New cofactor supports α,β-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition

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The bacterial *ubiD* and *ubiX* or the homologous fungal *fdc1* and *pad1* genes have been implicated in the non-oxidative reversible decarboxylation of aromatic substrates, and play a pivotal role in bacterial ubiquinone (also known as coenzyme Q) biosynthesis1–3 or microbial biodegradation of aromatic compounds4–6, respectively. Despite biochemical studies on individual gene products, the composition and cofactor requirement of the enzyme responsible for *in vivo* decarboxylase activity remained unclear4–7. Here we show that Fdc1 is solely responsible for the reversible decarboxylase activity, and that it requires a new type of cofactor: a prenylated flavin synthesized by the associated UbiX/Pad14,10. Atomic resolution crystal structures reveal that two distinct isomers of the oxidized cofactor can be observed, an isoalloxazine N5-iminium adduct and a N5 secondary ketimine species with markedly altered ring structure, both having azomethine ylide character. Substrate binding positions the dipolarophile enolic acid group directly above the azomethine ylide group. The structure of a covalent inhibitor–cofactor adduct suggests that 1,3-dipolar cycloaddition chemistry supports reversible decarboxylation in these enzymes. Although 1,3-dipolar cycloaddition is commonly used in organic chemistry11,12, we propose that this presents the first example, to our knowledge, of an enzymatic 1,3-dipolar cycloaddition reaction. Our model for Fdc1/UbiX catalysis offers new routes in alkenyl hydrocarbon production or aryl (de)carboxylation.

Decarboxylation is among one of the most common reactions in nature, despite the fact it is inherently difficult to achieve at ambient conditions. This is due to the high energy of the carbanion intermediate that is formed concomitant with carbon dioxide formation. To overcome this challenge, the majority of decarboxylases13 make use of cofactors, including organic cofactors such as flavins, pyridoxal phosphate (PLP), thiamine pyrophosphate (TPP) or metal ions such as Mg2+, Fe2+ or Mn2+. Decarboxylation is also frequently coupled to substrate oxidation. Comparatively few decarboxylases have been shown to require no cofactor and in these selected cases catalysis involves non-oxidative decarboxylation of those substrates for which the corresponding carbanion species can be stabilized using simple inorganic salts, or microbial biodegradation of aromatic compounds4–6, respectively. Despite biochemical studies on individual gene products, the composition and cofactor requirement of the enzyme responsible for *in vivo* decarboxylase activity remained unclear4–7. Here we show that Fdc1 is solely responsible for the reversible decarboxylase activity, and that it requires a new type of cofactor: a prenylated flavin synthesized by the associated UbiX/Pad14,10. Atomic resolution crystal structures reveal that two distinct isomers of the oxidized cofactor can be observed, an isoalloxazine N5-iminium adduct and a N5 secondary ketimine species with markedly altered ring structure, both having azomethine ylide character. Substrate binding positions the dipolarophile enolic acid group directly above the azomethine ylide group. The structure of a covalent inhibitor–cofactor adduct suggests that 1,3-dipolar cycloaddition chemistry supports reversible decarboxylation in these enzymes. Although 1,3-dipolar cycloaddition is commonly used in organic chemistry11,12, we propose that this presents the first example, to our knowledge, of an enzymatic 1,3-dipolar cycloaddition reaction. Our model for Fdc1/UbiX catalysis offers new routes in alkenyl hydrocarbon production or aryl (de)carboxylation.

The *ubiX* and *ubiD* or the related *pad1* and *fdc1* genes have been shown to be responsible for non-oxidative reversible decarboxylation of aromatic substrates4–6 (Fig. 1a). These genes are widely distributed in archaeal, bacterial and fungal genomes7, and *ubiX* and *ubiD* feature in the prokaryotic ubiquinone biosynthetic pathway8–10. Genetic studies have led to the suggestion that both genes encode (redundant) decarboxylases11. However, although UbiX/Pad1 proteins are distantly related to flavin-containing cysteine decarboxylases and have been shown to bind FMN, no *in vitro* decarboxylase activity has been detected12–14. Furthermore, most biochemical studies of UbiD/Fdc1 have also failed to detect *in vitro* activity15,16. Although recent UbiD/Fdc1 structures reveal distinct structural homology to a family of NADH–FMN oxidoreductases, only metal ion binding rather than flavin binding has been reported17. Hence, the composition and putative cofactor requirement of the enzyme responsible for the observed *in vivo* decarboxylase activity remained unclear.

We co-expressed the *Aspergillus niger* *fdc1* gene in *E. coli* with either the associated *A. niger* *pad1* or the homologous *ubiX* from *E. coli*. Although no direct interaction could be detected between the purified Fdc1 and either UbiX or Pad1 proteins, clear differences could be observed between single expressed Fdc1 and the corresponding *ubiX* or *pad1* co-expressed Fdc1 protein (denoted Fdc118–20). Purified Fdc118–20 has distinct spectral properties (Fig. 1b) and catalyses reversible decarboxylation of a wide range of aromatic carboxylic acids *in vitro* (Fig. 1c, d and Extended Data Fig. 1), Similar observations were made for *Saccharomyces cerevisiae* Fdc118–20, although in this case the corresponding Fdc1 singly expressed protein (but not Fdc118–20) weakly binds FMN (Extended Data Fig. 2). During the review stage for this publication, similar observations for the *S. cerevisiae* Fdc1 were reported elsewhere, although the Fdc1 cofactor was not identified20.

