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The UbiX-UbiD system: the biosynthesis and use of prenylated flavin (prFMN).

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The UbiX-UbiD system consists of the flavin prenyltransferase UbiX that produces prenylated FMN that serves as the cofactor for the (de)carboxylase UbiD. Recent developments have provided structural insights into the mechanism of both enzymes, detailing unusual chemistry in each case. The proposed reversible 1,3-dipolar cycloaddition between the cofactor and substrate serves as a model to explain many of the key UbiD family features. However, considerable variation exists in the many branches of the UbiD family tree.

Keywords: prenylated FMN, (de)carboxylase, prenyl transferase, UbiX, UbiD, cycloaddition

Highlights

- **The UbiX-UbiD system presents a rich source of novel flavin chemistry**
- **UbiX is a prenyl transferase generating the prenylated FMN cofactor (prFMN)**
- **Oxidative maturation of prFMN in the UbiD enzyme generates an azomethine ylide**
- **Cycloaddition between oxidized prFMN and substrate supports (de)carboxylation**

Introduction

Under ambient conditions, the decarboxylation of organic acids is driven by the release of CO₂. In fact, certain reactions in biochemistry are coupled to decarboxylation as a means of driving the reaction to completion (1-3). However, most organic acids are highly stable molecules, a consequence of the inherent kinetic barrier to decarboxylation. Indeed, the C-C bond heterolysis resulting in CO₂ formation also generates a highly nucleophilic carbanion species (**Fig 1a**). Spontaneous decarboxylation at ambient conditions only occurs for those reactions where the latter can be stabilized. A myriad of decarboxylase enzymes have evolved in Nature, reflecting the wide variety of chemical approaches that can be used to stabilize or bypass formation of the carbanion intermediate and thereby catalyse this reaction (2,3). While not exclusively so, many decarboxylases depend on the use of cofactors, ranging from inorganic metals ions to organic molecules such as thiamine pyrophosphate (TPP) (4) or pyridoxal phosphate (PLP) (5). The latter are used as transient electron sinks during decarboxylation of the substrate-cofactor adduct (**Fig 1b**).

While many decarboxylases couple the decarboxylation to oxidation of the alkyl-moiety, others catalyse non-oxidative decarboxylation (2,3,6,7). In case of the latter enzymes, the carboxylic acid functionality is replaced by a proton. A well-studied example of a non-oxidative decarboxylase is the family of phenolic acid decarboxylases (also confusingly named PAD; (7). These use acid-base chemistry to stabilize the phenolate form during decarboxylation and do not require a cofactor. Instead, the substrate phenol group is recruited to fulfill the role of stabilisation of the carbanion species via formation of a quinoid intermediate preceding the decarboxylation reaction (**Fig 1c**). Hence, the PAD enzymes have the obligate requirement of a phenolic substrate.

In contrast, decarboxylases exist that also catalyse non-oxidative phenolic acid decarboxylation, but do not have a strict requirement for a phenolic substrate. These enzymes are found in bacteria and fungi, and are evolutionary unrelated to PAD and, in contrast, strictly require alpha-beta unsaturated carboxylic acid substrates (**Fig 2**). Early studies identified two distinct genes associated with

this activity, leading to the proposal these encoded redundant decarboxylases (8-12). This proposal has turned out incorrect (*vide infra*), but has given rise to confusing terminology associated with these enzymes. The smaller gene encodes for UbiX (*E. coli* nomenclature, (8,9) or phenylacrylic acid decarboxylase (fungal nomenclature, 10,11; confusingly also using the PAD acronym), a protein distantly related to the flavin-dependent cysteine decarboxylase family (13,14). The larger gene encodes for UbiD (*E. coli* nomenclature) or Fdc (yeast nomenclature, ferulic acid decarboxylase), a previously biochemically largely uncharacterized enzyme family. The UbiX/UbiD nomenclature (which we will use for UbiX) is derived from the presence of both genes in the bacterial ubiquinone biosynthesis pathway, while the Pad/Fdc nomenclature is derived from the proposed functionality in yeast (we will use both UbiD and Fdc terminology here, to denote Fdc represents a distinct clade within the UbiD-family). Yeast Pad is more closely related to UbiX than Fdc is to the corresponding UbiD (UbiX/Pad 50% identity, UbiD/Fdc 26% identity), and the *pad* gene can restore the ubiquinone production in an *E. coli ubiX* mutant (9).

Discovery of a novel modified flavin cofactor: prFMN

Several UbiX family members have been studied in detail, demonstrating that it binds flavin mononucleotide (FMN) as other members of the flavin-dependent cysteine decarboxylase family (15-17). While several crystal structures have been published, there are no reports of the isolation of a UbiX-associated decarboxylase active *in vitro*. Structural data for UbiD has been a more recent development, with the first crystal structure for UbiD deposited in the PDB database by a structural genomics consortium, followed by the first structures of related UbiD-family members (18,19). The various structures reported did not contain any organic cofactor, but some suggested the presence of a bound metal ion. However, no accompanying *in vitro* decarboxylase activity could be detected. In fact, previous literature did suggest UbiD activity was dependent on Mn²⁺ and a small, unidentified component derived from cellular extracts (20). Distant structural homology of the C-terminal UbiD domain to metal and FMN-binding proteins hints as to the nature of this component. The lack of *in vitro* decarboxylase activity for both the isolated UbiX or UbiD proteins, and the fact

both *ubiX* and *ubiD* genes are required for *in vivo* decarboxylation activity, can be explained by the observation that in yeast UbiX is involved in formation of a modified flavin required for activity by Fdc (21). Hence, UbiX is not a decarboxylase as was previously assumed (despite the distant relationship to flavin-dependent cysteine decarboxylases), but serves to modify FMN. In the absence of the UbiX-modified FMN, UbiD/Fdc1 is inactive.

