Oxidative Maturation and Structural Characterization of Prenylated FMN Binding by UbiD, a Decarboxylase Involved in Bacterial Ubiquinone Biosynthesis*

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The activity of the reversible decarboxylase enzyme Fdc1 is dependent on prenylated FMN (prFMN), a recently discovered cofactor. The oxidized prFMN supports a 1,3-dipolar cycloaddition mechanism that underpins reversible decarboxylation. Fdc1 is a distinct member of the UbiD family of enzymes, with the canonical UbiD catalyzing the (de)carboxylation of para-hydroxybenzoic acid-type substrates. Here we show that the Escherichia coli UbiD enzyme, which is implicated in ubiquinone biosynthesis, cannot be isolated in an active holoenzyme form despite the fact active holoFdc1 is readily obtained. Formation of holoUbiD requires reconstitution in vitro of the apoUbiD with reduced prFMN. Furthermore, although the Fdc1 apoenzyme can be readily reconstituted and activated, in vitro oxidation to the mature prFMN cofactor stalls at formation of a radical prFMN species in holoUbiD. Further oxidative maturation in vitro occurs only at alkaline pH, suggesting a proton-coupled electron transfer precedes formation of the fully oxidized prFMN. Crystal structures of holoUbiD reveal a relatively open active site potentially occluded from solvent through domain motion. The presence of a prFMN sulfite-adduct in one of the UbiD crystal structures confirms oxidative maturation does occur at ambient pH on a slow time scale. Activity could not be detected for a range of putative para-hydroxybenzoic acid substrates tested. However, the lack of an obvious hydrophobic binding pocket for the octaprenyl tail of the proposed ubiquinone precursor substrate does suggest UbiD might act on a non-prenylated precursor. Our data reveals an unexpected variation occurs in domain mobility, prFMN binding, and maturation by the UbiD enzyme family.

The decarboxylation of organic acids is a common reaction in biochemistry, and a wide range of enzymes has evolved to catalyze this reaction (1, 2). Several decarboxylases make use of cofactors to achieve catalysis either by stabilizing the developing negative charge on the non-carboxyl substrate moiety (3), by coupling decarboxylation to substrate oxidation (4), or by providing a transient electron sink (5). The widespread UbiD family of enzymes catalyzes the reversible decarboxylation of α,β-unsaturated acids, a chemical feat that is accomplished by the use of the recently discovered cofactor prenylated FMN (prFMN) (6). The latter is synthesized from FMNH2 and dimethylallyl monophosphate (DMAP) by the non-canonical terpene cyclase UbiX, producing a highly modified FMN with linkages via the N5 and C6 positions to the DMAP dimethylallyl moiety (7). This effectively creates an additional non-aromatic ring to the FMN isoalloxazine ring system. In the case of the fungal enzyme Fdc1 (a member of the UbiD family), the N5-allyl linkage from the UbiX prFMN product is oxidized to the corresponding N5═C iminium species, generating an azomethine ylide species required for activity (Fig. 1A). The exact mechanism of Fdc1-mediated prFMN oxidation is unclear, but it occurs readily in the presence of oxygen, suggesting a stepwise radical process (6, 7). The fully oxidized prFMN has azomethine ylide character, and azomethine ylides are frequently used in organic chemistry in 1,3 dipolar cycloaddition reactions with dipolarophiles, generating a pyrrolidine ring (8). It is proposed that Fdc1 and by extension other UbiD enzymes make use of a 1,3 dipolar cycloaddition reaction between prFMN and the α, β-unsaturated acid substrate to generate a transient pyrrolidine substrate cofactor adduct (Fig. 1B, species 6) (6). The latter can readily undergo decarboxylation concomitant with pyrrolidine ring opening. Indirect evidence for this mechanism has been reported for the fungal decarboxylase Fdc1, which catalyzes the decarboxylation of cinnamic acid-type substrates (6, 9). However, Fdc1 belongs to a distinct branch of the UbiD family of enzymes (sequence identity between Escherichia coli UbiD and Aspergillus niger Fdc1 is

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4 The abbreviations used are: prFMN, prenylated FMN; PHB, 4-hydroxybenzoic acid; DMAP, dimethylallyl monophosphate; ENDOR, electron nuclear double resonance; SOMO, singly occupied molecular orbital; 1,3-Bis-Tris propane, bis[tris(hydroxymethyl)methylamino]propane.

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known about UbiD cofactor binding, maturation, or activity have been postulated to occur in the UbiD enzymes (17), little is for Ubi enzymes have been reported, including UbiC, UbiA, and UbiX (7, 15, 16). decarboxylation, and studies have shown that either aromatic ring modifications, catalyzed by UbiD-mediated (PHB) (13, 14). PHB is thought to be prenylated before further solubility (11, 12). The pathway is proposed to start from cho-

Identification of putative intermediates in the Q biosynthetic pathway has made use of the fact that prenylation confers lipid mobility, prFMN binding, and maturation by the UbiD enzyme despite the available structure of the apoform of the E. coli UbiD (PDB code 2IDB).

Here we present a method for in vitro reconstitution of UbiD and present crystal structures of UbiD in complex with prFMN. A comparison of apoUbiD and holoUbiD (we refer to holoUbiD as UbiD in complex with prFMN, which has several redox states) structures combined with ligand binding data suggests an Mn\(^{2+}\)-dependent cofactor binding mechanism, similar to what is observed in Fdc1. However, surprisingly, in vitro oxidative maturation of the cofactor stalls at a radical prFMN intermediate (Fig. 1A, species 2) at ambient pH. Crystal structures also reveal domain motion by the prFMN binding domain occurs in UbiD, previously not observed in Fdc1. Hence, our data reveal that an unexpected variation occurs in domain mobility, prFMN binding, and maturation by the UbiD enzyme family.

