The role of conserved residues in Fdc decarboxylase in prenylated flavin mononucleotide oxidative maturation, cofactor isomerisation and catalysis

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The UbiD family of reversible decarboxylases act on aromatic, heteroaromatic, and unsaturated aliphatic acids and utilize a prenylated flavin mononucleotide (prFMN) as cofactor, bound adjacent to a conserved Glu-Arg-Glu/Asp ionic network in the enzyme’s active site. It is proposed that UbiD activation requires oxidative maturation of the cofactor, for which two distinct isomers, prFMNketimine and prFMNiminium have been observed. It also has been suggested that only the prFMNiminium form is relevant to catalysis, which requires transient cycloaddition between substrate and cofactor. Using Aspergillus niger Fdc1 as a model system, we reveal isomerization of prFMNiminium to prFMNketimine is a light-dependent process that is largely independent of the Glu277-Arg173-Glu282 network and accompanied by irreversible loss of activity. On the other hand, efficient catalysis was highly dependent on an intact Glu-Ar-Glu network, as only Glu to Asp substitutions retain activity. Surprisingly, oxidative maturation to form the prFMNiminium species is severely affected only for the R173A variant. In summary, the unusual irreversible isomerization of prFMN is light dependent and likely proceeds via high-energy intermediates, but is independent of the Glu-Arg-Glu network. Our results from mutagenesis, crystallographic, spectroscopic and kinetic experiments indicate a clear role for the Glu-Arg-Glu network in both catalysis and oxidative maturation.

A wide range of enzymes are known to catalyze decarboxylation, many requiring cofactors such as PLP, metal ions or flavin for catalytic activity (1, 2). The flavins FMN and FAD represent arguably one of the most versatile cofactors, responsible not only for a range of redox reactions, but also for light-dependent catalysis (3, 4). The chemical properties of a flavin are influenced by the microenvironment inside the enzyme active site, and are occasionally altered by covalent modification at the C6 or C8 position of the isoalloxazine dimethylbenzene moiety (5). Recently, a highly modified form of flavin, prenylated FMN (prFMN), incorporated by decarboxylases belonging to the UbiD family of enzymes was described (6–8). The UbiD superfamily is composed of a wide variety of (de)carboxylases acting on aromatic, heteroaromatic and unsaturated aliphatic acids (9). Prenylated FMN consists of a FMN molecule modified by the addition of a fourth non-aromatic ring joined via N5-C1’ and C6-C3’
The enzyme responsible for the biosynthesis of prFMN is UbiX (6), which forms the fourth ring via the addition of the isoprene moiety of dimethylallyl-monophosphate to FMNH$_2$. It is proposed that prFMN is released from UbiX and bound by apo-UbiD enzymes in a reduced form (prFMN$^{\text{red}}$). Following binding of prFMN$^{\text{red}}$ and formation of holo-UbiD, the cofactor undergoes oxidation to form the catalytically relevant oxidized prFMN species (Fig. 1A).

Surprisingly, atomic resolution crystal structures of Aspergillus niger Fdc1, a UbiD-type (de)carboxylase, revealed two forms of the oxidized cofactor. These correspond to an isoalloxazine N5-iminium form (prFMN$^{\text{iminium}}$) and the isomeric N5-secondary ketimine form (prFMN$^{\text{ketimine}}$), the latter having a very distinct ring structure derived from the isoalloxazine ring system (Fig. 1A). While a putative mechanism for decarboxylation has been postulated for both these forms, there are several indications prFMN$^{\text{iminium}}$ is the catalytically relevant form (7, 10, 11). Mechanistic insights have been obtained from the structure of a covalent substrate-cofactor adduct of A. niger Fdc1 with α-hydroxycinnamic acid, a close mimic of the natural substrate cinnamic acid. This adduct reveals a covalent bond between the prenyl-C1′ of the prFMN$^{\text{iminium}}$ cofactor and a molecular species derived from the decarboxylation of α-hydroxycinnamic acid. Combined with solution data, this led to the suggestion prFMN$^{\text{iminium}}$ catalyzes substrate (de)carboxylation via formation of a covalent prFMN-substrate cycloadduct through 1,3-dipolar cycloaddition (Fig. 1B). Indeed, the prFMN$^{\text{iminium}}$ cofactor has azomethine ylide characteristics (i.e. the dipole), while many of the UbiD substrates can be classified as dipolarophiles. Isotope effect experiments (10) and theoretical studies (11) have also suggested prFMN$^{\text{iminium}}$ is responsible for 1,3-dipolar cycloaddition-based catalysis. More recently a mechanism-based inhibitor, 2-fluoro-2-nitrovinylbenzene, was used to detect a cycloadduct via MS, adding to the growing body of evidence for 1,3-dipolar cycloaddition in UbiD enzymes (12). The question remains how the prFMN$^{\text{ketimine}}$ is formed and whether it can play any catalytic role.

To what extent the UbiD active site contributes to oxidative maturation, cofactor isomerization and catalysis is also unclear. In the UbiD enzyme family, R173, E277 and E282 (A. niger Fdc1 numbering) form a conserved ionic network. Crystal structures of A. niger Fdc1 reveal E282 is positioned close to the prFMN C1′, and is therefore most likely to act as the key acid-base. Unlike R173 and E277, the E282 side chain can occupy distinct positions, effectively competing with the substrate carboxylate group or CO$_2$ for a site adjacent to R173. This suggests R173 is essential for substrate binding. The role of E277, which is located in the periphery of the active site, is less clear. Preliminary studies of R173A, E277Q and E282Q variants suggested all were catalytically inactive and possess altered UV-vis spectra (7). This implies the role of this network could be twofold: firstly to facilitate oxidative maturation of the cofactor to the iminium form (Fig. 1A), and secondly to act as a key acid-base during catalysis (Fig. 1B).

To further elucidate the roles of these residues, we created an additional three Fdc1 variants (R173K, E277D and E282D) and assessed the effect of each of the six mutations on cofactor maturation and substrate decarboxylation for both the A. niger and Saccharomyces cerevisiae Fdc1 enzymes. We describe crystal structures and activity measurements of Fdc1 and the variants that demonstrate the light-dependent isomerization of prFMN$^{\text{iminium}}$ to prFMN$^{\text{ketimine}}$. In addition to the high-resolution crystal structures, mass spectrometric, spectroscopic, kinetic and hydrogen/deuterium exchange studies of 6 Fdc1 variants provide clear evidence for the role of the E-R-E motif in both oxidative maturation and catalysis.