The nature of the modification could not be resolved by this study, but was determined by atomic resolution crystal structures of the active form of the *A. niger* Fdc1 enzyme (22). The *holo*-enzyme form of the latter was obtained by co-expression with *E. coli ubiX*. The *A. niger* Fdc1 crystal structure revealed extensive modifications to both the FMN N5 and C6 positions, resulting in the addition of a fourth non-aromatic ring consisting of 5 carbon atoms and including a quaternary carbon (**Fig 3**). While covalent modification of flavins has been observed before (involved in covalent tethering of the cofactor to the enzyme, and/or alteration of the redox potential; (23)), the extent of this particular modification and the likely impact on the inherent flavin chemical properties was unprecedented. While the linkage to the C6 atom could be unambiguously defined as a single C-C bond, the N5-C1' linkage was suggested to be an iminium. This could be indirectly confirmed by high resolution mass spectrometry (which confirmed the total formula of the modified FMN), and also by the fact that incubation with the mild reducing agent cyanoborohydride (routinely used to specifically reduce iminium species) inactivated the enzyme. While *apo*-Fdc could not be crystallised, mass spectrometry revealed rearrangements on cofactor binding occur throughout the protein (24).

UbiX acts as a flavin prenyltransferase

The fact the flavin modification consisted of a C5-moiety that included a quaternary carbon suggests similarities to prenyltransferase chemistry (25), a reaction presumably catalyzed by UbiX (26). Furthermore, a single phosphate/sulphate ion was present in previously published UbiX crystal structures in relatively close proximity of the FMN substrate (15-17). Initial attempts at demonstrating UbiX prenyltransferase activity using the standard

terpenoid precursors (27) dimethylallyldiphosphate (DMAPP) or isopentenylidiphosphate (IPP) as co-substrates with FMN failed. Inspection of the available crystal structures for putative DMAPP/IPP binding sites suggested the diphosphate moiety of DMAPP/IPP could not be accommodated by the active site. In contrast, binding of the smaller dimethylallyl-*mono*-phosphate (DMAP) close to the UbiX bound FMN could be observed by virtue of its effect on the oxidized FMN spectrum, but no prenyltransferase activity was observed under aerobic conditions (26). Crystal structures of the *P. aeruginosa* UbiX enzyme in complex with oxidized FMN and DMAP confirm direct positioning of the latter above the isoalloxazine ring, apparently poised for catalysis (**Fig 3**). Activity was obtained when incubating UbiX with DMAP and reduced FMNH₂ under anaerobic conditions, resulting in the formation of a modified FMNH₂ that rapidly oxidized under aerobic conditions to form a purple semiquinone-like species. The crystal structure of UbiX in complex with this purple product confirmed that a modification similar to that observed in the *A. niger holo*-Fdc1 enzyme had occurred (26). This confirms UbiX functions as a prenyltransferase, and that the novel cofactor used by UbiD is prenylated FMN (prFMN).

Further insights into the UbiX reaction mechanism could be obtained using kinetic crystallography, making use of the fact the reaction proceeds slowly and via a readily detectable intermediate species (as observed using stopped-flow; 26). These revealed formation of the N5-C1' bond occurs first, leading to a long-lived adduct with an *sp*³ hybridized N5 position presumably corresponding to the intermediate observed in solution (Species III, **Fig 4**). The phosphate-C1' bond breakage is assisted by the presence of a so-called pi-cage, consisting of various aromatic groups and the reduced flavin isoalloxazine ring system. A conserved Glu (E140, Species I to II in **Fig 4**) is proposed to serve as proton donor to the phosphate leaving group. While still uncommon in flavoenzyme chemistry, the use of the reduced FMNH₂ N5 as a nucleophile (as occurs in UbiX) has now been demonstrated in UDP-galactopyranose mutase and the non-canonical thymidylate synthase ThyX (28,29) (see also other reviews in this special issue).

The Friedel-Crafts C-C bond formation reaction (species III to V, **Fig 4**) is proposed to require protonation of the dimethylallyl adduct by the bound phosphate, leading to formation of a transient ternary carbocation located directly above the FMN C6 (Species IV, **Fig 4**). Mutagenesis of key active site residues indirectly supports this proposal: distinct intermediates are seen to accumulate in corresponding crystal structures. The difference with the WT structures consists in the configuration of the observed dimethylallyl-adduct, a change that can be explained by assuming reversible protonation and ternary carbocation formation occurs within the active site (26).