Results

Overexpression and FMN Binding by ApoUbiD—A method used for expression of holoFdc1 in E. coli was used to express ubiD (6). Despite several attempts to purify holoUbiD, purified UbiD fractions consistently lacked any of the spectral features consistent with prFMN binding as observed for Fdc1. Despite expression of various tagged forms as well as the untagged ubiD, only apoUbiD could be purified. This either suggests a very labile nature of the UbiD-prFMN complex or the inability to form this complex in vivo. Although prFMN could not be observed for the purified UbiD fractions, we could establish UbiD readily binds FMN in the presence of Mn\(^{2+}\) but not Mg\(^{2+}\). This is reminiscent of the Mn\(^{2+}\)- and K\(^{+}\)-dependent binding of prFMN by Fdc1 (6), although the presence of K\(^{+}\)
Cofactor Binding in UbiD

A

B

FIGURE 3. UbiD reconstitution with prFMN. A, UV-visible spectra of apoUbiD after incubation with UbiX generated reduced prFMN in the presence of Mn2+ and K+ and a desalting step to remove non-specifically bound prFMN. After exposure to oxygen, a purple-colored species rapidly developed that remained stable over a prolonged period of time. Spectra in red are the corresponding UV-visible spectra of Fdc1 as reported in Payne et al. (6). B, UV-visible spectra of radical prFMN-UbiD and prFMN-UbiX samples prepared for ENDOR spectroscopy. HprFMN denotes protiated prFMN, and DprFMN denotes prFMN deuterated at the C1 position.

does not appear essential for FMN binding in UbiD (Fig. 2A). We determined the FMN dissociation constant in the presence of various cations using tryptophan fluorescence quenching (Fig. 2B). No Trp fluorescence quenching was observed when titrating UbiD with FMN in the presence of Mg2+. In contrast, binding is clearly observed using tryptophan fluorescence quenching for titrations with FMN in the presence of Mn2+. The presence of K+ did not seem to affect binding, with a $K_d$ of 1.33 μM ± 0.09 μM observed in the presence of Mn2+ only and $K_d$ 1.77 μM ± 0.13 μM in presence of Mn2+ and K+ (Fig. 2B).

Anaerobic Reconstitution of UbiD—Given the fact that we could observe formation of the UbiD-FMN complex in the presence of Mn2+, we set out to reconstitute apoUbiD with prFMN. To prepare prFMN, Pseudomonas aeruginosa UbiX was purified and mixed with FMNH2 and DMAP under anaerobic conditions. FMNH2 was obtained either through a chemical reduction with sodium dithionite or through enzymatic reduction using E. coli Fre and NADH. The use of Fre reductase utilizing NADH for the reduction of FMN can be justified by the gene organization in E. coli, where the fre gene utilized is located adjacent to ubiD. After incubation and removal of the UbiX enzyme, apoUbiD and MnCl2 were added to the reaction mixture under anaerobic conditions. After desalting, the UbiD fraction displayed clear spectral features with a main feature centered at 425 nm likely associated with reduced prFMN binding (Fig. 3).

Oxidative Maturation of the Reconstituted UbiD-reduced prFMN Complex—The reconstituted UbiD-reduced prFMN complex was exposed to oxygen, leading to the rapid formation of a purple-colored species. The UV-visible spectrum of the latter reveals features at 357 and 541 nm (Fig. 3), and these closely resembled those observed for the prenyl transferase UbiX in complex with a radical prFMN. In that case, exposure of the UbiX-reduced prFMN product complex to oxygen leads to formation of a semiquinone-like prFMN radical (7). Previous reconstitution experiments with the fungal Fdc1 did not reveal ready accumulation of a radical species, although the oxidative maturation process was proposed to proceed via such intermediates. In fact, inactivation of holoFdc1 with the mild reductant NaBH4CN under aerobic conditions does lead to formation of a purple species (6, Fig. 3A). Crucially, the latter was readily further oxidized to the active form upon desalting, leading to Fdc1 activation (6).