Crystal structures of *A. niger*, *Candida dubliniensis* and *S. cerevisiae* Fdc118–20 reveal a heavily modified FMN cofactor is bound by these enzymes (Fig. 2 and Extended Data Fig. 3). The modified FMN phosphoester is bound in complex with metal ions, similar to the distantly related FMN-binding protein from *Methanobacterium thermoautotrophicum*21. The electron density suggests both Mn2+ and K+ ions are present, and Mn2+ binding can be detected using EPR (Extended Data Fig. 4). Early studies on *E. coli* UbiD revealed activation by both Mn2+ and an unidentified cofactor22. The electron density for the modified FMN reveals extensive modification has occurred at both N5 and C6 positions, effectively adding a fourth ring to the isoalloxazine moiety (Fig. 2b). The atomic resolution obtained for *A. niger* Fdc118–20 allows identification of the cofactor structure as an isopentenyl-adduct to the flavin N5–C6 or prenylated FMN (prFMN) (Fig. 2c). The branched nature of the isopentenyl adduct and the position of the covalent linkages with the flavin suggest this modification is achieved through prenylation. In the accompanying paper20, we show that UbiX indeed acts as a flavin prenylase enzyme that can support Fdc1 activation *in vitro*. Activation is dependent on the presence of oxygen, suggesting the reduced prFMN UbiX product is oxidized to the corresponding flavo-N5 iminium adduct (prFMNiminium)20, (Fig. 2d). The presence of the oxidized prFMN was confirmed by high-resolution mass spectrometry of both the isolated cofactor and native mass spectrometry of the Fdc118–20 protein (Extended Data Fig. 5). Furthermore, complete inactivation of Fdc118–20 was achieved using the mild reductant sodium cyanoborohydride. Upon reoxidation of the sodium cyanoborohydride inactivated enzyme, a radical species was formed with spectral and EPR properties similar to that of the prFMN− radical species detected during non-physiological oxidation of the UbiX-prFMN complex10 (Fig. 2e and Extended Data Fig. 4).

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Furthermore, the amount of prFMN• radical formed is proportional to the levels of enzyme activity obtained before cyanoborohydride inactivation (Fig. 2f), suggesting that enzyme inactivation occurs by modification (probably hydrolysis) of the active prFMN species.

Marked heterogeneity of the prFMN\textsuperscript{ox} cofactor is observed in the \textit{A. niger} Fdc1\textsubscript{UbiX} wild-type crystal structures, with older crystal samples revealing an increased population of a hydroxylated prFMN\textsuperscript{minimum}. The latter species could also be observed by high-resolution mass spectrometry (Extended Data Fig. 5a). Surprisingly, a distinct prFMN\textsuperscript{ox} isomer (prFMN\textsuperscript{ketimine}) can be observed for most \textit{A. niger} Fdc1 crystals, although it is not detected in the \textit{C. dubliniensis} or \textit{S. cerevisiae} crystals (Fig. 3a). This form contains an altered isoalloxazine ring structure, a likely consequence of isomerization of the extended ring system during oxidative maturation of the prFMN cofactor. We propose this isomerization step occurs during the stepwise oxidation of the reduced prFMN UbiX product, via a radical mechanism (Extended Data Fig. 6). The isomerization leads to insertion of the prenyl C1' carbon into the flavin isoalloxazine ring, creating a central seven-membered ring and converting the N5-iminium into a secondary ketimine. The conformation derived from density functional theory (DFT) calculations for the proposed prFMN\textsuperscript{ketimine} species is in close agreement with that observed in the crystal structure. The calculations indicate this species is 30–40 kJ mol\textsuperscript{–1} lower in gas phase free energy than the prFMN\textsuperscript{minimum} species and can exist in two butterfly bent-like conformations of similar energy (Extended Data Fig. 7).

To obtain insights into the catalytic mechanism, \textit{A. niger} Fdc1\textsubscript{UbiX} crystals were soaked with a range of \textit{trans}-cinnamic-acid-related compounds. The corresponding enzyme–substrate complexes clearly

Figure 1 | Fdc1 solution data. \textbf{a}, Schematic overview of the reaction catalysed by Fdc1/Pad1 and the related UbID/UbiX proteins. \textbf{b}, UV-visible spectra obtained for heterologously expressed \textit{A. niger} Fdc1, with and without co-expression of a \textit{ubix} gene. \textbf{c}, UV-visible observation of the enzymatic conversion of cinnamic acid to styrene via decarboxylation by Fdc1\textsubscript{UbiX}. The initial spectrum of cinnamic acid shows a $\lambda_{\text{max}}$ of 270 nm. Over time, successive spectra show reduction of the 270 nm peak and appearance of a peak at 245 nm corresponding to styrene formation. \textbf{d}, Steady-state kinetic parameters obtained for Fdc1\textsubscript{UbiX} for sorbic acid and a variety of cinnamic acid-type compounds (error bars are s.e.m., $n = 3$).