**Results**

Light-dependent cofactor isomerization and enzyme inactivation. Following purification, incubation of wild-type holo-Fdc1 on ice leads to loss of decarboxylase activity with a half-life of approximately 30 minutes (Fig. 2A). The distantly related AroY also exhibits rapid loss of activity, but only when incubated under aerobic conditions (13).
However, when Fdc1 is incubated in the dark, activity remains constant for many hours, even under aerobic conditions. Hence, we repeated the purification of Fdc1 in the dark, revealing the latter exhibits slightly higher activity ($k_{cat} = 9.3 \pm 0.1$ s$^{-1}$) compared with the protein purified under ambient light ($k_{cat} = 7.6 \pm 0.2$ s$^{-1}$). The respective enzyme preparations exhibit different UV-vis spectra (Fig. 2B), with the “dark” protein preparation displaying a prominent feature at 380 nm and showing subtle differences between 340 nm and 385 nm compared to protein purified under normal light conditions. Exposure of the “dark” protein preparation to 365 nm UV light from an LED source for five minutes results in complete loss of activity, and a change in the UV-vis spectrum (Fig. 2B).

High-resolution crystal structures of Fdc1 purified and crystallized in the dark reveal the prFMN cofactor is exclusively in the iminium form (Fig. 2C). Following brief exposure of these crystals to UV light, the corresponding crystal structure reveals isomerization of at least 50% of the prFMN cofactor to the previously observed ketimine form (Fig. 2D). Returning the illuminated Fdc1 crystals to the dark for an extended period of time does not revert the prFMN$^{\text{ketimine}}$ to prFMN$^{\text{iminium}}$, indicating the isomerization is irreversible. As illumination leads to inactivation, these observations directly confirm that prFMN$^{\text{iminium}}$, rather than prFMN$^{\text{ketimine}}$, is the active form of the cofactor.

**UV-vis spectrophotometric characterization of Fdc1 E277 and E282 variants reveal minor variation in prFMN incorporation and maturation.** Four Fdc1 variants (E277D, E277Q, E282D, E282Q) were successfully co-expressed with UbiX to produce the corresponding holo-enzymes. The UV-vis spectrum of each variant indicated the presence of prFMN, as indicated by broad absorbance features between 340-390 nm and at 550 nm (Fig. 3). However, the exact spectral properties showed subtle variation between the various species. While E282D shows similar features between 340 nm and 390 nm as the wild type enzyme, there is also evidence for the presence of an additional minor species, as indicated by the weak feature at 550 nm. The latter has been shown to correspond to an inactive radical form of the cofactor (7). In the case of E282Q, the level of incorporation appears lower than in the wild type and other variants.

ESI-MS was used to confirm prFMN incorporation, and ESI-MS of both E277D and E282D shows a main MH$^+$ ion mass of 525.16, corresponding to prFMN$^{\text{imimum}}$ (Fig. 4). Unfortunately, no mass corresponding to the bound prFMN could be observed for either E277Q or E282Q variants.

An acidic residue at both position 277 and 282 is required for activity. The decarboxylation activity of the four Glu variants was tested using cinnamic acid as a substrate. While the more conservative E282D variant is still able to catalyze decarboxylation of cinnamic acid, the corresponding E282Q variant is completely inactive, as shown by both end point HPLC (Fig. 5) and UV-vis spectrophotometric activity assays. Where activity was detected, we determined the corresponding apparent values, as the relative occupancy of the prFMN$^{\text{imimum}}$ might be subject to variation. For E282D, $k_{cat}^{\text{app}}$ was found to be 0.63 ± 0.04 s$^{-1}$ and $K_M^{\text{app}}$ 50 ± 10 µM, compared to the wild-type Fdc1 $k_{cat}^{\text{app}}$ of 9.4 ± 0.1 s$^{-1}$ and $K_M^{\text{app}}$ of 10.0 ± 0.6 µM. Similarly, activity for E277D can be detected by both UV/vis decarboxylation assays and HPLC, with a $k_{cat}^{\text{app}}$ of 1.2 ± 0.2 s$^{-1}$. An accurate value for $K_M$ could not be obtained for this variant, as it does not follow Michaelis-Menten kinetics (Fig. 6 A). In contrast, only very low levels of decarboxylase activity could be detected for the E277Q variant by analyzing for product formation using HPLC, suggesting an upper limit for $k_{cat}^{\text{app}}$ of 0.3 min$^{-1}$.

The effect of pH on the rate of decarboxylation by E282D and E277D was investigated (Fig. 6 B-D). In both cases, fitting the data to a bell curve reveals a pH optimum of 7 similar to the wild type protein.

**Enzyme catalyzed styrene H/D exchange confirms the need for acidic residues at 277 and 282.** Hydrogen/deuterium exchange of styrene assessed using NMR spectroscopy was used to further examine the effect of the four E to D/Q mutations on catalysis (Fig 7 B). Deuterium incorporation at the trans-position of styrene C1 (indicated by position 1 on Fig. 7 A) indicates proton abstraction of
the substrate is able to occur (as a first step towards carboxylation), and that the cofactor must be in a catalytically active state. Disappearance of the resonance at 5.3 ppm, as well as simplification of the resonance at 6.9 ppm from a doublet of doublets to a doublet, indicates exchange has taken place. Hydrogen/deuterium exchange occurs for wild type and E282D within the 20-minute dead time of the experiment. Exchange also occurs in the presence of the E277D variant, although much more slowly than observed with the wild type protein, incubation for 2 hours was required for ~ 90% conversion. No significant exchange can be observed for E277Q, despite the detection of low levels of activity by HPLC. Similarly, H/D exchange was not observed for E282Q.

Light-dependent cofactor isomerization is not dependent on the E-R-E motif. The prFMN_{iminium} to prFMN_{ketimine} isomerization is likely to occur following deprotonation of the C1’ position, which could occur through either the adjacent prFMN O4 or through the E282 side chain (Fig. 8). To probe whether the enzyme active site is involved in this unusual isomerization, we tested the effect of light on key Fdc1 variants. Incubation of E282D and E277D (the only active variants) in the light leads to loss of activity over a similar time scale to wild type, indicating the formation of prFMN_{ketimine} is also possible in these variants (Fig. 2A). We furthermore determined the 1.28 Å crystal structure of E282Q (an inactive variant, vide infra) following UV light exposure (Fig. 2F), revealing the prFMN_{ketimine} form can also be observed (albeit at under 50% occupied) in the absence of the key catalytic acid-base residue.