EPR and ENDOR Spectroscopic Characterization of the UbiD-Radical prFMN Complex—To confirm the exact nature of the radical prFMN species that accumulates during oxidation of the UbiD-reduced prFMN complex, we performed EPR and ENDOR spectroscopic studies on both the UbiD-radical prFMN complex and a deuterated sample obtained by reconstitution with C1-deuterated DMAP, leading to prFMN deuterated at the N5-C1’ position (DprFMN). To allow for a comparative analysis, the same experiments were also performed on the UbiX-radical prFMN complex initially characterized using EPR/ENDOR in White et al. (7). The EPR spectrum of the UbiX-radical prFMN complex (Fig. 4A) exhibits an isotropic spectrum, as is typical for many radicals in frozen solution, with $g = 2.0033$, some partially resolved hyperfine coupling, and a peak-to-trough line width of 36.1 G. The use of DMAP deuterated at C1 in the production of the UbiX-radical prFMN complex produces an EPR spectrum (Fig. 4B) lacking partially resolved hyperfine coupling and with the much reduced line width of 16.1 G. This suggests that hyperfine coupling to the C1-protons of prFMN is a significant determinant of the line width of the EPR spectrum of the UbiX-radical prFMN complex and the source of the partially resolved hyperfine coupling. The Davies ENDOR spectrum of the UbiX-radical prFMN complex (Fig. 5, A and C) reveals hyperfine coupling to two protons that are lost when the radical is formed using C1-deuterated DMAP, leading to the N5-C1’ position (DprFMN). To allow for a comparative analysis, the same experiments were also performed on the UbiX-radical prFMN complex initially characterized using EPR/ENDOR in White et al. (7). The EPR spectrum of the UbiX-radical prFMN complex (Fig. 4A) exhibits an isotropic spectrum, as is typical for many radicals in frozen solution, with $g = 2.0033$, some partially resolved hyperfine coupling, and a peak-to-trough line width of 36.1 G. The use of DMAP deuterated at C1 in the production of the UbiX-radical prFMN complex produces an EPR spectrum (Fig. 4B) lacking partially resolved hyperfine coupling and with the much reduced line width of 16.1 G. This suggests that hyperfine coupling to the C1-protons of prFMN is a significant determinant of the line width of the EPR spectrum of the UbiX-radical prFMN complex and the source of the partially resolved hyperfine coupling. The Davies ENDOR spectrum of the UbiX-radical prFMN complex (Fig. 5, A and C) reveals hyperfine coupling to two protons that are lost when the radical is formed using C1-deuterated DMAP, leading to the N5-C1’ position (DprFMN). To allow for a comparative analysis, the same experiments were also performed on the UbiX-radical prFMN complex initially characterized using EPR/ENDOR in White et al. (7). The EPR spectrum of the UbiX-radical prFMN complex (Fig. 4A) exhibits an isotropic spectrum, as is typical for many radicals in frozen solution, with $g = 2.0033$, some partially resolved hyperfine coupling, and a peak-to-trough line width of 36.1 G. The use of DMAP deuterated at C1 in the production of the UbiX-radical prFMN complex produces an EPR spectrum (Fig. 4B) lacking partially resolved hyperfine coupling and with the much reduced line width of 16.1 G. This suggests that hyperfine coupling to the C1-protons of prFMN is a significant determinant of the line width of the EPR spectrum of the UbiX-radical prFMN complex and the source of the partially resolved hyperfine coupling. The Davies ENDOR spectrum of the UbiX-radical prFMN complex (Fig. 5, A and C) reveals hyperfine coupling to two protons that are lost when the radical is formed using C1-deuterated DMAP, leading to the N5-C1’ position (DprFMN). To allow for a comparative analysis, the same experiments were also performed on the UbiX-radical prFMN complex initially characterized using EPR/ENDOR in White et al. (7). The EPR spectrum of the UbiX-radical prFMN complex (Fig. 4A) exhibits an isotropic spectrum, as is typical for many radicals in frozen solution, with $g = 2.0033$, some partially resolved hyperfine coupling, and a peak-to-trough line width of 36.1 G. The use of DMAP deuterated at C1 in the production of the UbiX-radical prFMN complex produces an EPR spectrum (Fig. 4B) lacking partially resolved hyperfine coupling and with the much reduced line width of 16.1 G. This suggests that hyperfine coupling to the C1-protons of prFMN is a significant determinant of the line width of the EPR spectrum of the UbiX-radical prFMN complex and the source of the partially resolved hyperfine coupling. The Davies ENDOR spectrum of the UbiX-radical prFMN complex (Fig. 5, A and C) reveals hyperfine coupling to two protons that are lost when the radical is formed using C1-deuterated DMAP, leading to the N5-C1’ position (DprFMN). To allow for a comparative analysis, the same experiments were also performed on the UbiX-radical prFMN complex initially characterized using EPR/ENDOR in White et al. (7).
and an unpaired electron spin density at N5 of 0.3, which is typical for the N5 atom of both neutral and anionic flavosemiquinones. The only other resolved hyperfine couplings outside the matrix feature around $g_{9263}$ are assigned to the 8-methyl protons of the UbiX-radical prFMN complex (Fig. 5, A and B). Flavin semiquinone radicals typically exhibit only hyperfine couplings to the 8-methyl and C6 protons in frozen solutions (19) (although coupling to the C1-$H_{1103}$-protons of the ribityl side chain attached at N10 and the N5 proton has been reported for some neutral flavin semiquinones; Ref. (19); however, the C6 proton is not present in prFMN. The 8-methyl proton hyperfine coupling differs between neutral (“blue”) and anionic (“red”) flavin semiquinones, being larger in the latter. The value of 9.81 MHz ($A_{9261}$) exhibited by the UbiX-radical prFMN complex is somewhat intermediate between those reported previously for neutral and anionic flavin semiquinones (19). Thus EPR and ENDOR analysis of the UbiX-radical prFMN complex is completely consistent with a radical in which the unpaired electron is delocalized over the three flavin rings of prFMN. The deuterium hyperfine couplings to the C1’-deuterons in UbiX-radical prFMN formed using C1-deuterated DMAP support this assignment as $A_{9261}$ = 6.4 MHz and $A_{9262}$ = 4.6 MHz. This is as expected given that the ratio of the deuterium-to-proton nuclear gyromagnetic ratio is 0.1535.

The EPR spectrum of the UbiD-radical prFMN complex (Fig. 4C) is also isotropic with $g = 2.0033$ and has a peak-to-trough line width of 37.6 G. However, it lacks the partially resolved hyperfine coupling observed for the equivalent UbiX complex. Such an EPR spectrum was also observed for the NaBH$_4$CN-reduced and subsequently air-oxidized holoFdc1 (6). The use of C1-deuterated DMAP (Fig. 4D) reduced the line width by only 7.4 G to 29.4 G, a reduction of only 8.2 G compared with the 20-G reduction observed for the UbiX-radical prFMN complex on DMAP C1 deuteration. The UbiD-radical prFMN complex is difficult to quantitate accurately because of the influence of the manganese ion discussed below, but double integration of the EPR signal and comparison with radical standards suggests that the radical is formed in at least 60% of prFMN mole-
The ENDOR spectrum of the UbiD-radical prFMN complex (Fig. 5D) revealed a Gaussian line at 33.5 MHz that DMAP C1 deuteration (Fig. 5E) shows arises from a proton with an apparent hyperfine coupling of 37.5 MHz. This is intermediate between the two C1′-proton hyperfine couplings determined for the UbiX-radical prFMN complex. The deuterium hyperfine coupling for the UbiD-radical prFMN complex made using C1-deuterated DMAP is \( A_{\text{iso}} = 5.7 \) MHz. Thus the EPR and ENDOR properties of the UbiD-radical prFMN complex resemble, but are not identical to, those of the UbiX-radical prFMN complex, and some of the behavior of the former are anomalous, particularly the line width change in the EPR spectrum on DMAP C1 deuteration and presence of only one Gaussian line attributable to a C1′ proton in the ENDOR spectrum. These anomalies are explained by the presence of the manganese ion in close proximity to prFMN in UbiD that UbiX lacks. Resolution enhancement of the UbiD-radical prFMN sample clearly fall within the envelope of the three lines arising from hyperfine coupling to the three separate 8-methyl protons in the ENDOR spectrum of UbiD-radical prFMN (Fig. 5C). Iminium formation would also be expected to perturb the SOMO and thus lead to a change in the 8-methyl proton hyperfine coupling in the UbiD-radical prFMN complex when compared with the UbiX equivalent.