Figure 2 | Fdc1\textsubscript{UbiX} crystal structures. \textbf{a}, Crystal structure of the \textit{A. niger} Fdc1\textsubscript{UbiX}. The monomer present in the asymmetric unit is depicted in cartoon format (helices in green, sheets in blue) while the symmetry related monomer forming the Fdc1\textsubscript{UbiX} dimer is shown in grey. \textbf{b}, Detailed view of Mn\textsuperscript{2+} binding site, linking the modified FMN phosphate group to the protein. \textbf{c}, Detailed view of the modified FMN cofactor. Omit electron density map corresponding to the bound cofactor contoured at 5σ. A fourth ring (non-isoalloxazine derived atoms are shown in green) can clearly be observed. An asterisk indicates the position of additional weak electron density at the C1 position, which can be accounted for by partial hydrolysis. Key residues involved in polar interactions with the isoalloxazine derived cofactor moiety are shown as sticks. \textbf{d}, Chemical structure of the modified FMN bound by \textit{A. niger} Fdc1\textsubscript{UbiX} as derived from atomic resolution density and high-resolution mass spectrometry (Extended Data Fig. 5). Atoms derived from dimethylallyl-monophosphate are shown in red\textsuperscript{10}. The azomethine ylide resonance form is shown below. \textbf{e}, Oxidation of cyanoborohydride inactivated Fdc1\textsubscript{UbiX} leads to formation of a purple species, similar in UV-visible and EPR spectral properties (Extended Data Fig. 4) to that observed for the oxidized UbiX–prFMN complex\textsuperscript{10}. \textbf{f}, Following purification, Fdc1\textsubscript{UbiX} activity gradually decreases when incubated on ice, and formation of the purple prFMN radical following NaBH\textsubscript{4}CN treatment follows a similar trend (error bars are s.d., $n = 3$).
reveal substrates are stacked directly above the prFMN\textsuperscript{ox} ring system (Fig. 3b, c). Electron density for both the iminium and ketimine species can be detected in the atomic resolution density maps, the latter adopting a more planar conformation of the extended ring system in order to accommodate substrate binding (Fig. 3b, c and Extended Data Fig. 7). The substrate enoic acid double bond is positioned directly above the C4a of both the prFMN\textsuperscript{ox} species. Although the aryl-group of all substrates tested occupies a similar position directly above the prFMN N1 atom, electron density corresponding to the carboxylate moiety is distinct for each substrate (Fig. 3c). Although clear electron density for the pentafluorocinnamic acid carboxylate is lacking, both \(\alpha\)-methylcinnamic acid and \(\alpha\)-fluorocinnamic acid carboxylate groups can be observed. Both occupy a position vacated by reorientation of the conserved E282, and establish polar contacts with R173 and the amide nitrogen of M283. In the case of \(\alpha\)-methylcinnamic acid, the carboxylate is within hydrogen bonding distance of the N5 secondary ketimine group of the prFMN\textsuperscript{ketimine} form. A complex with the decarboxylate is within hydrogen bonding distance of the N5 secondary amide nitrogen of M283. In the case of \(\alpha\)-methyl-cinnamic acid, the \(\alpha\)-hydroxystyrene prFMNiminium adduct superimposes with a bound CO\(_2\) molecule.

At first glance, the position of the substrate \(\alpha,\beta\)-unsaturated carbonyl directly above the prFMN\textsuperscript{ox} C4a suggests the possibility of transient formation of a prFMN\textsuperscript{ox} C4a substrate \(\beta\)-carbon bond reminiscent of Michael addition-like chemistry and other flavin-catalysed reactions\textsuperscript{22}. This mechanism has similarities to the amidohydrolase-type decarboxylases, where nucleophilic attack by a metal-bound hydroxide ion is postulated to lead to transient C=\(\equiv\)C bond cleavage of an enolic acid moiety leading to decarboxylation\textsuperscript{13,23}. In the case of Fdc1, C=\(\equiv\)C bond cleavage should proceed with concomitant protonation of the \(\alpha\)-carbon to allow decarboxylation. Our structural data suggests the N5 secondary ketimine of prFMN\textsuperscript{ketimine} could act as a probable acid-base catalyst, providing a rationale for both FMN modification and rearrangement (Fig. 4a and Extended Data Fig. 7). However, although our solution mass spectrometry and chemical inhibition data cannot distinguish between the iminium and ketimine cofactor isomers, solution data presented in Fig. 2e, f suggests prFMN\textsuperscript{iminum} is the catalytically relevant species.

To determine which prFMN\textsuperscript{ox} isomer supports catalysis by Fdc1\textsuperscript{UbiX} and provide further insight into the likely mechanism, we sought to determine the structure of a covalent substrate–cofactor adduct. When \textit{A. niger} Fdc1\textsuperscript{UbiX} protein is incubated with phenylpyruvate, for which the corresponding enol-tautomer \(\alpha\)-hydroxycinnamic acid (present as a minor population in solution) closely resembles the cinnamic acid substrate, the UV-visible spectrum is altered (Fig. 4b). Similar observations are made when incubating with phenylacetaldehyde. Furthermore, incubation with phenylpyruvate or phenylacetaldehyde leads to reversible enzyme inhibition, with a gradual increase of enzyme inhibition data cannot distinguish between the iminium and ketimine cofactor isomers, solution data presented in Fig. 2e, f suggests prFMN\textsuperscript{iminum} is the catalytically relevant species.

Figure 3 | Fdc1\textsuperscript{UbiX} cofactor structure and ligand complexes. a, Omit electron density map corresponding to a distinct isomer of the prFMN contoured at 5\(\sigma\). An expansion of the central ring of the isoalloxazine system can clearly be observed, with the distinct butterfly bent conformation accompanied by altered conformation of the ribityl moiety. b, Detailed view of prFMN\textsuperscript{ox} isoforms in the Fdc1\textsuperscript{UbiX}–substrate complexes. The omit electron density maps corresponding respectively to the prFMN\textsuperscript{iminium} (in blue) and prFMN\textsuperscript{ketimine} (in red) species each contoured at 5\(\sigma\) are shown for the \(\alpha\)-methyl-cinnamic acid complex. c, A series of Fdc1\textsuperscript{UbiX} substrate/product complexes. Selected active site residues of Fdc1\textsuperscript{UbiX} are shown in atom colour sticks, with the omit electron density contoured at 5\(\sigma\) corresponding to \(\alpha\)-methyl-cinnamic acid, pentafluorocinnamic acid, \(\alpha\)-fluorocinnamic acid and 4-vinyl-guaiacol, respectively.
This observation further confirms that prFMN$^{\text{iminium}}$ is the catalytically relevant species, and the unexpected presence of a C1$^-$$^-$ substrate $^b$-carbon bond suggests a distinct mechanism from that of the amidohydrolase-type decarboxylases occurs (Fig. 4d). Fluorination of the substrate $^b$-carbon or the aromatic group has only a modest effect on catalysis (Fig. 1d). Furthermore, simple enoic acids also act as substrates, as prolonged incubation of Fdc1UbiX with these substrates does lead to formation of the corresponding terminal alkenes ($^a$-olefins) at modest levels (Extended Data Fig. 8). Both observations argue against formation of transient ionic species.

