UbiX is a flavin prenyltransferase required for bacterial ubiquinone biosynthesis

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Ubiquinone (also known as coenzyme Q) is a ubiquitous lipid-soluble redox cofactor that is an essential component of electron transfer chains2. Eleven genes have been implicated in bacterial ubiquinone biosynthesis, including ubiX and ubiD, which are responsible for decarboxylation of the 3-octaprenyl-4-hydroxybenzoate precursor3,4. Despite structural and biochemical characterization of UbiX as a flavin mononucleotide (FMN)-binding protein, no decarboxylase activity has been detected3,4. Here we report that UbiX produces a novel flavin-derived cofactor required for the decarboxylase activity of UbiD5. UbiX acts as a flavin prenyltransferase, linking a dimethylallyl moiety to the flavin N5 and C6 atoms. This adds a fourth non-aromatic ring to the flavin isoalloxazine group. In contrast to other prenyltransferases6,7, UbiX is metal-independent and requires dimethylallyl-monophosphate as substrate. Kinetic crystallography reveals that the prenyltransferase mechanism of UbiX resembles that of the terpene synthases8. The active site environment is dominated by π systems, which assist phosphate-C1 bond cleavage following FMN reduction, leading to formation of the N5-C1 bond. UbiX then acts as a chaperone for adduct reorientation, via transient carbocation species, leading ultimately to formation of the dimethylallyl C3-C6 bond. Our findings establish the mechanism for formation of a new flavin-derived cofactor, extending both flavin and terpenoid biochemical repertoires.

Flavin is a common cofactor responsible for highly versatile (redox) chemistry9, its properties modified or fine-tuned by the protein scaffold, occasionally through covalent attachment to the protein matrix via the C6 or C8 atoms of the isoalloxazine dimethylbenzene ring10. We have shown that the reversible decarboxylases belonging to the UbiD or the homologous Fdc enzyme family require a heavily modified FMN cofactor2. In these enzymes, the FMN is linked to a C5-alkyl group via N5-C1 and C6-C3 linkages, adding a fourth (non-aromatic) ring to the isoalloxazine ring system. Knockout of ubiD/ fdc1 or the associated ubiX (or the homologous pad1) genes leads to similar phenotypes, and the latter have been shown to encode for FMN-modifying proteins for which no decarboxylase activity has been detected in vitro11,12. Recently it was found that UbiX/Pad1 is responsible for activation of Saccharomyces cerevisiae Fdc112, suggesting that UbiX/Pad1 is responsible for the synthesis of the UbiD/Fdc1 cofactor. The observed FMN modification by a C5-unit suggests this occurs through prenyltransferase activity (Fig. 1a).

We tested this hypothesis by incubating Pseudomonas aeruginosa UbiX13 with oxidized FMN and the universal isoprene precursors dimethylallyl-pyrophosphate (DMAPP) or isopentenyl-pyrophosphate (IPP). However, spectroscopic evidence for formation of a ternary isoprene precursor–FMN–UbiX complex or of covalent FMN modification could not be obtained. By contrast, perturbation in the FMN ultraviolet (UV)-visible spectrum was observed in the presence of dimethylallyl-monophosphate (DMAP), leading to an apparent Kd of 12.0 ± 0.4 μM (Fig. 1b). Although the minor spectral perturbation is indicative of binding close to the FMN isoalloxazine, it does not reflect FMN modification. However, reduction of the ternary FMN–DMAP–UbiX complex using sodium dithionite followed by re-oxidation under aerobic conditions leads to formation of a stable purple-coloured intermediate (Fig. 1c). Ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry of extracts from both the reduced and re-oxidized ternary FMN–DMAP–UbiX complex reveal a molecular species with mass corresponding to the reduced (prenylated-FMNreduced or prFMNreduced) or radical (prFMNreduced) form of the UbiD/Fdc1 cofactor, respectively (Extended Data Fig. 1) and electron paramagnetic resonance (EPR) spectroscopy confirms the formation of a radical in ~95% yield (relative to FMN) in the reoxidized ternary complex (Fig. 1d). Furthermore, the radical species has EPR properties consistent with that of a N5, C5 alkylated flavin semiquinone (Extended Data Figs 2–4). Activity of Aspergillus niger apo-Fdc1 could be reconstituted in vitro following anaerobic incubation with prFMNreduced–UbiX. Decarboxylase activity could only be observed after exposure of the reconstituted Fdc1 to oxygen (Fig. 1e), but appeared independent of the presence of UbiX (Extended Data Fig. 5a). No activity could be observed when incubating apo-Fdc1 with the prFMNradical–UbiX. This suggests only the prFMNreduced form can be correctly oxidized by UbiD/Fdc1 to the corresponding reactive N5-iminium species (prFMNiminium).