Mass spectrometry of the isolated cofactor from both the unlabeled and C1′-deuterated UbiD-radical prFMN complex confirms that the C1′ of prFMN in UbiD has not been deprotonated to the active iminium form of the cofactor (Fig. 6).

Alkaline pH Assists with Further Oxidative Maturation in Vitro—We tested whether redox cycling might allow formation of the prFMN\textsubscript{iminium} species in UbiD by reducing the UbiD-radical prFMN complex with sodium dithionite followed by reoxidation under aerobic conditions. Although sodium dithionite bleaches the UV-visible spectrum of the UbiD-radical prFMN sample, reoxidation leads to formation of the same UbiD-radical prFMN species (Fig. 7A). After the observation that UbiD-radical prFMN could be reduced by sodium dithionite, the redox potential of the cofactor was measured (22). The peak at 546 nm was used to determine the redox state of prFMN and after subtraction of the background at 620 nm was plotted against the potential \textit{versus} normal hydrogen electrode (NHE). The curve was fitted using the Nernst equation for a single electron reduction (22), giving a midpoint potential of \(-240 \text{ mV} \pm 2 \text{ mV}\) for the reduced prFMN-to-radical prFMN transition (Fig. 7B). No further oxidation of the prFMN radical could be observed when under aerobic conditions.

We then sought to test whether further oxidation to the prFMN\textsubscript{iminium} species is blocked by the C1′—H bond breakage. After incubation of the UbiD-radical prFMN at an alkaline pH of 9.2, the UV-visible spectrum changed over the minute time scale (Fig. 7C). After 90 min of incubation at pH 9.2, no further spectral developments are observed. This suggests that at alkaline pH the C1′—H bond can be broken, concomitant with further oxidation. In addition, when incubating the UbiX-radical prFMN under similar alkaline conditions, no significant UV-visible spectral changes were observed, suggesting the changes observed in the UbiD enzyme are specific to this protein (Fig. 7D). Unfortunately, no decarboxylation product could be detected using HPLC when the UbiD-prFMN pH 9.2 sample was incubated with a range of \textit{para}-hydroxybenzoate-like substrates. Activity at pH 7 could not be readily determined, as the
majority of the bound cofactor is lost upon desalting the sample at pH 7.2 (Fig. 7E). Mass spectrometry of the isolated cofactor from the pH 9.2-incubated sample confirmed the presence of a species corresponding to the prFMNiminium species. In addition, a range of other prFMN species was detected, which can be interpreted as corresponding to further oxidation and/or hydrolysis products (Fig. 8).

**Crystal Structures of ApoUbiD**—Crystals of both the N-terminal-tagged UbiD and C-terminal-tagged UbiD could be obtained in distinct space groups. Both structures contain a trimer in the asymmetric unit, and Pisa analysis (23) shows that the likely oligomeric state of UbiD is a hexamer (Fig. 9). This correlates with the observed behavior of UbiD during gel filtration and with the reported hexameric nature for other UbiD homologues (24). Individual UbiD monomers consist of three domains, an N-terminal prFMN binding domain (residues 1–333), a multimerization domain (334–465), and a C-terminal helix that interacts with the prFMN binding domain of an adjacent monomer (466–491). An overlay of the various UbiD monomers from the apoUbiD structures using the prFMN binding domain as reference reveals the relative orientation of the prFMN binding and multimerization domains is variable to an extent (Fig. 10). Both the UbiD trimers (from the various space groups) contain a single monomer that displays a closed configuration (Fig. 10B), likely obtained through a hinge motion at position 333, whereas the other monomers adopt a more open conformation (Fig 10A). As the active site is situated at the domain interface, motion of the prFMN binding domain affects the active site structure. In fact, a comparison with the related Fdc1 structure reveals the prFMN binding domain of the latter positioned closer to the multimerization domain compared with the apoUbiD structures (Fig 10C). As the active site is situated at the domain interface, motion of the prFMN binding domain affects the active site structure.

**Crystal Structures of HoloUbiD and UbiD-FMN Complex**—To gain further insight into the UbiD active site structure and to determine the effects of FMN/prFMN binding on UbiD, we determined the structure of the UbiD-FMN complex as well as the holoUbiD using co-crystallization for the better diffracting N-terminally tagged form. Neither FMN nor prFMN binding significantly affected the position of the prFMN binding domain when compared with the apoUbiD structure. However, a rearrangement of loop 230–248 occurs that allows the binding of FMN/prFMN and the associated metal ion (Fig. 11). In the apo structure, electron density for the 230–248 loop was weak in two of the three subunits, indicative of local disorder. Conversely, in holoUbiD structures, the loop can be clearly defined. Additional electron density for both the prFMN/FMN as well as the associated Mn$^{2+}$ is readily visible, the latter linking the prFMN/FMN phosphate with various conserved amino
acids acting as metal ligands. Unlike the Fdc1 structure, no clear density indicative of K<sup>+</sup> binding can be observed (despite the presence of K<sup>+</sup>), consistent with the fact FMN binding appeared independent of K<sup>+</sup> concentration.

Crystal Structure of a Sulfite holoUbiD Adduct—Although the redox state of the prFMN in the holoUbiD structure cannot be verified from the electron density, crystals appeared colorless, suggesting it is either in the reduced or the fully oxidized state. The fact that reduced prFMN readily oxidizes under aerobic conditions suggests the crystal structures correspond to the mature holoUbiD form. Further evidence to support this interpretation is obtained when using chemically reduced FMNH<sub>2</sub> to prepare the prFMN cofactor for co-crystallization. Unlike the holoUbiD structure containing an unmodified prFMN cofactor (when using Fre reductase to generate prFMN), the prFMN-UbiD complex structure obtained using prFMN in the presence of sodium dithionite contains clear electron density for an adduct to the C1′ of the N5—C1′ bond (Fig. 12). The size and density of this additional feature can be modeled by a sulfite adduct; similar adducts to the isoalloxa-

zine ring have been previously observed in flavoproteins (25). Hence, this suggests nucleophilic attack of the sulfite ion generated from dithionite oxidation occurred to the iminium form of prFMN.