The neutral form of the prFMN$^{\text{iminium}}$ has distinct azomethine ylide character (a well-known 1,3-dipole) that is positioned directly adjacent to the substrate $^a$$^-$-$^b$-unsaturated carbonyl (a dipolarophile) by the enzyme. Thus, we propose a 1,3-dipolar cycloaddition between the substrate and the prFMN$^{\text{iminium}}$ azomethine ylide form leads to a transient covalent substrate–prFMN$^{\text{iminium}}$ pyrrolidine adduct, establishing covalent bonds with both C1$^a$ and C4a (species II). Ample precedent is available for the concerted 1,3-dipolar cycloaddition between azomethine ylides and $^a$$^-$-$^b$-unsaturated carbonyls in organic chemistry.$^{11,12}$ Whether the proposed Fdc1-mediated cycloaddition occurs through a single pericyclic transition state cannot be established at this stage. Fragmentative decarboxylation (Grob-type fragmentation$^{24}$) of the pyrrolidine adduct II can occur coupled to breaking the $^b$-carbon-prFMN C4a bond. This leaves a single bond connecting the substrate $^a$-carbon with the C1$^a$ of prFMN$^{\text{iminium}}$ (species IIIa)

We propose protonation by E282 concomitant with formation of a second pyrrolidine adduct (species V) leads to product formation (species VI) via a retro 1,3-dipolar cycloaddition. Together with R172 and E277, E282 forms a network of polar interactions that is conserved throughout the UbiD/Fdc1 family. The R173A, E277Q and E282Q variants of Fdc1UbiX are all inactive, and display altered UV-visible spectrum properties, suggesting these mutations also affect cofactor maturation (Extended Data Fig. 9).

The modification of the flavin isoalloxazine ring through prenylation at N5 and C6, followed by oxidation strikingly alters and expands the flavin chemical repertoire.$^{22}$ Oxidation of the N5-prenyl adduct bond leads either to the corresponding iminium adduct or, when coupled to ring isomerization, a secondary ketimine adduct. Both species have azomethine ylide character and distinct catalytic

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**Figure 4** Insights into the Fdc1$^{\text{UbiX}}$ mechanism. a, Proposed mechanism for Fdc1$^{\text{UbiX}}$ catalysis using the prFMN ketimine form. b, Incubation of Fdc1$^{\text{UbiX}}$ with phenylpyruvate leads to changes in the UV-visible spectrum. The inset shows conversion of cinnamic acid to styrene (monitored at 270 nm) after addition of phenylpyruvate treated Fdc1$^{\text{UbiX}}$. c, Active site of Fdc1$^{\text{UbiX}}$ in complex with a phenylpyruvate derived adduct. The omit electron density map (contoured at 7$\sigma$, in blue) corresponding to the prFMN$^{\text{iminium}}$-phenylpyruvate adduct is shown. Atoms derived from phenylpyruvate are depicted in green. d, Proposed mechanism for Fdc1$^{\text{UbiX}}$ catalysis and phenylacetaldehyde adduct formation of the prFMN$^{\text{iminium}}$ form.
potential. In the Fdc1/UbiD enzyme family, the prFMX_{minimum} form supports reversible decarboxylation of a wide range of (aromatic) substrates. Although the [3+2] reaction between azomethine ylide dipoles and alkene dipolarophiles has been extensively used in organic chemistry,\(^{1,12}\) the mechanism proposed here would present the first example, to our knowledge, of a biological [3+2] reaction. Combined with the recent description of both natural\(^{3}\) and artificial\(^{36}\) bona fide [4+2] cycloaddition catalysing enzymes, this hints at more widespread use of pericyclic reaction chemistry in nature. As Fdc1/UbiD enzymes have evolved from an NADH-FMN oxidoreductase module,\(^{2,11}\) the possibility exists that other unrelated prFMN-dependent enzymes might have developed from distinct flavin-binding modules. Such distinct prFMN-dependent enzymes could make use of different aspects of the prFMN chemistry, as occurs with other organic cofactors.\(^{27}\) Artificial flavoenzymes containing N5-alkylated flavins have been created that are capable of \(\text{H}_2\text{O}_2\)-driven enantioselective sulfoxidations,\(^{28}\) using the fact that N5-alkylated flavins can form remarkably stable \(4\alpha\)-peroxylflavins and are powerful oxidising catalysts.\(^{29}\) It is possible similar enzymes already exist in nature.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Contributions** K.A.P.P. carried out molecular biology, biophysical and structural biology studies of A. niger Fdc1. C.B.K. carried out molecular biology experiments underpinning biological and structural biology studies of *S. cerevisiae* Fdc1 performed by M.D.W. K.F. and S.E.J.R. performed and analysed EPR experiments. S.H. performed DFT calculations. N.J.W.R., D.K.T. and R.G. undertook liquid chromatography–mass spectrometry of extracts and interpreted the data on substrate–product species. R.B. and P.B. performed native mass spectrometry. S.S.B. solved the C. dublinensis Fdc1 structure. All authors discussed the results with N.S.S. and D.P. and all participated in writing the manuscript. D.L. initiated and directed this research.