Formation of active *holo*-UbiD requires oxidative maturation

While this data supports a general mechanism for prenyltransferase activity by UbiX, the redox state of the prFMN formed in the UbiX reaction is distinct from that observed in *holo*-Fdc1. The latter is oxidized at the N5-C1' position with respect to the UbiX product. Indeed, while reconstitution of *apo*-Fdc1 using the UbiX prFMN product (acquired under anaerobic reaction conditions) can be achieved, accompanying activation of the decarboxylase activity requires the presence of oxygen following prFMN-Fdc1 complex formation (22,26). This is suggestive of oxidative maturation occurring within the *holo*-Fdc1 enzyme (**Fig 3**). The exact mechanism for this has yet to be revealed, but is likely to bear some similarity to flavin oxidation under aerobic conditions.

Confusingly, the oxidized form of prFMN could be observed in two distinct isomeric forms in some (but not all) of the *holo*-Fdc1 crystal structures. While one species clearly corresponds to an oxidized version of the UbiX product (containing an iminium at the N5-C1' position), the other prFMN species visible appears to have undergone an unusual reorganization resulting in formation of a 7-membered central ring and an associated 5-membered non-aromatic ring (22). These are derived from the central 6-membered ring of the isoalloxazine ring system and the prenylation derived 6-membered ring. Hence, this unusual prFMN species contains a ketamine rather than an iminium group at the N5-C1' and displays a marked butterfly configuration in absence of substrate. It is unclear what the origin or the mechanism for the conversion between both

prFMN species is, but further studies strongly indicate the prFMN^{ketimine} form is not relevant to catalysis (*vide infra*).

Fdc1 uses prFMN for reversible 1,3-dipolar cycloaddition

Crystal structures of *holo*-Fdc1 in complex with cinnamic acid-type substrates reveals these are bound directly above the prFMN ring system, with the prFMN^{ketimine} form adopting a more planar configuration to accommodate ligand binding (**Fig 5**). This places the alpha-beta unsaturated C=C directly above the N5 and C4a of the prFMN (of both prFMN^{iminium} and prFMN^{ketimine} forms). The substrate carboxylic acid group is located close to the conserved R173 (*A. niger* Fdc numbering), a member of a triad of residues that form a salt bridge network (E277-R173-E282) conserved within the UbiD family (**Fig 5**). No covalent adduct between substrate and prFMN could be observed in the crystal structures. However, when incubating the Fdc1 enzyme with phenylpyruvate, the protein UV-visible spectrum is subtly altered, with associated inhibition of activity. The crystal structure of a phenylpyruvate-Fdc1 complex reveals a covalent adduct is made in this case, with a linkage between the C1' of the prFMN^{iminium} (the prFMN^{ketimine} form was not detected) and the alpha carbon of phenylpyruvate (22; **Fig 5** inset). Furthermore, this adduct has undergone decarboxylation, resulting in a phenylacetaldehyde adduct. This strongly suggest the prFMN^{iminium} form is relevant to catalysis, with transient formation of a substrate alpha-C1' prFMN^{iminium} adduct.

Therefore, a plausible mechanism for Fdc1 (and by extension the wider UbiD-family) needs to explain the obligate need of the UbiD-family for alpha-beta unsaturated carboxylic acids as substrate, the need for the prFMN^{iminium} as a cofactor (as opposed to FMN or reduced prFMN) and the adduct formation between the substrate alpha and the C1' of prFMN^{iminium}. Such a mechanism could be proposed inspired by the analogy of the prFMN^{iminium} cofactor to azomethine ylides (**Fig 5**). The latter are routinely used in 1,3-dipolar cycloaddition chemistry with dipolarophiles, which can include alpha-beta unsaturated carboxylic acids (30). Cycloaddition reactions are rare in biochemistry (31,32), and 1,3-dipolar cycloaddition has only been inferred to

occur by virtue of the structure of complex secondary metabolites (33). However, a 1,3-dipolar cycloadduct formed between the prFMN^{iminium} and the alpha-beta unsaturated carboxylic acids (giving rise to a pyrrolidine ring; cycloaddition step in Fig 5) can explain how Fdc1 catalyses decarboxylation: the pyrrolidine ring adduct can readily undergo decarboxylation as this can occur concomitant with ring opening and formal reduction of the isoalloxazine ring system (decarboxylation step, **Fig 5**). Similar to the PLP- or TPP-dependent decarboxylases (4,5), the covalent substrate-cofactor adduct formation allows delocalization of the negative charge as a consequence of heterolytic C-C bond breakage onto the aromatic moiety of the cofactor (**Fig 1b**). Crucially, this gives rise to an intermediate that consists of a C1'-alpha carbon adduct between prFMN^{iminium} and the substrate. In the case of the phenylpyruvate inhibitor, it is proposed that the tautomeric enol form (*i.e.* alpha-hydroxycinnamic acid) functions as the actual substrate, forming an intermediate adduct that, following decarboxylation, can undergo enol-keto tautomerisation, leading to a relatively stable phenylacetaldehyde adduct and enzyme inhibition (22).