Modeling of the holoUbiD Substrate Complex—We were unable to obtain the structure of a holoUbiD substrate complex despite soaking holoUbiD crystals in with PHB or PHB-like molecules. However, an overlay of the various Fdc1 complexes with holoUbiD suggests a plausible binding mode for the PHB moiety (Fig. 13). Using the position of the cinnamic acid-like substrates in Fdc1 as a guide to place PHB, a model for the holoUbiD-substrate complex can be generated. The latter suggests conserved Arg-180 binds the PHB acid moiety, with the benzyl group stacked above the prFMN ring system. The para-hydroxyl group is in close proximity of Arg-197. In contrast to Fdc1, prFMN adduct formation and decarboxylation in UbiD should lead to an intermediate where the substrate benzoyl moiety is oriented perpendicular to the prFMN plane. Such a drastic reorientation of the substrate aromatic group is not required in Fdc1, as decarboxylation occurs on a non-aromatic

FIGURE 7. The effect of redox cycling and pH on the UbiD-prFMN oxidative maturation. A, UV-visible spectra of the UbiD-radical prFMN complex after reduction with sodium dithionite and subsequent reoxidation. B, redox potentiometry of UbiD-radical prFMN. The absorption at 546 nm at different reduction potentials allows the midpoint potential to be determined with fitting to the Nernst equation. The midpoint potential is 240 mV ± 2 mV. C, UV-visible spectral changes occurring after incubation at pH 9.2 of the UbiD-radical prFMN complex. D, UV-visible spectral changes occurring after incubation at pH 9.2 of the UbiX-radical prFMN complex. E, UV-visible spectrum of the UbiD-radical prFMN complex at pH 7.2 after 90 min of incubation at pH 9.2 and after subsequent desalting.
sp² carbon. The more open active site configuration observed in UbiD and the fact that a relatively bulky sulfite adduct to the C₁/H₁032 position appears readily accommodated in the UbiD active site suggest the 90° reorientation of the benzoyl group is possible. A tentative model of the latter can be made by placing a benzoyl group at the position where C₁/H₁032 adducts are observed (in both Fdc1 and UbiD). In the case of UbiD, putative interactions between the benzoyl-prFMN adduct para-hydroxyl group and the protein are made with Arg-192 and His-291.

Discussion

The discovery of the prenylated FMN as cofactor for the UbiD family enzymes allows for more in-depth study of these decarboxylases and potentially their future application. Initial studies have been reported using the Fdc1 enzyme, a distinct member of the UbiD family that catalyzes decarboxylation at a non-aromatic position. In contrast, canonical UbiD enzymes have been reported to act on benzoate-like substrates. It is at present unclear to what extend the properties and mechanistic details of Fdc1 can be attributed to the wider UbiD family. If both Fdc1 and UbiD make use of a similar 1,3 dipolar cycloaddition-based mechanism, the latter enzymes would require a more malleable active site given the projected motions of the substrate during catalysis (Fig. 1B).

A first indication that the properties of Fdc1 do not fully reflect the wider UbiD family was obtained by the fact that we were unable to produce the bona fide holoUbiD in E. coli despite the presence of a highly co-expressed ubiX and the fact E. coli is the physiological host. The latter approach worked for several Fdc1 enzymes (6). Although it is possible the UbiD-prFMN complex is particularly unstable during protein purification, we found that upon reconstitution the latter complex appeared relatively stable (at least in the UbiD-prFMN radical state). Alternatively, an as yet unknown factor might be required for UbiD prFMN incorporation in vivo, the levels of which were insufficient for overproduction of holoUbiD.
Indeed, several UbiD homologues have been reported to contain additional smaller subunits. Notably, *Streptomyces* species require three genes for vanillic acid decarboxylase activity: *vdcB*, *vdcC*, and *vdcD* (26, 27). In this gene cluster, *vdcB* and *vdcC* correspond to *ubiX*- and *ubiD*-like genes, whereas *vdcD* is a small gene of unknown function that is required for activity in vivo. It is possible *vdcD* and similar genes might play a role in prFMN incorporation (and possibly maturation and/or catalysis) in vivo. However, we have not found *E. coli* genes homologous to any of the characterized small subunits that would allow us to verify this hypothesis nor did we find any *E. coli* protein to co-purify with apoUbiD.

Using the purified apoUbiD, we set out to reconstitute the holoenzyme using prFMN prepared in vitro using the purified prenyl transferase UbiX. This approach proved successful in generating the reduced UbiD-prFMN complex. No activity could be detected for this complex with a range of aromatic acids (data not shown), consistent with the idea that oxidative maturation of the bound prFMN (to the iminium form) is required for activity as observed for Fdc1 enzymes. Upon exposure to oxygen, the reduced prFMN-UbiD complex rapidly forms a semiquinone-like radical species, consistent with the redox potential of \(-240 \text{ mV}\). Both UV-visible and EPR spectral features are of the UbiD-prFMN radical complex are similar to that observed for the prenyl transferase UbiX-product complex after exposure to oxygen. In the case of Fdc1, such a radical species was postulated to occur during oxidative maturation, but it was only observed as a minor population for Fdc1 after cyanoborohydride reduction and consequent reoxidation in vitro (6, 7). Even more surprisingly, the UbiD-radical prFMN species does not further oxidize with any measurable rate at ambient pH. This suggests the *in vitro* reconstituted UbiD is unable to affect the C1’-\(\text{bond}\)H bond breakage required for formation of the fully oxidized N5=Cl’ iminium species and that maturation occurs via path (b) in Fig. 1A. The conserved acidic residue at position 290 (Asp in UbiD, Glu-285 in Fdc1) is a prime candidate to act as a base given the close proximity to the C1’ position. Crystal structures of apo and holoUbiD reveal the latter in a similar position to that observed for the mature Fdc1 enzyme, establishing a similar conserved network of ionic interactions with Arg-180 (Arg-173 in *A. niger* Fdc1) and Glu-285 (Glu-277 in *A. niger* Fdc1). Nevertheless, when comparing...
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FIGURE 11. Induced fit flavin and metal ion binding in UbiD. A, stereoview of the metal binding region of the apoUbiD (in green) and FMN-Mn$^{2+}$-UbiD complexes (in blue). Key residues are shown in colored sticks. The omit density for the Mn$^{2+}$ is shown in red, contoured at 5 $\sigma$. B, stereoview of the metal binding region of UbiD (in blue, labels in black) and Fdc1 (in gray, labels in gray and italics). No clear density corresponding to a K$^+$ could be observed in UbiD. A domain motion as identified in Fig 10 would position Arg-390 closer to the prFMN phosphate, mimicking the interaction between Lys-391 and prFMN in Fdc1.