**Author Information** Coordinates and structure factors have been deposited in the Protein Data Bank under accession numbers 4Z2A, 4Z4S, 4Z4T, 4Z4V, 4Z4B, 4Z4A, 4Z4C and 4Z2D. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.L. (david.leys@manchester.ac.uk).

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METHODS

Cloning. The S. cerevisiae. C. dubliniensis and A. niger fdc1 genes were codon optimized and synthesized (GenScript). A. niger fdc1 gene was cloned into the Ndel and XhoI sites of PET30a and the S. cerevisiae and C. dubliniensis genes were cloned into the Ndel and XhoI sites of PET21b. E. coli ubxK was cloned into the Ndel and XhoI sites of PET21b and PET30a. A. niger fdc1 PET30a was transformed into E. coli BL21(DE3) with and without ubx PET21b and S. cerevisiae and C. dubliniensis fdc1 PET21b were transformed into E. coli BL21(DE3) with and without ubx PET30a.

Mutagenesis. Mutagenesis primers were designed using the QuikChange Primer Optimizer at 260 nm, sorbic acid at 260 nm. Extinction coefficients for each substrate were calculated with a DB-WAX column (30 m × 0.32 mm i.d., 5 pm). The rate of 1-alkenes produced during the enzyme reactions. The column temperature was programmed as follows: 40 °C hold for 2 min, to 150 °C at 20 °C min⁻¹. The injector temperature was set at 250 °C. The carrier gas was helium at a flow rate of 1 ml min⁻¹. Product identification was achieved by comparison with pure 1-alkenes standards.

Carboxylation HPLC assays. Assays containing 100 mM sodium nitrite, 100 mM NaPi at pH 6, 3 M NaCl, centrifuged at 16,100 g for 10 min and analyzed by HPLC as previously described.

UV-visible spectroscopy/protein quantification. UV-visible absorbance spectra were recorded on a Cary UV-2500 spectrophotometer. Protein concentration of A. niger Fdc1 and S. cerevisiae Fdc1 was estimated using the E280 equation: E280 = 68,870 M⁻¹ cm⁻¹ and E280 = 63,830 M⁻¹ cm⁻¹ (calculated from the primary amino acid sequence using the ProtParam program on the ExpASy proteomics server).

UV-visible decarboxylation assays. For substrates with two or more double bonds conjugated to the acid group initial rates were determined by following consumption of substrate by UV-visible spectroscopy using a Cary 50 Bio-spectrophotometer (Varian). Assays were performed with different 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 2,3,4,5,6-penta-fluorocinnamic acid consumption was monitored at 260 nm, Δν-fluorocinnamic acid at 265 nm, cinnamic acid at 270 nm and ultraviolet absorption spectra recorded.

UV-visible spectra and the EPR spectra were recorded. A. niger Fdc1 samples were added to an EPR tube and frozen in liquid nitrogen. EPR spectra were obtained using a Bruker ELEXYS E500 EPR spectrometer equipped with a Super High Q (ER 4118-SHO) resonator. Temperature control was achieved using an Oxford Instruments ESR900 cryostat connected to an ITC 503 temperature controller from the same manufacturer. Protein crystallization and structure determination. Crystallization was performed by vapour diffusion. A. niger Fdc1 in 100 mM NaCl, 25 mM Tris pH 7.5 and 0.3 mM 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 to prepare an optimization screen based around 0.2 M potassium thiocyanate, Bis-Tris propane 6.5, 20% w/v PEG 3350 mixing 0.05 μl seed stock, 0.25 μl protein solution and 0.3 μl reservoir solution at 4 °C. For S. cerevisiae Fdc1, crystals were obtained using 0.5 μl 2 mg ml⁻¹ in 20 mM potassium phosphate, 200 mM NaPi pH 7.5 and 0.3 mM of 0.2 M calcium acetate, 20% PEG 3000 at 4 °C. For C. dubliniensis Fdc1 were obtained at 4 °C using 0.3 μl 18 mg ml⁻¹ C. dubliniensis Fdc1 in 100 mM NaCl, 1 mM MnCl₂, 25 mM Tris pH 7.5 and 0.3 μl of 0.2 M potassium thiocyanate, sodium acetate pH 5.5 and 15% w/v PEG 4000.

All crystals were co-crystallized in mother liquor supplemented with 10% PEG 200 and flash cooled in liquid nitrogen. Diffraction data was collected at Diamond beamlines at 100K and processed using the CCP4 suite33. Data was reduced and scaled using XDS34. Following molecular replacement using IP2 as a starting model, the structures of wild-type A. niger and S. cerevisiae Fdc1 and complexes were refined using REFMAC5 (ref. 32) and refined by cycles of manual rebuilding in COOT. Ligand coordinates and definitions were generated using the GlycoBioChem PRODRG2 server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg). The data and refinement statistics are available in Extended Data Table 1. Native mass spectrometry. Protein was buffered exchanged into 100 mM ammonium acetate using a 10-μg desalting column (BioRad). Native mass spectrometry experiments were carried out on a Synapt G2 instrument (Waters, Manchester, UK) with a nanoelectrospray ionization (nESI) source. Mass calibration was performed by a separate infusion of Nal cluster ions. Solutions were ionized through a positive potential applied to a platinum wire of thickness 0.125 mm (Goodfellow) inserted into a thin-walled glass capillary (inner diameter 0.9 mm, outer diameter 1.2 mm, World Precision Instruments, Stevenage, UK) that was pulled to provide a nESI tip in house with a flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA). Fdc1 samples (5–10 μM) were sprayed from 100 mM ammonium acetate pH 6.8. Capillary voltage 1.6 kV, sample cone voltage 80–90 V, extractor cone voltage 3 V, backing pressure 5 mbar, source temperature 50 °C, trap gas flow 120 ml min⁻¹. Data were processed using Masslynx V4.1 software and Originlab 9.0.

