Hydroxyphenylpyruvate dioxygenase and hydroxymandelate synthase.

methylene group of 4-hydroxyphenylacetate to form hydroxymandelate (in HMS) or it catalyses the 'NIH shift' in which substituent migration and ring hydroxylation produce homogentisate (in HPPD). Isotope effect studies have provided keen insights into how the intermediate partitions to form the two products.[372] Four key residues in the active sites of these enzymes are critical for defining product specificity, and an HPPD has been engineered to exhibit HMS activity.[373] Several catalytic intermediates have been detected by using spectroscopic approaches with these enzymes.[374,375] The structure of HMS from *Amycolatopsis orientalis*[376] exhibits the same fold as found in HPPD, first reported for the enzyme from *Pseudomonas fluorescens*;[377] however, their folds are unrelated to the typical 2OG oxygenase fold. Further information on this pair of enzymes is provided in Chapter 21.

Conspectus: The 2OG-dependent oxygenases catalyze a diverse array of reactions that have profound implications in biology. The structures and mechanisms of these fascinating enzymes are discussed in Chapters 2–4 and representative topics are detailed more fully in Chapters 5–21.

Note added in proof

A large number of publications related to this topic have appeared since submission of this chapter, only three of which are cited here. A prolyl residue in prokaryotic elongation factor Tu is hydroxylated by a 2OG-dependent oxygenase, perhaps serving as an evolutionary precursor to prolyl hydroxylases used in oxygen sensing.[379] A review describing these enzymes in coumarin synthesis has appeared.[380] Finally, Wel05 was shown to be a 2OG-dependent halogenase acting on a free substrate (*i.e.* not tethered to a peptidyl carrier protein).[381]

Acknowledgements

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