R173 variants reveal deficiencies in cofactor maturation. The UV-vis spectra of one of the purified R173 variants, R173A, exhibits a modest 550 nm spectral feature that has previously been associated with the prFMN_{radical} form of the cofactor (Fig. 3C). ESI-MS experiments using the R173A variant were able to detect MH+ cofactor masses of 525.16 Da and 526.16 Da corresponding to the mature prFMN_{iminium} and prFMN_{radical} forms of the cofactor respectively. In addition, a 541.16 Da species was also observed, which we attribute to prFMN_{iminium} having undergone subsequent hydroxylation and further oxidation (Fig. 4). HPLC activity measurements confirm R173A retains low levels of activity as purified from cells co-expressing UbiX, confirming the presence of prFMN_{iminium} (Fig. 5).

To further investigate cofactor maturation in R173A Fdc1, we undertook an in vivo reconstitution of this variant. The apo-R173A variant was obtained by expressing in the absence of UbiX. Apo-R173A was anaerobically reconstituted in vitro by adding prFMN_{reduced} produced by incubation of FMNH2 and DMAP with UbiX, to apo-R173A in an anaerobic environment as reported previously (8). After removal of excess prFMN, unreacted FMN and DMAP, followed by oxidation via brief exposure to air, reconstituted R173A Fdc1 exhibits a UV-vis spectrum dominated by the 550 nm spectral feature (Fig. 9A), consistent with a large proportion of prFMN_{radical} being present. EPR spectroscopy also indicates the presence of the prFMN_{radical} (Fig. 9B) (6) with further support for this assignment arising from the narrowing of the EPR signal when deuterrated DMAP was used in the biosynthesis of prFMN (Fig. 9C), as observed for E. coli UbiD (7). Since such relatively featureless radical EPR signals can be difficult to assign to specific radicals, we also employed electron nuclear double resonance (ENDOR) spectroscopy that provides a ‘fingerprint’ for a radical through measurement of the hyperfine interactions between the unpaired electron of the radical and its constituent magnetic nuclei (in this instance hydrogen and deuterium atoms). The ENDOR spectra of air-oxidised reconstituted R173A Fdc1 are essentially identical to those we reported previously for prFMN_{radical} in E. coli UbiD (Fig. 9D-G) (7) confirming the assignment.

In contrast to R173A, the R173K variant did not appear to contain a significant amount prFMN_{radical}, indicated by a very weak feature at 550 nm. Furthermore, no activity could be detected for R173K, despite the identification of a MH+ ion mass of 525.18 Da by ESI-MS (Fig. 4). H/D exchange experiments using styrene (Fig 7B) confirmed limited activity for R173A, detectable at very low levels after incubation for 4 hours, whilst no detectable
exchange could be observed in the presence of R173K<sub>2</sub>.

**Crystal structure of R173A confirms cofactor maturation occurs over longer timescales.** The 1.19 Å crystal structure of R173A co-expressed with UbiX was obtained revealing clear additional density on the prFMN C1’ confirming hydroxylation readily occurred at that position (Fig. 10E). This suggests the oxidative maturation of the cofactor did complete (prior to hydroxylation), albeit over long timescales. A similar observation was made for the *E. coli* UbiD enzyme, where a sulfite adduct was observed in the crystals (7).

In the R173A crystal structure, residue E282 has been modeled in two conformations, a prFMN facing conformation (labeled I in Fig. 10J) and a second conformation that brings the carboxyl group of E282 to within hydrogen bonding distance (2.7 Å) of H284 (labeled II in Fig. 10J). In addition, there is a minor movement of residues 184-187 resulting in L185 moving away from the active site. These results, combined with the low levels of decarboxylase activity detectable by HPLC, and small proportion of H/D exchange observed on styrene in the presence of R173A confirm cofactor maturation can occur in the R173A variant. Unfortunately repeated efforts to crystallize R173K were unsuccessful, so we cannot assess the effect of this mutation on the architecture of the active site.

**Crystal structures of Fdc1 E282 variants reveal minor structural variation.** The high-resolution crystal structure for E282D (1.06 Å) reveals residue D282 occupies position I (Fig. 10A), with slight movements in the protein backbone allowing the D282 carboxyl group to occupy a similar position to E282 in the wild type enzyme (Fig. 10J). In this case, no C1’ adducts are visible in the electron density. All other residues occupy the same position as in the wild type, with hydrogen bonding distances remaining constant.

Residues R173 and E277 also occupy the same position as wild type in the high-resolution crystal structures for E282Q (1.13 Å/1.28 Å) (Fig. 10G,H). One structure shows the presence of a minor species corresponding to a prFMN hydroxylated at C1’ (Fig. 10H), whilst another structure, obtained from a separate protein preparation, shows unmodified prFMN (Fig. 10G). Residue Q282 is observed in a conformation within hydrogen-bonding distance (2.9 Å) of H284, (position II). Additional density in the active site, in the position usually occupied by the carboxyl group of E282, has been interpreted as a molecule of thiocyanate, (a component of the crystallization solution) mimicking the CO<sub>2</sub> product. To assess whether the E282Q mutant prevents substrate binding, which could cause the lack of decarboxylase activity, we performed ligand soaks with pentafluorocinnamic acid. In the corresponding crystal structure, partial occupancy of pentafluorocinnamic acid could be observed, indicating substrate binding remains possible (Fig. 10I).

**Crystal structures of Fdc1 E277 variants reveal significant C1’ adduct formation.** The crystal structure of E277D (1.64 Å) also shows electron density corresponding to partial hydroxylation (~30%) of the C1’ (Fig. 10C). Additional electron density located close to the C1’ could be representative of further modification of the C1’ at a very low occupancy. There is a slight shift in the protein backbone from residues 274-278 compared to wild type; this allows the carboxyl group of D277 to occupy almost the same position as E277, however, a slight rotation of the acid group leads to an extension of one hydrogen bond from 2.8 Å to 3.3 Å (Fig. 10C). Residue E282 faces away from the active site and prFMN cofactor (labeled III in Fig. 10J).