Mass spectrometry analysis of the extracted Fdc1 cofactor. Protein was buffer exchanged into deionised H2O using a 10-μg column. 4 μl 1 M ammonium acetate was added to 200 μl protein solution. The protein was precipitated and cofactor released with addition of 200 μl acetonitrile and incubation at 70 °C for 5 min before centrifugation at 16,100 g to remove the precipitation.

All solvents were of HPLC-MS grade purity purchased from Sigma-Aldrich (Gillingham, UK). HPLC grade formic acid was purchased from Fisher Scientific (Loughborough, UK). Mass spectrometer calibration solution and chromatography columns were purchased from Thermo-Fisher Scientific (Hemel Hempstead, UK).

All UHPLC-MS work was carried out on a Thermo-Finnigan Orbitrap-ITQ XL hybrid mass spectrometer operated in negative ionization mode coupled to a Thermo Accela autosampler (Fisher Scientific, Bremen, Germany). Chromatographic separations were adapted from work carried out by Fu and co-workers35 and performed on a Thermo Hypersil Gold 2.1 mm C18 column at a solvent flow rate of 400 μl min⁻¹. For initial profiling tests the column was eluted with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The solvent composition during gradient elution was initiated with 5% (B) for 5 min and subsequently ramped to 95% (B) over 15 min, followed by a 5 min isocratic elution at 95% (B) before a return to 95% (A) held for further 5 min for column equilibration. All samples were maintained at 4 °C within the autosampler refrigerator while the column was maintained at 50 °C within the autosampler oven. Mass calibration was carried out in accordance with the manufacturer’s guidelines using
caffeine (20 μg ml⁻¹), the tetrapeptide MRFA (1 μg ml⁻¹) and Ultramark 1621 (0.001%) in an aqueous solution of acetonitrile (50%), methanol (25%) and acetic acid (1%). Acquisition settings for initial profiling were carried out at 60,000 resolution in centroid and ran at 1 μ-scan per 400 ms in the 100–1,000 m/z range with source gasses set at sheath gas = 40 arbitrary units, aux gas = 5 arbitrary units, sweep gas = 5 arbitrary units. The ESI source voltage was set to 4.2 V, and capillary ion transfer tube temperature set at 275 °C.

Mass fragmentation analysis was carried out with the same column chemistry, source settings and flow rate but with an isocratic solvent elution of 55% (A)/45% (B), an optimum composition determined for eluting the analyte of interest, based on the initial elution profile described above. Mass spectrometry source, samples storage/column was kept under identical conditions. Collision induced dissociation (CID) settings were set up to trap ions 523 m/z with an isolation width of 1.0 m/z, normalized collision energy of 35, activation Q of 0.250 and activation time of 30 ms.

DFT calculations. Density function theory (DFT) models were optimized in the gas phase using the B3LYP/6-311++G(d,p) or BH&H/6-311++G(dp) level of theory implemented in Gaussian 09 (ref. 35). Models are shown in Extended Data Fig. 7 and structural alignments to the crystal coordinates were performed using Swiss-PdbViewer version 4.1 (ref. 36). Harmonic vibrational frequencies calculated using normal mode analysis were used to confirm that optimized geometries were always in local or global minima, and to calculate relative free energies.