FIGURE 12. Crystal structures of UbiD in complex with prFMN. A, UbiD active site structure (of the closed monomer) with key residues shown in colored sticks. Omit electron density is shown for the prFMN UbiD cofactor contoured at 3 $\sigma$. B, a similar view to A, but for the sulfite-prFMN adduct. C, structure of the related Fdc1 enzyme in similar orientation. In this case an inhibitor bound form of the prFMN cofactor is shown for a direct comparison with the UbiD sulfite-prFMN adduct. Residues that are affected in their relative position with respect to the prFMN cofactor by UbiD domain motion are indicated by an asterisk (*).
the relative orientation of the UbiD prFMN binding and multimerization domains with that observed for Fdc1 enzymes, it appears the UbiD enzyme is in a more open state. It is possible domain motion in UbiD (as observed by comparing various apoUbiD crystal structures; Fig 10) might lead to a formation of a closed, Fdc1-like UbiD state. The latter might affect the pKa of prFMN and/or Asp-290, leading to efficient deprotonation of the C1' position concomitant with further oxidation.

Incubation of the UbiD-radical prFMN at pH 9.2 (but not the UbiX-radical prFMN species) leads to spectral changes and formation of a range of prFMN species as detected by mass spectrometry. Although the catalytically relevant prFMN$_{\text{iminium}}$ species appears to be present among these, a wide range of oxidation and/or hydrolysis products was also present, a likely consequence of the prolonged incubation at alkaline pH. This observation strongly suggested the prFMN$_{\text{iminium}}$ species is only formed when in complex with UbiD (and cannot be formed by UbiX). It furthermore suggests a proton-coupled electron transfer precedes formation of the fully oxidized prFMN. Although further oxidation to prFMN$_{\text{iminium}}$ cannot be observed at ambient pH, it is possible this might occur at a very slow rate.

Indeed, holoUbiD crystals that appear after days of incubation are colorless, suggesting slow maturation does occur over such time scales. The fact that a sulfite adduct to the C1’ can be observed in certain crystals (which can only occur when an iminium form of the cofactor would have been present before sulfite nucleophilic attack) further confirms the idea that in vitro oxidative maturation in UbiD stalls at the radical prFMN species but ultimately can proceed to the catalytically relevant species.

It is possible that the physiological in vivo oxidative maturation of prFMN in UbiD proceeds in a manner different from Fdc1. Flavoenzymes, for example, are also known to make use of different oxidants depending on the exact active site architecture (28, 29). It is possible that, in UbiD, maturation in vivo proceeds with the loss of a hydride from C1’ (i.e. path (a) in Fig. 1A) as opposed to two sequential one electron oxidation events. We have not found the presence of either PHB or PHB-like substrate analogues or that of NAD$^+$ or NADP$^+$ to affect formation of the radical prFMN species in vitro (data not shown). Alternatively, the presence of an as-yet unidentified subunit/ligand might be required for oxidative maturation as well as prFMN incorporation in UbiD.

Although active UbiD could not be obtained here, UbiD crystal structures in complex with prFMN provide some insights into the likely mode of action. In comparison with Fdc1, the relative position of the prFMN and multimerization domains of UbiD is subtly different, leading to a more accessible active site in UbiD, with a large cavity above the prFMN ring system. A putative domain motion is observed in UbiD through a hinge motion at Gly-333 that could lead to a conformation similar that observed in Fdc1. However, various key residues close to the prFMN C1’ (i.e. Asp-290) are located close to the Gly-333 hinge and appear, therefore, largely unaffected in position by closure of the active site. In contrast, residues lining the putative substrate binding cavity above the prFMN ring system are distant from the hinge point, suggesting that part of the active site is indeed malleable (Fig. 13). Hence, in canonical UbiD enzymes a domain motion might be coupled to catalysis to support the various reorientations of the substrate. It is, however, unclear whether such a motion has any effect on prFMN maturation.

Surprisingly, no obvious hydrophobic binding site can be detected in UbiD that would correspond to an octaprenyl-tail binding pocket for the insoluble ubiquinone precursor substrate. The enzyme itself does not contain any obvious surface features that might affect membrane association, and the protein appears readily isolated in a soluble state despite a previous report suggesting a membrane association (30). It is possible the present model for ubiquinone biosynthesis requires correction, as it would seem more plausible for the physiological UbiD
substrate to be soluble and thus not prenylated, contrary to present literature. Indeed, this apparent contradiction may be explained by the fact that original reports on UbiD discovery only tested the membrane fraction to determine the likely intermediates of the pathway, focusing only on accumulating prenylated intermediates. This has led to the present model that all intermediates of ubiquinone biosynthesis are lipid-soluble and that the prenyltransferase UbiA acts early in the pathway.

Recent reports show that in yeast, certain knock-out mutants are able to synthesize CoQ6 when supplemented with non-prenylated intermediate (31, 32). It was shown that a Coq6 knock-out (the first monooxygenase in the pathway) is able to produce ubiquinone when supplemented with dihydroxybenzoic acid or vanillic acid (32). Coq6 is equivalent to E. coli Ubil and is thought to be active with a carboxylated, prenylated intermediate. The fact that non-prenylated intermediates can salvage the pathway shows that ring modifications are possible before prenylation. In fact, the prenyltransferase UbiA is capable of prenylating ring-substituted aromatic acids (33). It thus seems a distinct possibility that prenylation occurs late in the pathway rather than early and that lipid soluble intermediates accumulating in knock-out mutants do not necessarily reflect key steps along the pathway but merely the accumulation of soluble intermediates combined with a degree of non-discriminate UbiA prenyltransferase activity. In vitro studies of ubi enzymes will assist with determining the likely sequence of enzymatic reactions in the ubiquinone biosynthetic pathway. At present, our studies with UbiD suggest an as-yet hypothetical additional (small) subunit or allosteric effector might be required for prFMN incorporation and maturation, similar to reports for other UbiD enzymes requiring additional subunits for activity in vivo. It is possible a small UbiD-associated subunit might also affect activity. Thus, our data reveal unexpected variation occurs in domain mobility, prFMN binding, and maturation by the UbiD enzyme family. Further study will be needed to fully determine both the general and protein specific features of the UbiD enzyme family.