Two crystal structures were obtained for E277Q, derived from two separate enzyme preparations. In the first structure (1.64 Å), extensive modification on the C1’ can be observed that is similar to the phenylpyruvate-derived inhibitor adduct observed for the WT enzyme (7) (Fig. 10B). It appears that under certain conditions, *E. coli* produces sufficient amounts of phenylpyruvate leading to adduct formation in vivo, resulting in distinct enzyme preparations in case of E277Q. In the E277Q phenylpyruvate adduct structure, the rotation of the Q277 amide group and altered position of R173 disrupt the local hydrogen-bonding network between Q277 and R173. The
second crystal structure of E277Q (1.03 Å) only reveals partial hydroxylation of the C1’ (Fig. 10D). In the latter structure, the amide group of Q277 occupies a similar position to the carboxyl of E277 in the wild type protein (Fig. 10J). The presence of both these adducts indicate oxidative maturation of prFMN is able to take place for E277Q.

Similar trends are observed for Saccharomyces cerevisiae Fdc1 variants. The corresponding 6 variants were also made in the related S. cerevisiae Fdc1, with the corresponding UV-vis spectra, decarboxylation assays (of cinnamic acid), and crystal structures revealing similar trends to those observed for the A. niger variants. Again, incubation of wild-type protein that has been co-expressed with UbiX on ice leads to a decrease in activity over time, with a half-life of approximately 30 minutes (Fig. 11D), however, when incubated in the dark activity remains stable for many hours. Whilst there is little evidence for prFMN$^{\text{ketimine}}$ in any S. cerevisiae Fdc1 crystal structures, it is likely that the mechanism of inactivation is the same.

The UV-vis spectra for S. cerevisiae Fdc1 are complicated by the incorporation of unmodified flavin, indicated by the 450 nm peak visible in the wild type spectrum. The S. cerevisiae Fdc1 variants studied bind a lower proportion of FMN, indicated by a reduction of the 450 nm peak, with features between 300 – 400 nm suggesting prFMN incorporation. E280 (equivalent to E277 of A. niger Fdc1) variants appear to bind the lowest proportion of unmodified flavin and exhibit different levels of prFMN binding, with differences in ~ 340 nm centered features. As observed for A. niger Fdc1, the R175A variant (S. cerevisiae numbering) shows a prominent 550 nm peak, indicating the presence of prFMN$^{\text{radical}}$, however this feature is absent in the R175K variant.

Decarboxylase activity can be detected by UV-vis spectrophotometric assay for the wild type protein and E285D, E280D, and E280Q variants. Wild type S. cerevisiae Fdc1 exhibits a $k_{\text{cat}}^{\text{app}}$ of 10.5 ± 0.4 s$^{-1}$ and $K_M^{\text{app}}$ of 25.5 ± 3.6 µM. For E285D $k_{\text{cat}}^{\text{app}}$ has been measured as 0.28 ± 0.01 s$^{-1}$ and $K_M^{\text{app}}$ as 20.4 ± 3.6 µM. For E280D $k_{\text{cat}}^{\text{app}}$ has been measured as 0.92 ± 0.03 s$^{-1}$ and $K_M^{\text{app}}$ as 8.6 ± 1.5 µM. For E280Q, $k_{\text{cat}}^{\text{app}}$ has been measured as 0.09 ± 0.02 s$^{-1}$, a value for $K_M$ could not be obtained (Fig. 6). Activity cannot be detected for R175A, which is consistent with the presence of prFMN$^{\text{radical}}$ indicated by the UV-vis spectrum. Activity cannot be detected for R175K or E285Q despite there being no obvious 550 nm feature in these variants.

Crystallization of wild type, E285D and R175A S. cerevisiae Fdc1 variants confirms cofactor incorporation (Fig. 12). E285D exhibits minimal disruption to the active site architecture (Fig. 12 A, C), with hydrogen bonding distances remaining very similar to wild type and prFMN lacking modification on the C1’. The crystal structure of the R175A variant again shows prFMN in an unmodified form, here disruption of the ERE hydrogen bonding network has resulted in residue E285 moving away from the active site to within hydrogen bonding distance (2.7 Å) of residue H287 (Fig. 12 B-C).

Discussion

The UbiD family contains a highly conserved R-EX$_4$E/D sequence motif, which is believed to play a role in catalysis. However, in view of the UbiD cofactor requirement, there are in fact three individual processes in which the R-EX$_4$E/D motif could play a role that affects activity. These include the oxidative maturation of the cofactor, the light induced isomerization of the iminium to the ketimine form, and acid-base catalysis during the (de)carboxylation by Fdc1.

Despite the fact a mechanism for catalysis has been proposed for the prFMN$^{\text{ketimine}}$ (7), indirect evidence supports the hypothesis that prFMN$^{\text{iminium}}$ is the catalytically active form of the cofactor (7, 10, 11). The observation that light leads to irreversible inactivation of Fdc1 in solution, and that illumination is associated with prFMN$^{\text{ketimine}}$ formation in crystallo, strongly suggests prFMN$^{\text{ketimine}}$ is not relevant to catalysis. The unusual isomerization from the prFMN$^{\text{iminium}}$ to the prFMN$^{\text{ketimine}}$ form is likely to occur following C1’ deprotonation. This particular step is similar to the prFMN oxidative maturation, where C1’ deprotonation could in principle occur with either the adjacent
prFMN O4 acting as the base, or with the closely located E282, acting as the base, similar to what is proposed to occur during (de)carboxylation. As prFMN$^{\text{ketimine}}$ can be observed in UV-illuminated E282Q crystals, it appears likely that O4 is able to act as an internal base, with no direct role E282 in the light dependent isomerization of prFMN.

This suggests prFMN light dependent isomerization might be a general feature of the prFMN$^{\text{iminium}}$ form. However, we have only demonstrated light sensitivity in two Fdc1 enzymes, and light sensitivity has not previously been reported for other members of the UbiD superfamily. In fact, oxygen sensitivity has been reported for some members of the UbiD superfamily such as 3,4-dihydroxybenzoate decarboxylase (8, 14), indole-3-carboxylate decarboxylase (15), and phthaloyl-CoA decarboxylase (16). It is possible the latter enzymes might undergo a 1,3-dipolar cycloaddition with singlet oxygen, a potent dipolarophile that can be formed as a consequence of illumination (17). Further investigation will be required to determine whether this or another oxidative process is responsible for enzyme inactivation in these cases.