No such tautomerisation can occur for standard substrates such as cinnamic acids, and it is proposed that as a first step to resolving the C1'-C α decarboxylation adduct, proton addition from the conserved Glu282 concomitant with reformation of the pyrrolidine ring occurs (CO₂/E282 exchange, **Fig 5**). In the substrate free enzyme, the side chain of Glu282 can be observed to occupy a position similar to that of the substrate carboxylic acid group and, following release of CO₂, it is proposed the C4 α -beta carbon bond is reformed with formal oxidation of the prFMN^{iminium} isoalloxazine ring system and protonation at the substrate alpha position. A *retro* 1,3-dipolar cycloaddition step (or cyclo-elimination; **Fig 5**) from this decarboxylated pyrrolidine adduct is then proposed to result in the release of the styrene product from the prFMN^{iminium} cofactor.

This is the first report of an enzyme capable of utilizing a 1,3-dipolar cycloaddition chemistry (33). There are some enzymes that are proposed to perform cyclization of the substrate via a [4+2] cycloaddition (Diels-Alder) reaction (31,34,35) or through a 1,3-dipolar cycloaddition reaction (36,37).

However, these reactions are all intramolecular and essentially irreversible cycloadditions. In contrast, the proposed Fdc1 mechanism uses a cycloadduct as a transient intermediate, making use of reversible 1,3-dipolar cycloaddition. Crucially, this mechanism explains the observation that Fdc1 can catalyse the reverse carboxylation reaction (at higher [CO₂]), and the obligate need of the UbiD-family for alpha-beta unsaturated carboxylic acids as substrate. It also suggests there are no strict requirements for the nature of the gamma position, and slow decarboxylation of short-chain alpha-beta unsaturated carboxylic acids by the *A. niger holo*-Fdc1 further supports this (22).

Recent solution studies on the yeast Fdc1 enzyme indicated that proton transfer to the product occurs before the rate-determining step (38). A linear free-energy analysis of the reaction using *para*- and *meta*-substituted phenylacrylic acids revealed a negative ρ value, consistent with the rate-determining step being the formation of styrene from the prenylated flavin-product adduct through a cyclo-elimination reaction. Computational studies have provided further insights into the Fdc1 mechanism. Density functional theory (DFT) calculations supported the proposed 1,3-dipolar cycloaddition mechanism and the role of Glu282 as a proton donor (39). Other researchers have used a combined quantum mechanics/molecular mechanics (QM/MM) approach to investigate the Fdc1 mechanism (40). Their calculations indicate that the prFMN^{iminium} is the catalytically relevant species and that, after the 1,3-dipolar cycloaddition, the carboxylic group can readily split off from the prFMN^{iminium}-substrate adduct. According to the energy barrier analysis, the protonation step is rate-limiting.

Unlike many of the proteins in the UbiD superfamily, Fdc1 acts on substrates where the acid is not directly attached to an aromatic ring system, (de)carboxylating unsaturated aliphatic acids instead. Fdc1 was first discovered in spoilage yeasts and fungi (10,11,41,42) capable of decarboxylating the food preservatives sorbic acid and cinnamic acid. The corresponding products have a distinctive aromas of kerosene or burnt plastic and are often associated with food and drinks having an unusual and undesirable flavor (11). The exception occurs for some beer brewing processes, especially from Germany, where a

smoked flavor is selected for by using fermentation yeasts which possess *pad1* and *fdc1* genes capable of producing 4-vinylguaiacol from ferulic acid (43).

The only other members of the UbiD-family known to be capable of performing aliphatic acid decarboxylation are a poorly characterised group of enzymes involved in polyketide synthesis in *Streptomyces* species (44,45), whereas all other members of the family reported to date act on substrates where the acid group is bound directly to an aromatic ring. While the proposed reversible cycloaddition to aliphatic acids is considered plausible, a corresponding reaction with an aromatic substrate presents the issue of transient dearomatisation. It is therefore unknown to what extent the properties and mechanism of the fungal Fdc1 can be applied to other UbiD-family members.

Structure and mechanism of the canonical UbiD enzyme

The UbiD-family was first identified and named after the *E. coli* UbiD protein, which is proposed to decarboxylate a prenylated hydroxybenzoic acid in the biosynthesis of ubiquinone (20,46). Prokaryotic ubiquinone biosynthesis is proposed to start with the production of 4-hydroxybenzoic acid from chorismate catalyzed by UbiC, a chorismate pyruvate lyase (47-49). A membrane embedded prenyltransferase, UbiA, then uses 4-hydroxybenzoic acid as a substrate and prenylates the substrate C3 resulting in a polyprenyl attachment (50,51). The length of the isoprenoid tail is dependent upon the species being studied: *E. coli* contains an octaprenylated ubiquinone, whereas other organisms contain ubiquinone with isoprenoid tails of 5-10 units.

UbiD (and UbiX) are suggested to catalyse the third in a series of nine reactions resulting in the production of the electron carrier ubiquinone (52,53). In prokaryotes, UbiD is responsible for the decarboxylation to 2-polyprenylphenol (20,46), which is then subjected to hydroxylation and methylation modifications (53). Surprisingly, UbiD (and UbiX) homologues have not been implicated in eukaryotic ubiquinone biosynthesis, despite the fact many of the other enzymes involved are homologous to corresponding prokaryotic proteins. It should also be noted that UbiD homologues have also been determined to be active in

cyanobacterial plastoquinone biosynthesis pathways (54), and are proposed to participate in an alternative menaquinone biosynthetic pathway (55).