Stopped-flow experiments mixing reduced FMNH2–UbiX with DMAP under anaerobic conditions reveal transient formation of a distinct spectral species (species 2 in Fig. 1f) before formation of the prFMNreduced product. The rate of species 2 formation showed a hyperbolic dependence on DMAP concentration with apparent Kd = 730 ± 70 μM and limiting rate of k1 = 177 ± 7 s−1 (Extended Data Fig. 5b, c). The decay was independent of DMAP concentration and occurred with k2 = 0.316 ± 0.002 s−1. Stopped-flow experiments mixing reduced prFMNreduced–UbiX with oxygen revealed that the rate of formation of the purple-coloured end product is linearly dependent on oxygen concentration (Extended Data Fig. 5d, e).

We obtained the 1.8 Å crystal structure of P. aeruginosa UbiX in complex with oxidized FMN and DMAP (Fig. 2a, b). The DMAP substrate is located directly above the FMN isoalloxazine re face, with the dimethylallyl moiety sandwiched between the FMN and A89–S90. The dimethylallyl group is furthermore surrounded by aromatic rings of W84, Y169 and W200 that, together with the FMN dimethylbenzene moiety, resemble the π-cage found in other prenyltransferases or terpene synthases4,6. The phosphate moiety is bound by several cationic residues, including conserved residues R122, K129, R139 and R185 in addition to poor interactions with S90, the backbone amide of G91, E140 and Y169 (Extended Data Fig. 6). The short N5 to substrate C1 distance (3.3 Å) and relative small N10–N5–C1’ angle of 98° are compatible with those observed for other flavin-substrate complexes4,6. No evidence of direct nucleophile/oxidative attack of the oxidized FMN N5 was observed. Substrate complex
crystals that are transiently reduced with sodium dithionite under aerobic conditions turned purple upon oxidation, closely resembling solution properties. The 1.6 Å crystal structure of a purple-coloured crystal reveals formation of the four-ring prFMN radical (Fig. 2d). Although product formation has little effect on the active site structure, the presence of the fourth ring is accompanied by a slight rotation when compared to the wild-type N5–C1 bond formation occurs first (Fig. 2c). The N5–C1’ adduct adopts a significant butterfly conformation, and the N5 is clearly sp2 hybridized. Although S15 and E49 are located in proximity of the N5, a (transient) reorientation of both side chains is required to establish a hydrogen-bonding network with the N5. The 1.9 Å structure of substrate complex crystals frozen 20–30 s following reduction reveals formation of the prFMN reduced product (Extended Data Fig. 7). Longer incubation times lead to formation of the prFMN radical species as indicated by a gradual purple colouration of crystals. No other intermediates were seen to accumulate within the wild-type crystals, in agreement with our stopped-flow solution data (Fig. 1f).

In order to determine whether S15 and/or E49 are involved in N5 deprotonation and/or formation of the C6–C3’ linkage, we created an E49Q variant. The mutation severely affects but does not abolish in vitro Fdc1 activation (Fig. 1e). Although the oxidized substrate E49Q complex is similar in structure to the corresponding wild-type structure (Fig. 3a), a distinct intermediate species was seen following reduction and rapid freezing within 1–5 s (Fig. 3b). Although the latter structure clearly contains a reduced FMNH2, no N5–C1’ bond has formed, in contrast to the corresponding wild-type intermediate structure obtained within 1–5 s following reduction (Fig. 2c). Furthermore, both S15 and Q49 establish a hydrogen-bonding network with the N5. This suggests N5 deprotonation through S15 and E49 is linked to N5–C1’ bond formation, a process largely rendered ineffective through the E49Q mutation (Fig. 4, species 1red). Structures of the E49Q variant derived for crystals frozen 0.5–10 min after reduction reveal that accumulation of an N5–C1’ dimethylallyl adduct does occur (Fig. 3c), albeit distinct in conformation from that observed in the 1–5 s wild-type structure (Fig. 2c).