**Experimental Procedures**

**Cloning of E. coli UbiD**—E. coli ubiD was cloned into pET28a between Ndel and Xhol restriction sites with an N-terminal hexahistidine tag using ligation independent cloning by In-Fusion (Clontech). A C-terminal hexahistidine tag construct was also made by cloning into pET30a between Ndel and Xhol using the same techniques. Genomic material was used as a template for PCR amplification of ubiD. Construct sequences were checked using Eurofins Genomics sequencing, and plasmids were transformed into E. coli BL21 (DE3) (New England Biolabs) for protein expression.

**Protein Expression and Cell Growth**—Cells were grown in 1 liter of Luria Bertani broth at 37 °C with 180 rpm shaking until the A600nm ≈ 0.6, at which point cells were induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside, and the temperature was reduced to 18 °C. Induced cells were grown overnight, typically 18 h, before centrifugation. For larger scale preparations, 22 L cells were grown in a fermentor using the same protocol as described for 1-liter flasks.

**UbiD Purification**—Cells were resuspended in 50 mM Tris, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol (buffer A), and a lysozyme, RNase, DNase, and protease inhibitor mixture (Roche Applied Science) added. Cells were lysed by sonication (Bandelin) at 30% amplitude in thirty 20-s intervals interspersed with 40 s on ice. Cell lysate was centrifuged at 145,000 × g for 1 h, and the supernatant was filtered through a 0.45-μm filter and applied to nickel-nitrilotriacetic acid resin (Qiagen) packed in a gravity flow column. The resin was washed with buffer A containing 10 mM imidazole followed by an additional wash step with buffer A containing 40 mM imidazole. Protein was eluted using buffer A containing 250 mM imidazole. UbiD was desalted using a 10DG column (Bio-Rad) into 20 mM Tris, pH 7.2, 500 mM NaCl, 5% (v/v) glycerol (buffer B) for further experiments. For samples produced for ENDOR analysis, buffers used contained 10% (v/v) glycerol to aid in stability at higher concentrations. All other aspects of purification remained constant.

**Reconstitution of UbiD with FMN**—UbiD was adjusted to a concentration of 200 μM before adding 500 μM FMN. MnCl2, KCl, and MgCl2 were added individually or in combinations, each to a concentration of 1 mM. Mixtures were incubated at room temperature for 20 min before desalting into buffer B through P6DG resin (Bio-Rad). Spectra were measured on a Cary WinUV 50 spectrophotometer (Agilent).

UbiD in buffer B was adjusted to a concentration of 1.6 μM (~0.1 mg ml−1). Using an Edinburgh Instruments FLS920 spectrofluorometer, the emission spectra of UbiD was measured between 300 and 420 nm after excitation at 295 nm. 2 mM MnCl2 and 2 mM KCl were added to UbiD in a 1-ml quartz cuvette where appropriate. FMN was added in 1-μl titrations. Measurements were taken in triplicate at 22 °C. The peak difference at 333 nm was used to plot the binding of FMN to UbiD and was fitted using a one-site binding curve using Prism.

**Anaerobic Reconstitution of UbiD with prFMN**—UbiD was reconstituted with prFMN produced in vitro using P. aerugi-nosa UbiX using two distinct reduction systems. UbiX was purified as previously described (7). Reactions consisting of buffer B containing 1 mM FMN, 2 mM DMAP (Sigma), and 50 μM Fre reductase, purified as previously described (34) (gifted by B. Menon), and 50 μM UbiX in anaerobic glovebox (Belle technology) were started by the addition of 5 mM NADH. Alternatively, reactions (in the absence of Fre reductase) were carried out using 1 mM FMNH2, obtained by titration with sodium dithionite and initiated by the addition of DMAP. Reactions were allowed to proceed for at least 3 h at room temperature. After incubation, the reaction mixture was filtered through 10k molecular weight cutoff centrifugal concentrators to remove UbiX and Fre proteins. The filtrate containing the prFMN product was used to reconstitute anaerobic UbiD in a 2:1 molar ratio (assuming complete conversion of FMN into prFMN). Both MnCl2 and KCl were added to UbiD in buffer B to a final concentration of 4 mM. The reconstituted UbiD was incubated for 10 min before desalting using a PD25 column (GE Healthcare) into buffer B for further experiments.

**Testing the Effect of Redox Cycling and pH on the UbiD-prFMN Oxidative Maturation**—UbiD containing radical prFMN was reduced by titrating in minimal volumes of dithio-
nited dissolved in buffer B. Reduction was followed by the change in the UV-visible spectrum. Upon full reduction, UbiD was desalted into buffer B and oxidized by exposure to atmospheric oxygen. UbiD was reconstituted as described above but was anaerobically desalted into 20 mM Bis-Tris propane, pH 7.2, 500 mM NaCl, 5% (v/v) glycerol rather than buffer B. After radical prFMN formation upon exposure to oxygen, minimal volumes of 1 mM Bis-Tris propane, pH 9.5, were added to attain pH 9.2. Spectral changes were observed over time. After no further changes were observed in the spectrum, UbiD was desalted into buffer B. The effect of pH on the cofactor in UbiX was tested in anaerobic desalted into 20 mM Bis-Tris propane, pH 7.2, 500 mM NaCl, 5% (v/v) glycerol before being oxidized.