Recent studies confirm *E. coli* UbiD also binds prFMN, although active *holo*-enzyme could not be obtained despite co-expression with UbiX (56). In this case, reconstitution experiments revealed oxidative maturation of the bound prFMN by UbiD did not proceed to the prFMN^{iminium} but stalls at the purple prFMN^{semiquinone} form. This species was previously observed as an oxidation product of the UbiX-prFMN^{reduced} product complex (26). Further oxidative maturation *in vitro* was observed at an unphysiological alkaline pH, indicating that a proton-coupled electron transfer might precede formation of the fully oxidized prFMN. It is unclear why *E. coli* UbiD cannot readily be converted to the active *holo*-form. Structure determination of the hexameric prFMN-UbiD complex reveals many similarities with the dimeric prFMN-Fdc1 structures, but also reveal a range of differences (**Fig 6**). The structure of the metal ions binding site associated with prFMN binding is subtly different, while the active site is much more solvent accessible compared to Fdc1. It is possible that occlusion from solvent occurs through domain motion by the prFMN binding domain. The latter might be linked to the need to catalyse 1,3-dipolar cycloaddition with an aromatic substrate, but that has yet to be established.

In the absence of detailed studies on structure and mechanism of a range of UbiD-family members, it remains unclear to what extent variation occurs in prFMN binding and maturation, domain mobility, and enzyme mechanism of the UbiD enzyme family. Significant variation does occur in the substrate specificity of the individual branches of the UbiD-family tree (**Fig 7**). In addition, previous studies have indicated certain UbiD-enzymes form hetero-oligomers, either with other UbiD-like or distinct subunits. Finally, some UbiD-enzymes also appear to serve as carboxylases *in vivo*.

An overview of the wider UbiD-family

Protocatechuic acid/Gallic acid decarboxylases

There have been multiple reports of reversible protocatechuic acid decarboxylases *in vivo* (57-59). The substrate range of these enzymes have been

reported to be narrow, with only protocatechuic acid and gallic acid being accepted as substrates. Protocatechuic acid decarboxylase is an important enzyme in directing abundant aromatic compounds from lignin into the TCA cycle. Gallic acid decarboxylases have been reported in some detail less recently, and it is unclear whether older reports of gallic acid decarboxylase correspond to members of the UbiD family due to a lack of sequencing data. However, reports of hexameric proteins of a similar molecular weight as protocatechuic acid decarboxylases are suggestive these too are UbiD-like proteins (60). In this case, iron was identified to be required, contrary to reports for other UbiD proteins demonstrating requirement for manganese. More recent reports of a gallic acid decarboxylase from *Lactobacillus plantarum* reveal a UbiD-like protein involved in decarboxylation (LpdC), and a small protein, LpdD, also implicated in decarboxylase activity (61,62). LpdC is 55% homologous to proteins annotated as protocatechuic acid decarboxylase, and capable of decarboxylating protocatechuic acid. *LpdD* is co-transcribed with the *ubiX*-homologue *lpdB*, indicating that LpdD might assist in either decarboxylation and/or cofactor production, although the gene has been shown to be non-essential for decarboxylase activity (62).

Vanillic acid decarboxylases

The vanillic acid decarboxylase (Vdc), also known as the 4-hydroxybenzoic acid decarboxylase (4-HBDC) family, has been shown to be able to decarboxylate 4-hydroxybenzoic acid as well as a range of benzoic acids possessing a *para*-hydroxyl group, including vanillic acid and protocatechuic acid (63-66). In the presence of high [CO₂] these enzymes are also capable of carboxylating phenol and guaiacol to their respective acids. One notable distinction between vanillic acid decarboxylases compared to other UbiD-like enzymes is that they not only require UbiD and UbiX homologues, but they also strictly require a third protein for activity, termed VdcD. The Vdc gene cluster is often composed of three genes, where *vdcb* equates to *ubiX*, *vdcc* corresponds to *ubiD*, and *vdcd* is a small gene encoding a protein with a molecular weight of ~ 10 kDa. While VdcD shares no discernable homology with any proteins of known function it has been reported to be essential for decarboxylation(12,64). The gene cluster often takes the form

of *vdcBCD*, however in some organisms the gene order may be altered (e.g. *vdcCDB* in *S. hydroxybenzoicus*), whilst in other cases the *ubiX* homologue *vdcB* is located elsewhere in the genome(12).

Hetero-aromatic acid decarboxylases

Multiple heteroaromatic acids have been reported to be decarboxylated by UbiD enzymes, including furan, pyrrole and indole aromatic compounds.