In the E49Q N5–C1’ dimethylallyl adduct, the dimethylallyl substrate-derived moiety has undergone a 180° rotation when compared to the wild-type N5-adduct species, along with a small rotation of the isoalloxazine ring (Figs 3c and 4, species IVa). Furthermore, both S15 and Q49 form a hydrogen-bonding network with N5, with the S15–N5 hydrogen bond maintaining the N5 in an sp3 state. This series of conformational changes achieves two objectives: a proton relay network is established between the N5 and solvent via S15 and E49 and additional space is created between the isoalloxazine dimethylbenzene moiety and Y169 to allow for formation of the fourth ring.
Many of the conformational changes observed in the E49Q N5-dimethylallyl adduct are unlikely to present a significant barrier to the reaction even within the crystals. However, the observed reorientation of the dimethylallyl moiety would require substantial protein breathing motions to occur if achieved through rotation along the dimethylallyl C1–C2 bond. An alternative route through olefin isomerization is possible, via protonation of the C2 atom (adding a pro-S hydrogen) with transient formation of a ternary C3 carbocation (Fig. 4, species III). The latter could reorient in the absence of active site deformation and be converted to the observed E49Q dimethylallyl–N5 adduct (Fig. 4, species IVa) by abstraction of the C2 pro-R proton. The substrate-derived phosphate is ideally positioned to establish a proton relay network to the substrate C2′, and the ternary C3′ carbocation resides within a π-cage made by the FMN dimethylbenzene moiety, Y169 and W200 that could assist carbocation formation.
Surprisingly, despite little difference in structure, crystals of a Y169F variant are severely compromised in their catalytic ability. A similar observation is made when using UbiX$^{Y169F}$ variant for in vitro reconstitution of Fdc1 activity (Fig. 1e). In the UbiXY$^{Y169F}$ crystals, an N5–C1’ dimethylallyl adduct nearly identical to that seen for the wild-type crystals can be readily obtained, but remains stable for several minutes (Extended Data Fig. 8). This suggests Y169 is key to the type crystals can be readily obtained, but remains stable for several minutes (Extended Data Fig. 8). This suggests Y169 is key to the conformational changes that follow formation of the first N5 adduct (Fig. 4, species III), possibly through assisting proton transfer via the substrate phosphate to the dimethylallyl C2’ or stabilization of the ternary C3’ carbocation.

We have been unable to trap any intermediates during formation of the dimethylallyl C3’–flavin C6 bond. We suggest C6 nucleophilic attack on the dimethylallyl C3’ carbocation occurs concomitant with or following protonation of the C2’ via the bound phosphate (Fig. 4, species IVb). The resulting cyclohexadiene adduct (species V) can form the final product (species VI) through aromatization concomitant with proton abstraction via S15 and E49 (Extended Data Fig. 9).

We suggest the E49Q mutation also affects this particular deprotonation, leading to the accumulation of an intermediate (species IVa) preceding this step as observed in the crystals. It is possible that formation of the cyclohexadiene adduct (V) occurs directly following conformational change of the C3’ carbocation species (III), via species IVb without intermediate deprotonation/protonation as outlined for species IVa.

In addition to UbiX, a distinct family of flavoenzymes (type 2 isoprenyl diphosphate isomerases) bind isoprene precursors. The latter have been suggested to use the flavin as an acid-base in the interconversion of IPP and DMAPP$^{M,15,16}$. Instead, our data reveal UbiX has many similarities to terpene synthases, achieving the required isoprenyl chemistry via similar strategies (Fig. 4)$.^{17,18}$ We suggest formation of an initial allylic carbocation through leaving of the phosphate group (pyrophosphate in the terpene synthases) is achieved through stabilization of the carbocation species within an active site dominated by π-systems (the π-cage). Furthermore, FMN reduction appears to act as the trigger for phosphate-C1’ bond breakage, increasing the electron density of the isoalloxazine ring that stacks with the dimethylallyl substrate. In contrast to the metal-requiring terpene synthases, the leaving group is stabilized by a multitude of ionic interactions. A direct contact with the conserved E140 furthermore suggests phosphate protonation occurs concomitant with C1’ allylic carbocation formation and/or N5 nucleophilic attack (Fig. 4, species I$^{reduced}$). Following formation of the N5–C1’ bond, UbiX appears to act as a chaperone for substrate reorientation (conversion of species III to IVb), similar to what is postulated to occur for terpene synthases. In UbiX, it appears both the leaving group phosphate and a conserved tyrosine residue (Y169) are involved in catalysing the conformational change of the N5 adduct required for completion of the reaction.