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

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Extended Data Figure 1 | HPLC chromatogram demonstrating enzymatic carboxylation of styrene by *A. niger* Fdc1^UbiX^. Chromatogram of a 10 mM styrene and saturating NaHCO₃ solution incubated in the absence (blue) and presence (red) of the *A. niger* Fdc1^UbiX^ enzyme.
Extended Data Figure 2 | *S. cerevisiae* Fdc1UbiX solution data. The top panel is a direct comparison to main Fig. 1b. In this case, the non-UbiX coexpressed Fdc1 binds FMN weakly. The bottom panel shows *S. cerevisiae* Fdc1UbiX steady-state kinetic data obtained with cinnamic acid. Error bars are s.e.m., *n* = 3.
Extended Data Figure 3 | A detailed view of the *S. cerevisiae* and *C. dubliniensis* Fdc1 active site. The omit map corresponding to the prFMN cofactor is shown in a blue mesh contoured at 3σ. Atoms derived from dimethylallyl-monophosphate are shown in green. Both Fdc1 crystals appeared colourless as observed for *A. niger* Fdc1, indicating negligible FMN binding.
Extended Data Figure 4 | EPR spectroscopy confirms Mn$^{2+}$ binding. Left panel shows X-band continuous wave frozen solution EPR spectra of (A) Fdc1$^{\text{UbiX}}$ and (B) Mn(H$_2$O)$_6^{2+}$ showing the characteristic six line pattern arising from the $m_I = \pm 1/2$ spin manifold of the $S = 5/2$ Mn$^{2+}$ ion. The six line pattern reflects hyperfine coupling to the $I = 5/2$ $^{55}$Mn nucleus and is sensitive to the environment of the ion, thus the differences between A and B indicate binding of Mn$^{2+}$ to the enzyme. Experimental conditions: microwave power 0.5 mW, modulation amplitude 7 G, temperature 20 K. The right panel shows X-band continuous wave frozen solution EPR spectra of (A) Fdc1$^{\text{UbiX}}$ reduced using sodium cyanoborohydride (NaBH$_3$CN) and subsequently exposed to air and (B) wild-type UbiX + DMAP reduced with dithionite and reoxidised with oxygen. Experimental conditions: microwave power 10 μW, modulation amplitude 1.5 G, temperature 20 K. Reduction using NaBH$_3$CN of the modified cofactor formed in Fdc1$^{\text{UbiX}}$ (prFMNiminium) gives rise to a radical on air oxidation with the same g value ($g_{\text{av}}$) and line width as that formed on the modified flavin (prFMN) in UbiX. However, the lack of the distinctive fine structure in A that is normally observed for the UbiX radical B, suggests heterogeneity in the Fdc1$^{\text{UbiX}}$ radical or possibly a magnetic interaction with the nearby Mn$^{2+}$ ion in Fdc1.
Extended Data Figure 5 | Mass spectrometry of the Fdc1\textsuperscript{UbiX} cofactor.  
a, (i) Structural elucidation of the Fdc1\textsuperscript{UbiX} cofactor. Full scan total ion current (TIC) created under a gradient elution using H\textsubscript{2}O/acetonitrile both containing 0.1% formic acid indicating a major peak apex at 9.53 min with a 52/48 solvent composition. Also shown is the proposed structure of 523 m/z. Mass spectrum taken at major peak apex in (ii) (9.53 min) indicating an associated full-scan molecular ion peak with $m/z = 523.1589$ (M$^+$ = C$_{22}$H$_{28}$N$_4$O$_9$P) at a resolution of 58,501 with a mass accuracy of 0.06 p.p.m. Also eluting alongside the target molecule are two isotopic variants containing $^{13}$C and $^{15}$N. The $^{13}$C peak is displaying a relative abundance of 22 to the 523 m/z peak that is in line with the number of carbon atoms contained within the structure. Fragmentation of the 523.16 m/z molecular ion peak in (iii) in an automated data dependent manner using helium based-chemical induced dissociation (CID level 35) generated a spectral tree that indicates the removal of the phosphate head group at the MS$^2$ level generating 425.18 m/z. Subsequent MS$^3$ level activation (CID 35) on 425.18 m/z partially (A) or completely (B) removing the tail group from the newly formed four-ring system generating 335.13 m/z or 309.09 m/z, respectively. The presence of a hydrolysed species with 541 m/z is also reported (iv). 
b, Native mass spectra of Fdc1 (top) and Fdc1\textsuperscript{UbiX} (bottom). Fdc1 presents in charge states 19$^+$ to 23$^+$, while Fdc1\textsuperscript{UbiX} presents in charge states 19$^+$ to 22$^+$. Right-hand spectrum; an enlarged view of the 21$^+$ charge state. The predicted masses are shown by blue dashed lines. The spectrum of Fdc1\textsuperscript{UbiX} shows that approximately two-thirds of the ions have two non-covalently bound cofactors, approximately one-third have one non-covalently bound cofactor and there is a small amount with no cofactor bound. Fdc1 contains no cofactor. The measured mass of the Fdc1 dimer is 112,265 Da (predicted mass from sequence is 112,270 Da). The measured mass for the apo form of Fdc1\textsuperscript{UbiX} is 112 345 Da, slightly higher than for Fdc1 which is attributed to an increased retention of salt. The mass difference of +80 Da corresponds to the mass of two potassium adducts. For the Fdc1\textsuperscript{UbiX} species with one bound cofactor, the measured mass (112,968 Da) is 178 Da higher than predicted. The predicted mass corresponds to the left hand side of the peak, which is the protein plus cofactor with no extra salt retained. The extra mass could be attributed to 2 Mn$^{2+}$ ions and 2 K$^+$ ions. The Fdc1\textsuperscript{UbiX} bound to two cofactors has a measured mass of 113,583 Da, which is 268 Da larger than expected. Again, however, the predicted mass corresponds to the left-hand side of the peak. These spectra indicate that the protein dimer carries either 1 or 2 cofactors of 525 Da, along with a variety of other salt adducts. The extent of adductation is higher for Fdc1\textsuperscript{UbiX} and increases with bound cofactors, indicating that the addition of the cofactor recruits counter ions.

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Extended Data Figure 6 | A proposed mechanism for cofactor maturation in Fdc1 UBIX through oxidation. We propose the prF MN\textsuperscript{red} cofactor produced by UbiX is bound by apo-Fdc1 and oxidized in a stepwise manner. Although the initial radical species resembles that observed during non-physiological oxidation of the prF MN\textsuperscript{red}–UbiX product complex\textsuperscript{10}, we propose that in the Fdc1 enzyme proton abstraction from the prF MN\textsuperscript{radical} C1\textsuperscript{'} leads to a distinct radical species. The latter can either be oxidized further to the corresponding prF MN\textsuperscript{iminium} or, via a radical based isomerization process, to form the central seven-membered ring ultimately leading to prF MN\textsuperscript{ketimine}. 
Extended Data Figure 7 | DFT models of the proposed prFMNi
ominum and prFMNketimine isomers and their substrate adducts. a, Chemical structures of the prFMNi
ominum and prFMNketimine models. DFT models (pink carbons) are overla
id with crystal coordinates (green carbons) of prFMNi
ominum (b) and the two butterfly bent conformations of prFMNketimine (c, d). c, d, The substrate-free (bent ‘up’) prFMNketimine is shown in c and the more planar substrate-bound (bent ‘down’) in d. For comparison of the extent of the butterfly bending of prFMNketimine, the two DFT models in c and d are aligned over the four-ring nitrogen atoms and overlaid in e, f. Chemical structures of the proposed initial prFMNi
ominum and prFMNketimine substrate adducts with the cinnamic acid substrate highlighted in red. The DFT-optimized structures of these species are overlaid in g with the prFMNi
ominum species shown with pink carbons and the prFMNketimine species with teal carbons. Three projections of overlaid DFT models of the prFMNi
ominum species with substrate bound (pink carbons; reproduced from g), after substrate decarboxylation (teal carbons; substrate double bond is cis) and upon protonation of the substrate β-carbon (green carbons) are shown in h. Note that the substrate carboxylate was artificially protonated in these models to maintain charge neutrality. Models were geometry optimized in the gas phase using either the B3LYP/6-311+ + G(d,p) (panels a–e) or BH&H/6-311+ + G(d,p) (panels f–h) level of theory. BH&H was chosen over B3LYP for the substrate adducts as BH&H has been shown to better describe π-stacking interactions, which are likely to occur between the modified isobaloxazine and substrate phenyl moieties37. Harmonic vibrational frequencies calculated using normal mode analysis were used to confirm that optimized geometries of all species were in local or global minima. Absolute energies and relative free energies of the substrate-free species, determined from the normal mode calculations, are given in the table, top right. Cartesian coordinates of the optimized structures are given in the Supplementary Information.
Extended Data Figure 8 | Fdc1 decarboxylase activity with simple acrylic acids. Gas chromatogram showing products formed from a solution of respectively 10 mM 2-hexenoic (1-pentene, blue; enzyme free control in black), 10 mM 2-heptenoic (1-hexene, green; enzyme free control in black) or 10 mM 2-octenoic acid (1-heptene, red; enzyme free control in black). Identification of 1-alkenes was based on known standards.
Extended Data Figure 9 | UV-visible spectral properties of Fdc1 variants. UV-visible spectra of Fdc1 (614 μM), Fdc1 Ubix (492 μM), Fdc1 R173A Ubix (749 μM) and Fdc1 E282Q Ubix (171 μM) normalised on the A280 peak. Inset, close up of the additional spectral features present in the 300–500 nm region.
Extended Data Table 1 | Data collection and refinement statistics