Similar to the isomerisation process described above, maturation of prFMN$^{\text{iminium}}$ to the catalytically relevant prFMN$^{\text{iminium}}$ requires C1’ proton abstraction concomitant with oxidation. Previous studies have suggested this process only occurs within the UbiD enzyme, suggesting a direct contribution of the enzyme to the oxidative process. Given the close proximity of E282, it is possible this residue is required for C1’ proton abstraction. The E282Q variant of Fdc1 is inactive, which could be due to the inability to catalyse (de)carboxylation, but could also result from defects in cofactor maturation. We reveal crystal structures of E282Q do contain additional density on the C1’ of prFMN corresponding to partial formation of a hydroxyl adduct. This strongly suggests prFMN$^{\text{iminium}}$ is formed. Furthermore, the reduced prFMN is not stable in the presence of oxygen, rapidly forming a purple coloured radical species (6, 8). Both the E282Q variant in solution as well as crystals of E282Q remain colourless under aerobic conditions, suggestive of prFMN$^{\text{iminium}}$ formation. Hence, E282 is not required for maturation of prFMN.

Furthermore, we reveal maturation is able to proceed in E282D, E277D and E277Q as indicated by enzyme activity, the identification of the ion mass corresponding to prFMN$^{\text{iminium}}$ by ESI-MS in ED variants and the formation of adducts on prFMN C1’ in corresponding crystal structures.

In contrast, both the UV-vis spectra of R173A and R173K variants exhibit a feature at 550 nm, characteristic of the presence of the purple semiquinone radical prFMN. Indeed, EPR experiments confirm the presence of this species in R173A. This suggests oxidative maturation is perturbed in these variants, similar to what has been observed for the E. coli UbiD (8). Crystal structures of the R173A variant do contain a hydroxyl adduct on the prFMN C1’, while a mass corresponding to prFMN$^{\text{iminium}}$ can also be detected for R173A, along with low levels of decarboxylase activity and H/D exchange. This indicates oxidative maturation is affected in R173A, but can still occur. In vitro reconstitution of apo-R173A leads to the prFMN$^{\text{radical}}$ – R173 complex that appears stable over several hours, suggesting that further oxidation/maturation events are indeed extremely slow.

Hence, cofactor maturation in Fdc1 does not require the presence of E282, but is affected by removal of the R173. However, the latter is located too far away from C1’ to directly be involved in proton abstraction. Instead, we propose prFMN oxidation by oxygen proceeds in a manner similar to that observed for flavins, with transfer of an electron to O$_2$ leading to formation of the observed semiquinone prFMN$^{\text{radical}}$ and superoxide as shown in figure 13. The loss of the superoxide would lead to a trapped semiquinone species as observed in UbiX (6), E. coli UbiD (8) and Fdc1 R173A. Rapid recombination of the superoxide and prFMN$^{\text{radical}}$ leads to a C4a peroxyoaduct, which can act to abstract a proton from C1’ during elimination of peroxide. A similar C4a peroxyoaduct has been synthesized in vitro by addition of hydrogen peroxide to N5-alkylated FMN (18). As the R173 side chain is likely positioned close to the postulated C4a peroxy moiety, mutation of this residue is likely to affect its formation as well as associated acid-base properties.

Thus, E282 appears only to play a role in catalysis, where it is postulated to act...
as acid-base during (de)carboxylation of the substrate-cofactor complex. In fact, a proportion of the UbiD family have a D at the position corresponding to E282, including the canonical UbiD enzyme. The requirement for an acidic residue at this position strongly suggests there is an enzyme mediated proton transfer step in the catalytic mechanism. Residue E282 is seen in three distinct positions as indicated in Figure 10J. Movement of residue 282 during the catalytic cycle of Fdc1 could therefore facilitate the shuttling of protons between solvent and the active site. Both the E282Q (A. niger) and E285Q (S. cerevisiae) variants are inactive, while E282D and E285D variants retain a considerable level of decarboxylase activity. Furthermore, the pH dependence of the reaction for both E to D variants is similar to that observed for the WT (Fig 6). This suggests the pKₐ of key groups involved in catalysis is unperturbed by the E to D substitution, with the lower kₐ values observed for E282/285D possibly resulting from increased proton transfer distances, as the carboxylate group is slightly shifted in crystal structures of these variants. Surprisingly, activity for the E277Q variant was very low, although crystal structures reveal no significant structural perturbation in the active site. This suggests E277 acts to tune the pKₐ of R173 and in turn E282. Unfortunately, due to the extremely low activity, it was not possible to accurately measure the pH dependence of the reaction. The negative charge provided by E/D277 is likely to increase the R173 pKₐ, in turn lowering the E282 pKₐ (Fig 13B). Furthermore, given the fact the acid substrate binds adjacent to R173, the R173-E277 duad is responsible for selective binding and positioning of the deprotonated substrate.

In conclusion, the UbiD R-EX₄E/D motif is required for efficient catalysis, but only the R173 position appears to influence cofactor maturation. The E(D)282 acts as the key-acid base, with the R173-E277 duad playing a role in fine tuning the E(D)282 properties as well as substrate binding. The exact mechanism for oxidative maturation remains unclear, but it seems plausible this resembles the mechanism for flavin oxidation, with R173 influencing the formation and properties of the C4a peroxoadduct. The unusual and irreversible isomerization of prFMN⁰ to prFMN⁺ is light-dependent, and likely proceeds via high-energy intermediates. While this does not appear dependent on the R-EX₄E/D motif, it is unclear to what extent prFMN⁺ formation might affect other UbiD family members. Flavins have been used to underpin light sensing systems in Nature (17), and it is possible the prFMN light dependent isomerization might feature in a biological process.

Experimental Procedures
Cloning - The A. niger fdc gene was codon optimized and synthesized (Genescript). The A. niger fdc1 gene was cloned into the Ndel and Xhol sites of pET30a, the S. cerevisiae fdc1 gene was cloned into the Ndel and Xhol sites of pET21b. E. coli ubiX was cloned into the Ndel and Xhol sites of pET21b and pET30a. A. niger fdc1 pET30a was transformed into E. coli BL21(DE3) with and without E. coli ubiX pET21b. S. cerevisiae fdc1 pET21b was transformed into E. coli BL21(DE3) with and without E. coli ubiX pET30a.

Mutagenesis - Mutagenesis primers were designed using the QuikChange Primer Design Program (http://www.genomics.agilent.com/primerDesignProgram.jsp). PCR was performed using Phusion Polymerase (NEB). Template was digested using DpnI and the PCR product transformed into E. coli NEB5a. Presence of the desired mutation was confirmed by sequencing (Eurofins). The plasmid was then co-transformed with the corresponding UbiX construct into E. coli BL21 (DE3).