2,5-furandicarboxylic acid (FDCA) decarboxylase, denoted as HmfF, was first identified from it's involvement in furfural and 5-hydroxymethylfurfural (HMF) catabolism in *Cupriavidus basilensis* HMF14 (67). Fufural and HMF are toxic furanic aldehydes generated during the thermoacidic pretreatment of lignocellulosic feed stocks that have been shown to be inhibitory to fermentive organisms. In *C. basilensis* HMF14, furfural is oxidized to furoic acid which is then ligated to coenzyme A, before undergoing hydroxylation, dearamotisation and ring opening to produce 2-oxogluteroyl-CoA which is fed into the TCA cycle. In the case of HMF, the hydroxyl and aldehyde groups undergo successive oxidations to produce FDCA which is then decarboxylated to produce furoic acid which feeds in to the main furfural degradation pathway. Gene knockouts showed that the decarboxylation of FDCA was dependent on two genes termed HmfF and HmfG which are homologues of UbiD and UbiX respectively, however at the time of writing no biochemical characterisation of these genes had been reported.

Reversible decarboxylases have also been reported that act on nitrogen containing heteroaromatics including pyrrole-2-carboxylate (P2C) decarboxylase from *Bacillus megaterium* PYR2910 (68) and indole-3-carboxylate from *Arthrobacter nicotianae* FI1612 (69). As well as decarboxylation both enzymes have been shown to catalyse carboxylation in the presence of high [CO₂] supplied as bicarbonate, or in the case of pyrrole-2-carboxylate decarboxylase the enzyme can also utilize CO₂ at supercritical concentrations (70). Pyrrole-2-carboxylate decarboxylase has been reported to have a subunit mass of 52 kDa (68), which is within the typical mass range expected of a UbiD-like enzyme, whilst indole-3-carboxylate decarboxylase has been shown to be

stimulated by divalent metal ions, including Mn^{2+} , which would be consistent with the role of such ions in anchoring the prFMN phosphate tail (69). It has been reported that these enzymes share homology with the UbiD family (71), however pyrrole-2-carboxylate decarboxylase activity was also shown to be dependent on the presence of organic acids leading to a mechanistic proposal based on acid-base catalysis using such acids. It is difficult to correlate such a mechanism with a role for prFMN.

Phthalate decarboxylases

Phthalate (1,2-dicarboxybenzoic acid) decarboxylases belonging to the UbiD family have been recently reported in obligate anaerobes (72,73). These organisms use succinyl-CoA-dependent succinyl-CoA:*o*-phthalate-CoA transferase (73) to create phthaloyl-CoA, that in turn is then the substrate for decarboxylation to benzoyl-CoA. The latter which can be reduced to dienoyl-CoA as part of the catabolic degradation pathway (74). Three species *Aromatoleum aromaticum* EbN1, *Azoarcus evansii* KB740 and *Thauera chlorobenzoica* 3CB1 displayed a prominent ~60 kDa band on a SDS-PAGE gel when grown on phthalate as opposed to benzoate which, following tryptic digest and mass spectrometric analysis was confirmed to be a UbiD-like enzyme. Analysis of the genome surround these genes showed that they were not only flanked by CoA transferase genes required for formation of phthaloyl-CoA, but also by a *ubiX* homologue. Neither isophthalate (1,3-dicarboxybenzoic acid) nor terephthalate (1,4-dicarboxybenzoic acid) were substrates for phthalate-CoA decarboxylase (72). UbiD-like terephthalate decarboxylases have been predicted to exist based upon homology and the ability of *Pelotomaculum* to utilize terephthalate (75), however no experimental evidence has been reported to confirm this.

Anaerobic Aromatic Carboxylases

Aerobic degradation of aromatic compounds is largely dependent on activation of the target compound by O_2 -dependent oxygenases. Such reactions are not usually viable under anaerobic conditions leading to a requirement for alternative methods of activating these compounds. In recent years carboxylation has emerged as an important initial reaction for a number of these

compounds (recently reviewed here(76)) with UbiD-like enzymes implicated in these processes.

Phenylphosphate carboxylase

Anaerobic organisms capable of growing on phenol have been shown to contain UbiD-like genes involved in carboxylation. Prior to carboxylation, phenol is phosphorylated with the concomitant consumption of ATP (77). The requirement of phenol phosphorylation is proposed to ensure carboxylation is energetically more favorable through the further generation of a phenolate ion (78).

The active *Thauera aromatica* phenylphosphate carboxylase is proposed to be a heterocomplex composed of two UbiD-like (α and β) subunits, a small γ subunit and a δ subunit involved in phosphatase activity (79,80). The latter has been shown to dissociate from the complex during purification, however net carboxylation activity could only be detected once the δ subunit had been added back to the core $\alpha\beta\gamma$ complex (79). Phenylphosphate carboxylation is proposed to require Mn^{2+} , analogous to Fdc1 and UbiD (78). The mass of the $\alpha\beta\gamma$ core complex was indicated to be ~360 kDa by size exclusion, but the stoichiometry of the individual components is unknown. The requirement for a small subunit (with an unknown function) does seem analogous to the Vdc family (79), however, the γ subunit has no homology to either VdcD or LpdD. In addition to the α and β subunits, there is a third UbiD homologue (termed orf7) located immediately down stream from the γ subunit, followed by orf8 that encodes a UbiX homologue. In light of recent insights, the role of the orf 8 UbiX homologue is likely to be prFMN biosynthesis, but the role of orf 7 remains unclear. Why two distinct UbiD-like subunits are required in the phenylphosphate carboxylase complex, and what their respective stoichiometries are remains enigmatic.