|            | A. niger Fdc1 (only perMg present) | A. niger Fdc1 (perMg + α-MAN) | A. niger Fdc1 + α-penta-fluoro cinnamate acid | A. niger Fdc1 + α-fluoro cinnamate acid | A. niger Fdc1 + γ-pheryl pyruvate | A. niger Fdc1 + γ-4-vinyl guaicol | S. cerevisiae Fdc1 | C. albicans Fdc1 |
|------------|-----------------------------------|--------------------------------|-----------------------------------------------|------------------------------------------|-----------------------------------|----------------------------------|------------------|----------------|
| **PDB code** | 4ZA4                             | 4ZA5                           | 4ZA7                                          | 4ZA8                                     | 4ZAB                              | 4ZA9                             | 4ZAC             | 4ZAD           |
| **Data collection** |                                  |                                |                                               |                                          |                                   |                                  |                  |                |
| Space group | P 2, 2 1/2                          | P 2, 2 1/2                     | P 2, 2 1/2                                    | P 2, 2 1/2                               | P 2, 2, 2                         | P 2, 2 1/2                      | P 2, 2 1/2      | P 2, 2 1/2     |
| α, β, γ (Å) | 95.99                             | 96.02                          | 95.93                                         | 95.93                                    | 95.91                             | 95.91                           | 96.05            | 96.59          |
| resolution (Å) | 63.93                             | 63.79                          | 64.16                                         | 64.20                                    | 64.20                             | 64.35                           | 64.47            | 96.18          |
| β (°)     | 87.63                             | 87.72                          | 87.73                                         | 87.79                                    | 87.79                             | 87.68                           | 87.89            | 116.64         |
| R_p (%)   | 48.01-1.10                         | 48.01-1.10                     | 48.01-1.10                                    | 48.01-1.10                               | 48.01-1.10                        | 48.01-1.10                      | 48.01-1.10       | 48.01-1.10     |
| R_factor (°) | 48.01-1.10                        | 48.01-1.10                     | 48.01-1.10                                    | 48.01-1.10                               | 48.01-1.10                        | 48.01-1.10                      | 48.01-1.10       | 48.01-1.10     |
| Completeness (%) | 32.38-1.16                         | 32.38-1.16                     | 32.38-1.16                                    | 32.38-1.16                               | 32.38-1.16                        | 32.38-1.16                      | 32.38-1.16       | 32.38-1.16     |
| Redundancy  | 61.1-1.10                          | 61.1-1.10                      | 61.1-1.10                                     | 61.1-1.10                                | 61.1-1.10                         | 61.1-1.10                      | 61.1-1.10        | 61.1-1.10     |
| **Refinement** |                                  |                                |                                               |                                          |                                   |                                  |                  |                |
| Resolution (Å) | 32.38-1.16                         | 32.38-1.16                     | 32.38-1.16                                    | 32.38-1.16                               | 32.38-1.16                        | 32.38-1.16                      | 32.38-1.16       | 32.38-1.16     |
| No. reflections | 152260                            | 152260                         | 152260                                        | 152260                                   | 152260                            | 152260                          | 152260           | 152260         |
| R_e (R_m) | 14.5/16.1                          | 14.5/16.1                      | 14.5/16.1                                     | 14.5/16.1                                | 14.5/16.1                         | 14.5/16.1                       | 14.5/16.1         | 14.5/16.1      |
| No. atoms  | 6952                              | 6952                           | 6952                                           | 6952                                     | 6952                              | 6952                            | 6952              | 6952           |
| R.m.s. deviations | 9.81                              | 10.71                          | 10.25                                          | 10.25                                    | 10.25                             | 9.83                            | 9.38              | 11.07          |
| Bond lengths (Å) | 0.028                             | 0.028                          | 0.028                                          | 0.028                                    | 0.028                             | 0.028                           | 0.028             | 0.028          |
| Bond angles (°)  | 2.384                             | 2.485                          | 2.469                                          | 2.429                                    | 2.622                             | 2.250                           | 2.304             | 2.467          |

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