Protein expression - Protein was expressed in E. coli BL21 (DE3) grown in LB media, supplemented with 50 µg mL⁻¹ ampicillin and 50 µg mL⁻¹ kanamycin at 37 °C, 180 rpm until mid log phase. The cultures were then cooled to 15 °C, supplemented with 1 mM MnCl₂, induced using 0.25 mM IPTG (Formedium) and grown overnight. Cells were harvested by centrifugation (4 °C, 7,000 g, 10 min).

Purification of A. niger Fdc1 - Cell pellets were re-suspended in Buffer A (50 mM Tris, 200 mM NaCl pH 7.5 in Milli-Q water) supplemented with complete EDTA-free
protease inhibitor cocktail (Roche), lysozyme, DNase and RNase (Sigma). The cells were lysed on ice using a Bandelin Sonoplus sonicator with a TT13/F2 tip, set to 30% power, 20 s on 40 s off for 30 minutes. Cellular debris was removed by ultracentrifugation using a Ti50.2 rotor in a Beckman optima LE-80k ultracentrifuge at 40k rpm for 1 hour at 4 °C. The supernatant was passed through a 0.45 μm filter. The clarified supernatant was applied to a gravity flow Ni-NTA agarose column (Qiagen). The column was washed with three column volumes of Buffer A supplemented with 10 mM imidazole, then three column volumes of Buffer A supplemented with 40 mM imidazole. Protein was eluted in 1 mL fractions using Buffer A supplemented with 250 mM imidazole. Fractions containing the purified protein were buffer exchanged into Buffer B (20 mM Tris, 100 mM NaCl, pH 7.5 in Milli-Q water) using a 10DG column (BioRad). Protein aliquots were flash frozen until required.

Purification of *S. cerevisiae* Fdc1 - Purification of *S. cerevisiae* Fdc1 was as above, using 50 mM KPO₄, 200 mM NaCl as Buffer A and 20 mM KPO₄ 100 mM NaCl as Buffer B.

In vitro reconstitution of apo-Fdc1 - R173A Fdc1 was reconstituted in vitro using prFMN produced by *P. aeruginosa* UbiX as previously described (8).

UV-visible spectroscopy and protein quantification - UV-visible absorbance spectra were recorded using a Cary 50 Bio spectrophotometer (Varian). Protein concentrations were calculated using $\varepsilon_{280nm} = 63,830$ M⁻¹ cm⁻¹ for *A. niger* Fdc1 variants and $\varepsilon_{280nm} = 68,870$ M⁻¹ cm⁻¹ for *S. cerevisiae* variants. All spectra have been normalized for protein content.

UV-visible spectrophotometric decarboxylation assays - The initial rate of decarboxylation was determined by following consumption of substrate by UV-vis spectroscopy using a Cary 50 Bio spectrophotometer (Varian). Assays were performed against various concentrations of substrate in 350 μL 50 mM KCl, 50 mM NaPi pH 6 in a 1 mm path length cuvette at 25 °C. The rate of cinnamic acid consumption was measured at 270 nm. The extinction coefficient for cinnamic acid is $\varepsilon_{270nm} = 20000$ M⁻¹ cm⁻¹, the extinction coefficient for styrene is $\varepsilon_{270nm} = 200$ M⁻¹ cm⁻¹. The rate of cinnamic acid consumption was calculated using $\Delta\varepsilon_{270nm}$. Protein concentration was determined using $A_{280}$, all $k_{cat}$ values are apparent due to variations in prFMN content. The final concentration of protein in each assay was varied to ensure measurements of cinnamic acid consumption could be measured within the linear portion of the experiment.

Light/dark studies - Time courses to compare loss of activity in the light and dark were carried out by incubating protein in either clear or black plastic microfuge tubes, with measurements of decarboxylase activity carried out as above. The UV-vis spectrum of UV-treated Fdc1 was acquired by exposing protein to UV-light from a 365 nm LED source (Thor Labs).

Hydrogen/Deuterium exchange assays - An excess of styrene was mixed with 50 mM KPi pH 6 in D₂O to obtain a saturated solution of styrene in D₂O (approx. 3% v/v). After addition of protein to a final concentration of 35 μM the ¹H NMR spectra were recorded at 298K on a Bruker 600 MHz AVI NMR spectrometer with a TXI cryoprobe equipped with z-gradients, using presaturation for water signal suppression (1.7s acquisition time, 2s interscan delay, 90° ¹H pulses). Dead time between enzyme addition and recording of spectra was approximately 20 minutes, except where indicated. Chemical shifts were referenced to trimethylsilyl-propionic acid (TSP).

HPLC decarboxylation assays - Assays containing 10 mM cinnamic acid, 50 mM KCl, 50 mM NaPi pH 6, were incubated for 2 hours at 25 °C with 50 μM enzyme. 100 μL of sample was added to 900 μL 50% v/v H₂O/acetonitrile with 0.1% trifluoroacetic acid (TFA). Sample analysis was performed using an Agilent 1100 Series HPLC equipped with a UV detector. The stationary phase was a Kinetex 5 μm C18 column, 250 X 4.6 mm. The mobile phase was acetonitrile/ H₂O
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(50/50), with 0.1% TFA. Detection was performed at 254 nm.

Protein crystallization and structure determination - Crystallization was performed by sitting drop vapor diffusion. An initial screening of 0.3 µL 14mg mL⁻¹ A. niger Fdc1 in 100 mM NaCl, 25 mM Tris pH 7.5 and 0.3 µL reservoir solution at 4 °C resulted in a number of hits including PACT condition F4 (Molecular Dimensions). Seed stock produced from these crystals was used in an optimization screen based around 0.2 M potassium thiocyanate, Bis-Tris propano pH 6.5, 20% w/v PEG 3350 by mixing 0.05 µL seed stock, 0.25 µL protein solution and 0.3 µL reservoir solution at 4 °C. For S. cerevisiae Fdc1 an optimization screen was developed based around CSII condition C6 (Molecular Dimensions) 0.1M sodium cacodylate pH 6.5, 0.25M Calcium Acetate, 15% PEG 4k. Crystals were obtained by mixing 0.05 µL seed stock, 0.25 µL protein solution and 0.3 µL reservoir solution at 4 °C. Crystals were cryoprotected in reservoir solution supplemented with 10% PEG 200 and flash cooled in liquid nitrogen. Diffraction data was collected at Diamond beamlines and processed using the CCP4 suite (19). Molecular replacement was undertaken in Phaser MR (20) using 4ZA4 as a model; further refinement was carried out using REFMAC5 (21) and manual rebuilding in COOT (22). Ligand coordinates and definitions were generated using AceDRG (23).