Benzene and naphthalene carboxylases

There have been reports of organisms capable of carboxylation of benzene and naphthalene (81). This is a very challenging reaction due to the stability of the aromatic ring(s), and the lack of functional groups to activate the aromatic group.

The utilization of benzene for growth in anaerobic iron or nitrate reducing conditions can proceed through a direct carboxylation reaction utilizing CO₂ and a UbiD-family protein (82,83). Naphthalene carboxylase was identified in a sulfate reducing bacterial culture (84,85). Although some anaerobic bacteria are thought to degrade naphthalene through an initial methylation step followed by oxidations (86), it has been discovered that some directly carboxylate the polyaromatic ring of naphthalene, with UbiD-like genes implicated in this process. In the case of a benzene degrading enrichment culture an apparent single band at ~60 kDa could be observed in cells grown on benzene, but not phenol or benzoate. Edman degradation matched this band to the gene product of orf138, that encodes a protein sharing 44% identity with the phenylphosphate carboxylase α -subunit. A protein with homology to the phenylphosphate carboxylase α -subunit was also shown to be upregulated upon growth on naphthalene by the sulfate-reducing enrichment culture N47. However, genes for other UbiD-like homologues were also reported to be upregulated and, in common with the phenylphosphate carboxylase operon, multiple UbiD-like genes were found in close proximity to the proposed naphthalene carboxylase α -subunit (87). *In vitro* data has not been obtained for purified aromatic carboxylases, and the exact composition of active enzyme is not known.

Outstanding questions and prospects in UbiX-UbiD biochemistry

The UbiX-UbiD system is wide spread in bacteria, archaea and fungi, with considerable variety in terms of substrate specificity, physiological role and subunit organization. To what extent this reflects variation in structure and mechanism within the UbiX and UbiD-families is as yet difficult to assess. The substrate for the UbiX reaction dimethylallylmonophosphate has not been previously reported, and it remains unclear whether it is derived from the commonly occurring dimethylallyldiphosphate. The UbiX prenylated FMN product requires oxidation prior to achieving the required azomethine ylide character necessary to support the reversible 1,3-dipolar cycloaddition reaction. It is not clear how this occurs in Fdc, nor how anaerobic organisms might achieve this transformation in the absence of molecular oxygen. Further elucidation of the maturation mechanism for the aliphatic producing Fdc1

enzyme as well as other UbiD enzymes, and the role of both active site and the cofactor in various aspects is under active investigation in our group. The aromatic substrate requiring UbiD enzyme can, in principle, utilize a similar reaction mechanism. This however would require transient dearomatization, likely presenting a considerable energetic barrier. To investigate this, model systems representing various members of the UbiD family tree will need to become available. Initial studies with the *E. coli* UbiD enzyme have revealed that enzyme maturation/activation can be difficult to achieve, and it is tempting to speculate that the role of smaller subunits associated with UbiD might be to assist with this process. While the UbiD catalyzed reaction can be run in reverse under high [CO₂], it is unclear how substrate dephosphorylation (or other exothermic processes) might be coupled to carboxylation under ambient conditions. Both decarboxylation and carboxylation reactions catalyzed by UbiD family members have potential biotechnological application: the enzyme family offers the possibility of interconversion between alkenes/aromatic compounds and the corresponding alpha-beta unsaturated acid/aromatic acids depending on [CO₂] without the need for any addition reagents. To what extent UbiD and/or the associated prFMN cofactor are stable under *in vitro* or *in vivo* conditions will need further investigation. Rapid inactivation under aerobic conditions has been reported for some UbiD members, while the prFMN cofactor has been shown to exist in a wide range of distinct forms, many not associated with activity. Finally, it remains intriguing to speculate whether other prFMN-dependent enzymes might exist. A wide range of flavin binding folds have evolved in Nature, one of which has been recruited to serve as the basis for the UbiD-scaffold. It seems plausible to assume this event might have occurred more than once, especially in view of the rich chemistry the modified prFMN cofactor might afford (**Fig 8**). Unfortunately, no sequence motif is associated with prFMN binding *per se*, making the search for novel non-UbiD prFMN-dependent enzymes a more challenging task.

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Figure legends

Fig 1. Biochemistry of decarboxylation reactions. A. A schematic representation of a generic decarboxylation reaction, illustrating the distinct outcomes of C-C bond breakage concomitant with decarboxylation depending on the nature of the molecule. The high energy associated with formation of an unstabilized carbanion species (indicated in square brackets) hinders decarboxylation. In contrast, the presence of an electrophilic group (indicated by magenta coloured E) allows spontaneous and fast decarboxylation. Biological catalysis often operates by forming an adduct between substrate and a cofactor that fulfills the role of E. B) Selected examples of cofactor-dependent decarboxylation. In each case a cofactor-adduct is formed prior to decarboxylation, which imposes restrictions on the substrate nature (as indicated by the green region). Consequent decarboxylation occurs by (transient) reduction of the cofactor moiety (as indicated by the magenta region). In contrast, panel C shows the reaction for phenolic acid decarboxylases, which use acid-base chemistry to guide a substrate-assisted mechanism. The prFMN-dependent reaction that is the subject of this review is shown with a blue background.