Redox Potentiometry of UbiD-Radical prFMN—Holo UbiD was desalted anaerobically into 20 mM Tris, pH 7.2, 500 mM NaCl, 5% (v/v) glycerol. 0.3 mM methyl viologen, 1 mM benzylic viologen, 7 mM hydroxyphthalazine and 2 mM phenazine methosulfate were added as reduct mediators. The absorption spectra were measured using a Cary WinUV 50 with probe attachment after the potential of solution was read using a Mettler Toledo potentiometer. Redox titration was carried out by stepwise addition of minimal volumes of sodium dithionite into UbiX fully occupied by FMN. 5 mM DMAP was added to initiate the reaction, which was allowed to proceed for 3 h. UbiD was desalted into 20 mM Bis-Tris propane, pH 7.2, 500 mM NaCl, 5% (v/v) glycerol before being oxidized.

| Crystallographic data collection and refinement statistics | Apo-UbiD N-tag/5M1C | Apo-UbiD C-tag/5M1B | Holo UbiD (prFMN)/5M1D | Holo UbiD (prFMN-SO3)/5M1E |
|-----------------------------------------------------------|---------------------|---------------------|-----------------------|--------------------------|
| Wavelength, Å                                            | 0.92818             | 0.9795              | 1.0444                | 1.0444                   |
| Space group                                              | P 4 1 2             | P 4 1 2             | P 4 1 2               | P 4 1 2                  |
| Cell dimensions, a, b, c (Å)                             | 196.435             | 210.187             | 194.199               | 194.52                   |
| Rmerge (%)                                               | 0.118 (0.936)       | 0.146 (0.976)       | 0.110 (1.232)         | 0.083 (1.088)            |
| Completeness (%)                                         | 100 (100)           | 99.9 (100)          | 99.9 (99.9)           | 99.5 (100)               |
| Redundancy (%)                                           | 13.1 (13.5)         | 6.5 (6.8)           | 7.3 (6.5)             | 6.7 (6.1)                |
| Wilson B-factor (Å²)                                     | 63.64               | 71.28               | 54.65                 | 63.27                    |
| Refinement Resolution (Å)                                | 87.85-2.75 (2.82-2.75) | 29.89-3.15 (3.23-3.15) | 137.3-2.7 (2.77-2.77) | 86.99-2.62 (2.69-2.62) |
| No. reflections                                          | 52,741 (3798)       | 51,401 (4807)       | 54,077 (3819)         | 58,691 (4292)            |
| Rmerge/Rfree                                            | 0.174/0.228 (0.372/0.396) | 0.221/0.276 (0.295/0.356) | 0.194/0.228 (0.324/0.367) | 0.176/0.212 (0.319/0.340) |
| No. non-hydrogen atoms                                   | 10,632              | 10,593              | 11,151                | 11,160                   |
| Mean B factor (Å²)                                       | 53.3                | 49.63               | 42.69                 | 48.8                     |
| Root mean square deviations                              | 0.013               | 0.010               | 0.012                 | 0.015                    |
| Bond lengths (Å)                                         | 1.60                | 1.63                | 1.57                  | 1.83                     |

Cofactor Binding in UbiD—Methyl 3-methyl-2-butenoate was reduced to the corresponding alcohol using LiAlD₄. This produced C¹-deuterated 3,3-dimethylallyl alcohol in a 86% yield (35). Both 3,3-dimethylallyl alcohol and C¹-deuterated 3,3-dimethylallyl alcohol were then subjected to phosphorylation with ditriethylammonium phosphate salt and trichloroacetonitrile in acetonitrile (36). This predominantly gives monophosphorylation to the corresponding DMAP and C¹-deuterated-DMAP derivatives 66–72% yield with ~15% of the pyrophosphorylated derivatives also observed. The monophosphorylated derivatives were isolated by column chromatography (silica gel, n-propyl alcohol/concentrated ammonia, 6/3/1). The isolated products were lyophilized to a white powder. ¹H, ¹³C, and ³¹P NMR were found to be in accordance with the literature.

EPR and ENDOR—UbiX reactions were carried out with 5 mM FMN, 20 mM (C¹-protriated) DMAP/(C¹-deuterated) DMAP, 100 µM Fre reductase, and 500 µM UbiX. Reconstitutions were started by the addition of 20 mM NADH. Reconstitutions of UbiD contained 10 mM MnSO₄ and 10 mM KCl with filtered reaction mixtures before desalting. Samples were oxidized by atmospheric oxygen and frozen immediately in 4-mm outer diameter quartz EPR tubes. EPR and ENDOR spectra were obtained using a Bruker E500/580 EPR spectrometer. Continuous wave X-band EPR spectra employed a Bruker “Super High Q” cavity (ER 4122SHQE) coupled to an Oxford Instruments ESR900 helium flow cryostat for temperature control, whereas X-band FID-detected Davies pulsed ENDOR spectra were collected using a Bruker EN 4118X-MD4 dielectric ENDOR resonator coupled to an ER 4118HV-CF100 CryoFree cooling system. Experimental parameters and temperatures were as given in the figure legends.

Crystallographic data collection and refinement statistics are given in Table 2.
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Mass Spectrometry—Samples were prepared by a 10-fold dilution of proteins into 100 mm ammonium acetate, pH 7.0. A 1200 series Agilent LC was used to inject 5 μl of sample into 5% acetonitrile (0.1% formic acid) and desalted inline. This was eluted over 1 min by 95% acetonitrile. The resulting multiply charged ion series was analyzed by an Agilent QTOF 6510 run in positive mode and deconvoluted using Agilent Masshunter Software.

Author Contributions—S. A. M. carried out molecular biology, protein purification, and reconstitution experiments. K. F. and S. E. J. R. carried out EPR spectra and analyzed the EPR spectroscopic data. S. A. M. crystallized the enzymes and solved the crystal structures. M. D. W. cloned and crystallized the C-tagged UbiD enzyme. A. N. C. synthesized the deuterated DMAP. K. A. P. P. collected the Fdc1 spectral data. S. A. M. and D. L. wrote the paper. All authors reviewed the results and approved the final version of the manuscript. D. L. conceived and coordinated the study with input from D. A. P.

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