EPR and ENDOR spectroscopy - 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. Spectra were acquired at 20 K using 10 µW microwave power, 100 KHz field modulation frequency and 1 G modulation amplitude. X-band FID-detected Davies pulsed ENDOR spectra were collected at 30 K and g = 2.0033 using a Bruker EN 4118X-MD4 dielectric ENDOR resonator coupled to an ER 4118HV-CF100 Cryo-Free cooling system. The length of the initial inversion pulse was 400 ns, the detection pulse 200 ns and the radiofrequency pulse 9 µs.

Mass Spectrometry - Samples were prepared by a desalting of proteins into 100 mM ammonium acetate, pH 7.0. A 1200 series Agilent LC was used to inject 5 µ L of sample into 5% acetonitrile (0.1% formic acid) and desalted inline to release the cofactor from the enzyme complex. This was eluted over 1 min by 95% acetonitrile. The resulting ions were analyzed by an Agilent QTOF 6510 run in positive mode and deconvoluted using Agilent Masshunter Software.

Author Contributions – S. S. B. and K. A. P. carried out molecular biology, protein purification, and solution studies. S. S. B. crystallized the enzymes and solved the crystal structures. K.F. and S. E. J. R. carried out EPR spectra and analyzed the EPR spectroscopic data. S. A. M. assisted with reconstitution experiments. M. C. recorded and analyzed NMR spectra. R. S. carried out mass spectrometry experiments. S. S. B. 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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Conflict of interest. The authors have no conflict of interest to declare.
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### Table 1. Crystallographic data collection and refinement statistics.

|                  | A. niger Fdc1 | Fdc1 E277D | S. cerevisiae wt Fdc1 | Fdc1 E277Q | Fdc1 E277Q with prFMN | Fdc1 E277Q with prFMN + ppyFAD | Fdc1 E277Q with prFMN + ppyFAD | A. niger Fdc1 E277Q with prFMN + ppyFAD | Fdc1 E277Q with prFMN + ppyFAD | Fdc1 E277D with prFMN + ppyFAD | Fdc1 E277Q with prFMN + ppyFAD | Fdc1 E282D with prFMN + ppyFAD | Fdc1 E282D with prFMN + ppyFAD |
|------------------|---------------|-------------|-----------------------|-------------|------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| **Resolution**   | 17.49 (1.64)  | 17.49 (1.64) | 17.49 (1.64)          | 17.49 (1.64)| 17.49 (1.64)           | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      | 17.49 (1.64)                      |
| **Space group**  | P 21 2 1      | P 21 2 1    | P 21 2 1              | P 21 2 1    | P 21 2 1               | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          | P 21 2 1                          |
| **Unit cell (Å)**| 95.95 63.40   | 95.95 63.40 | 95.95 63.40           | 95.95 63.40 | 95.95 63.40            | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       | 95.95 63.40                       |
| **RMS angles (°)**| 1.00 1.00 1.00 | 1.00 1.00 1.00     | 1.00 1.00 1.00        | 1.00 1.00 1.00 | 1.00 1.00 1.00          | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     | 1.00 1.00 1.00                     |
| **Average B-factor (Å²)** | 18.64         | 15.27       | 17.61                 | 14.16       | 15.97                   | 28.13                            | 29.57                            | 26.45                            | 27.59                            | 26.05                            | 28.13                            | 26.45                            | 27.59                            |
| **Refinement**   | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 |

**Note:** The table provides crystallographic data collection and refinement statistics for different Fdc1 variants, including resolution, space group, unit cell dimensions, and refinement statistics.
Figure 1. Overview of prFMN maturation and catalysis in Fdc1 A A schematic representation of the stepwise oxidation of reduced prFMN, which might involve deprotonation by Glu 282 in Fdc1 to form the iminium form. How the ketimine species is formed is unclear. B Proposed mechanism for Fdc1 catalysis involving 1,3 – dipolar addition between the substrate and the prFMN$_{iminium}$ azomethine ylide leading to a covalent substrate-prFMN$_{iminium}$ pyrrolidine adduct. Fragmentative decarboxylation is coupled to the breaking of the beta-carbon-prFMN C4a bond. The protonation of the substrate-cofactor complex by Glu 282, concurrent with formation of a second pyrrolidine adduct, leads to product release via a retro 1,3-dipolar cycloaddition.

Figure 2. Effect of illumination on A. niger Fdc1 activity A A plot of activity over time for A. niger Fdc1 wild type and variants in presence and absence of light exposure. Concentration of wild-type Fdc1 in assay: 45 nM. Concentration of E282D and E277D Fdc1 in assay: 600 nM and 300 nM respectively. B UV Vis spectra of A. niger wild type Fdc1 purified in the dark and following illumination with 365 nm UV light. For comparison the spectrum of protein purified under normal ambient light exposure conditions is also shown C Electron density corresponding to the prFMN cofactor from wild type A. niger Fdc1 that has been purified and crystallised in the dark. Omit map corresponding to prFMN$_{iminium}$ contoured to 3$\sigma$. D Electron density of the prFMN cofactor for wild type A. niger Fdc1 following illumination with UV light. Omit map corresponding to prFMN$_{iminium}$ (in red) and prFMN$_{ketimine}$ (in blue) contoured to 3$\sigma$. E Electron density corresponding to the prFMN cofactor for the E282Q A. niger Fdc1 purified and crystallised in the dark. Omit map corresponding to a mixture of prFMN$_{iminium}$, prFMN$_{hydroxylated}$ contoured to 3$\sigma$ F Electron density of the prFMN cofactor for E282Q A. niger Fdc1 crystals illuminated with UV light. Omit map corresponding to prFMN$_{hydroxylated}$ (in grey) and prFMN$_{ketimine}$ (in blue) contoured to 3$\sigma$.

Figure 3. UV-visible spectra for A. niger Fdc1 variants. An overlay of individual UV-vis spectra for each of the three conserved active site residues variants: E282 (A) E277 (B) and R173 (C). Spectra have been normalized for protein concentration using A$_{280}$.