Fig 2. The UbiX/UbiD and Pad1/Fdc1 reactions. The non-oxidative (de)carboxylation reactions catalysed by the UbiX/UbiD and Pad/Fdc systems are depicted.

Fig 3. The UbiX-UbiD/Fdc1 system. Biosynthesis and maturation of the prFMN cofactor is shown, with details of the respective UbiX (lower boxed region) and Fdc (upper boxed region) protein structures and active site regions involved shown. In the upper box region, representing Fdc and the oxidative maturation of the prFMN cofactor, the Fdc dimer is shown in cartoon with a detailed view of the active site region. Each monomer is coloured by domain, with the prFMN binding domain coloured in deep blue, the oligomerisation domain in cyan, and

the connecting helix in magenta. The C-terminal helix that binds to the prFMN domain of the opposite monomer is shown in red. In the low boxed region, representing UbiX structure and the UbiX catalyzed reaction, the three subunits of the UbiX dodecamer are shown in cartoon, the remainder as a grey solvent accessible surface. The active site is located at the interface between three subunits, and a detailed view of the active site is shown with amino acids coloured according to individual monomer. The colour change observed upon reduction and consequent oxidation of the UbiX:FMN:DMAP complex is shown as an inset.

Fig 4. The UbiX mechanism. Proposed mechanism for UbiX, with the prFMN/DMAP substrates of key UbiX crystal structures underpinning this proposal shown.

Fig 5. A 1,3-dipolar cycloaddition supports decarboxylation in Fdc1. Proposed mechanism for Fdc, with active site regions of key crystal structures of the Fdc substrate and inhibitor complexes that underpinning this proposal shown. The reaction is shown in the decarboxylation direction, but can also occur in the reverse direction as demonstrated by carboxylation under increased [CO₂] conditions.

Fig 6. Structural insights into UbiD. **A)** Colour coded solvent accessible surface of the *E. coli* UbiD hexamer (PDB code 5M1D): prFMN binding domain in blue, oligomerisation domain in cyan, the linker alpha-helix in magenta and the C-terminal alpha-helic in red. **B)** Putative domain motion leads to active site closure. The prFMN binding domain is observed in distinct positions with respect to the oligomerisation domain (see panel C for details), the hinge motion observed (hinge regions indicated by a yellow asterix) has the potential to occlude the prFMN (in green spheres) from solvent. **C)** Overlay of two distinct UbiD monomers, revealing a hinge motion occurs at the connecting alpha helix (in magenta) linking the prFMN domain (in blue) and the oligomerisation domain (in cyan). The direct link to catalysis has not been established, but it is interesting to note the active holo-Fdc1 enzyme is in a closed conformation whereas the inactive holo-UbiD enzyme is found in a range of open

conformations. **D)** Detailed view of the UbiD prFMN binding region. The orientation is similar to that of the Fdc1 prFMN binding region in Fig 3.

Fig 7. Overview of the UbiD enzyme family. Phylogenetic tree of the characterised UbiD homologues. *A. niger* Fdc1 (XP_001390534)(10), *S. cerevisiae* Fdc1 (NP_010828)(11), *C. dubliniensis* Fdc1 (XP_002421128)(22), *E. coli* UbiD (ACT45527)(88), *Synechocystis* sp. PCC 6803 4-Hydroxy-3-solaneylsbenzoate decarboxylase (NP_440197)(54), *P. aeruginosa* PA0254/HudA (WP_003115722)(18), *S. griseochromogenes* TtnD (ABV91289)(45), *S. himastatinicus* SmdK (WP_009713959)(44), *C. basilensis* HmfF (ADE20406)(67), *B. subtilis* VdcC (WP_003246683)(89), *M. thermoacetica* VdcC (WP_011391924)(90), *E. coli* O157 VdcC (WP_000863205)(12), *T. aromatica* phenylphosphate carboxylase alpha (CAC12690) and beta (CAC12688) subunits (79), *Clostridium* BF benzene carboxylase (ADJ94002)(82), *K. pneumoniae* AroY (WP_004224180)(91), *Lactobacillus plantarum* 3,4-dihydroxybenzoate decarboxylase LpdC (WP_003644796)(61) and *A. aromaticum* phthalyl-CoA decarboxylase (WP_011255033)(72). Different branches can be grouped by substrate specificity.

Fig 8. The prFMN chemical repertoire. An overview of the interconversion between the distinct prFMN species (DMAP derived carbon atoms in red). Similar to the extensive flavin chemical repertoire, each of the distinct prFMN states has the potential to contribute to a range of reactions. This suggests distinct prFMN dependent enzymes could exist.

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Fig 1. A

Fig 1. B

b) cofactor dependent decarboxylation

c) substrate assisted decarboxylation
p-coumaric acid

Fig 2

Fig 3.

Fig 4.

Fig 5.

Fig 6.

Fig 7.

Fig 8.