Figure 4. Mass spectrometry of prFMN extracted from A. niger Fdc1 variants. A A side by side comparison of Electrospray ionization-mass spectrometry spectra of prFMN extracted from A. niger Fdc1 variants B A proposed scheme for the maturation of prFMN in Fdc1, species prFMN$_{radical}$, prFMN$_{iminium}$, and prFMN$_{N5-amide}$ have been identified in ESI-MS spectra presented in panel A.

Figure 5. Detection of decarboxylation activity using HPLC. HPLC chromatogram of a 10 mM cinnamic acid solution in presence of 50 µM Fdc1 variants.

Figure 6. Characterization of the active variants E282D and E277D A Steady-state kinetics for mutant and wild type variants of Fdc1 with cinnamic acid. B, C, D pH-dependence of the decarboxylation of cinnamic acid rate for wild type and mutant forms of Fdc1. Concentration of E282D and E277D Fdc1 in assay: 600 nM and 300 nM respectively.

Figure 7. H/D exchange of styrene. $^1$H NMR spectra of styrene in D$_2$O with and without the addition of wild type and mutant variants of A. niger Fdc1. Enzyme catalyzed deuterium exchange on the trans-position of C1 results in disappearance of resonance at 5.3 ppm and the simplification of the signal at 6.9 ppm from a doublet of doublets to a doublet.

Figure 8. UV-induced tautomerisation of prFMN$_{iminium}$ to prFMN$_{ketimine}$. A proposed scheme for the tautomerisation reaction, arrows in blue represent a mechanism whereby O4 acts as an internal base. Arrows in red represent a mechanism whereby E282 acts as an external base in the first step.

Figure 9. UV-Visible absorbance, EPR and ENDOR spectra of prFMN-reconstituted
**Fdc1 R173A and as purified Fdc1 R173A.** A, UV-visible absorbance spectra of air oxidised Fdc1 R173A, ‘as purified’, blue, and following reconstitution with prFMN\textsuperscript{red}, red; B, X-band EPR spectrum of the reconstituted and air oxidised Fdc1 R173A; C, X-band EPR spectrum air-oxidised Fdc1 R173A reconstituted with prFMN\textsuperscript{red} deuterated at C1’; D, the X-band Davies ENDOR spectrum of prFMN\textsuperscript{radical} formed in WT *E. coli* UbiD (7); E, the X-band Davies ENDOR spectrum of the Fdc1 R173A radical shown in B; F the X-band Davies EPR spectrum of C1’-deuterated prFMN\textsuperscript{radical} formed in WT *E. coli* UbiD (7); G, the X-band Davies EPR spectrum of the Fdc1 R173A radical shown in C.

**Figure 10. Crystal structures of *A. niger* Fdc1 variants in complex with prFMN.** Panels A-E and G-H show the active site in atom colour sticks for Fdc1 variants, with corresponding omit electron density contoured at 3 \( \sigma \). The mutated residues are labeled in red in each case. Panel F shows an overlay of the two E277Q structures, the purple sticks corresponds to the phenylpyruvate derived adduct (as in panel B), while the orange sticks corresponds to the ligand free structure reported in panel D. Panel I shows the active site of the E282 variant in complex with pentafluoro cinnamic acid, with the omit density corresponding to the substrate contoured to 3 \( \sigma \). Panel J shows an overlay of the active site structures of the 5 variants of *A. niger* Fdc1 with the wild type structure (color code: red wild-type, purple E277Q, cyan E277D, blue R173A, green E282Q, yellow E282D), with the three conformations adopted by residue E282 labelled I, II, III. Hydrogen bonding is indicated by black dotted lines, and corresponding distances between key residues are shown for each panel, the distances shown for panel J correspond to wild-type structure.

**Figure 11. UV-visible spectra and kinetic analysis for *S. cerevisiae* Fdc1 mutants.** A UV-VIS spectrum is shown for each variant of the three conserved active site residues, order in individual panels: E285 variants (A), E280 variants (B) and R175 variants (C). Panel D shows a plot of activity over time for *S. cerevisiae* Fdc1 wild type in the presence and absence of light exposure. Panels G-H represent steady state kinetics for wild-type, E285D, E280D and E280Q *S. cerevisiae* Fdc1 variants. Values are reported as apparent due to possible variations in level of prFMN\textsuperscript{iminium} incorporation. Concentration of wild-type Fdc1 in assay: 65 nM. Concentration of E285D, E280D and E280Q Fdc1 in assay: 630 nM, 400 nM and 680 nM respectively.

**Figure 12. Crystal structures of *S. cerevisiae* Fdc1 variants in complex with prFMN.** Panels A and B show the prFMN and key residues in atom coloured sticks. The corresponding omit electron density is shown contoured at 3 \( \sigma \). Panel C shows an overlay of 2 Fdc1 variants (R175A in red, E285D in green) with the wild type structure (orange). Key hydrogen bonding distances between key residues are shown on each panel, the distances shown on panel C correspond to the wild-type structure.

**Figure 13. Mechanistic proposal for prFMN oxidative maturation and the role of E277 in catalysis.** A Proposed mechanism for maturation of prFMN\textsuperscript{red} to prFMN\textsuperscript{iminium} in Fdc1 B Schematic showing the influence of residue 277 on the charge state of residues 173 and 282 in *A. niger* Fdc1. Distinct positions of residue E282 corresponding to the presence or absence of a substrate carboxylate group are numbered I and III, and correspond to labels in figure 10J.
Fig 1.

A

B

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Fig 2.

A) Graph showing % Activity over Time/min for different conditions.

B) Graph showing Absorbance against Wavelength (nm) for different samples.

C) and D) Molecular models demonstrating structural changes.

E) and F) Enlarged views of the molecular structures.
Fig 3.
Fig 4A.

Fig 4B.
Role of Conserved Residues in Fdc1

Fig 5.
Fig 6.

A

B

C

D

Role of Conserved Residues in Fdc1

Wild Type

E277D

E282D

[cinnamic acid] (µM)

k_{cat app} (s^{-1})

k_{cat app} (s^{-1})

k_{cat app} (s^{-1})

k_{cat app} (s^{-1})
Role of Conserved Residues in Fdc1

Fig 7.
Fig 8.
Fig 9.
Fig 10.
Fig 11.
Role of Conserved Residues in Fdc1

Fig 12.
Fig 13.

A

B

II/III

I

II/III

I
The role of conserved residues in Fdc decarboxylase in prenylated flavin mononucleotide oxidative maturation, cofactor isomerisation and catalysis
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