The presence of ubiX/ubiD-like genes in the majority of microbes$^{5,19-21}$ and the essential role played in prokaryotic ubiquinone biosynthesis$^{2}$ suggest an ancient evolutionary origin for the UbiX flavin prenyltransferase chemistry. It remains unclear why dimethylallyl-monophosphate is used by UbiX, as opposed to the universal dimethylallyl-diphosphate$^{22}$. This might serve as a means to regulate product formation via availability of DMAP. Although the isomer isopentenyl monophosphate has recently been shown to occur in certain archaeae$^{23}$, the metabolic route to dimethylallyl-monophosphate remains unclear. The unusual biochemical strategy of using a reduced rather than oxidized flavin to act as a nucleophile$^{24,25}$ ensures formation of a relatively stable N5-alkyl adduct species (prFMN$^{reduced}$), as opposed to the corresponding N5-iminium adduct (prFMN$^{iminium}$). The latter would be prone to hydrolysis before transfer to apo-UbiD/Fdc1. Whether non-ubiD related enzymes make use of the novel chemical properties of the UbiX product remains to be established.

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**Figure 4 | Schematic representation of the proposed UbiX mechanism.**

DMAP derived atoms are shown in red. Roman numerals indicate the various intermediate species proposed (see main text). References to individual figures after roman numerals refer to corresponding crystal structures obtained for the wild type or UbiX$^{E49Q}$ mutant.
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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Supplementary Information is available in the online version of the paper.

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Author Contributions M.D.W. carried out molecular biology, biophysical and structural biology studies together with K.A.P., and D.L. M.D.W. and S.A.M. performed in vitro reconstitution experiments. K.F. and S.E.J.R. performed and analysed EPR experiments. S.H. performed DFT calculations. N.J.W.R. and D.K.T. undertook liquid chromatography–mass spectrometry of extracts and with R.G. interpreted the data on substrate–product species. All authors discussed the results with D.P and N.S.S. and 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 4ZXAF, 4ZAX, 4ZAZ, 4ZAY, 4ZAN and 4ZAZ. 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)
Preparation of cofactor extracts for mass identification. The oxidized product of UbiX turnover was extracted from *P. aeruginosa* UbiX under aerobic conditions by denaturing the protein with equal volumes of acetonitrile at 70 °C. Precipitate was removed by centrifugation at 16,000 g for 10 min. The resulting supernatant was sent for high-resolution mass analysis.

The reduced product of UbiX turnover was generated under anaerobic conditions from a steady-state reaction containing 1 mM FMNH₂, 1 mM DMAP and 20 mM *P. aeruginosa* UbiX. Protein was removed by precipitation at room temperature as described above. The resulting supernatant was sent for analysis by high-resolution mass spectrometry.

Stopped-flow kinetics of *P. aeruginosa* UbiX. The kinetics of a single turnover reaction between UbiX:FMNH₂ and DMAP was studied under anaerobic conditions on a SX.18M-V-R stopped-flow spectrophotometer (Applied Photophysics, dead time approximately 1 ms) by measuring changes in the flavin absorbance spectrum logarithmically over 60 s using a photodiode array (PDA) detector. 100 μM UbiX:FMNH₂ was rendered anaerobic in 20 mM Tris, 200 mM NaCl pH 8.0 and supplemented with 1 mM glucose, 10 μM HCl oxidation (Sigma) to guarantee an oxygen-free environment. The protein was reduced with sodium dithionite before being rapidly mixed with equal volumes of 500 μM–4 mM DMAP at room temperature. The spectral intermediates were resolved by singular value decomposition using the Pro-Kinetist program (Applied Photophysics).

Data could be fitted to a threestep model (A > B > C > D), which allowed the rate constants k_A, k_B, and k_C to be estimated. Species D was identified as a photoinduced adduct and considered an artefact of the prolonged illumination required.

The kinetic parameters of a single turnover reaction between UbiX:FMNH₂ reduced and oxygenated buffer were measured using a stopped-flow instrument (see above). Here 100 μM UbiX:FMNH₂ was rendered anaerobic (in the absence of glucose oxidase), before being reduced with dithionite and mixed with 1 mM DMAP. The protein was rapidly mixed with equal volumes of 0–100% oxygenated buffer, producing absorbance spectra that could be analysed by singular value decomposition. Data were fitted to a one-step model (A > B), which allowed the rate constant k_AB to be estimated.

In vitro reconstitution of *A. niger* Fdc1. For reconstitution under steady-state conditions, a solution containing 1 mM FMNH₂, 1 mM DMAP and, respectively, wild-type *P. aeruginosa* UbiX, E49Q or Y169F was incubated with 50 μM *A. niger* apo-Fdc1 in 20 mM Tris, 200 mM NaCl, 1 mM MnCl₂ pH 8.0 under anaerobic conditions. The specific activity of reconstituted Fdc1 with 50 μM cinnamic acid (in 50 mM potassium phosphate, 50 mM KCl pH 6.0; either aerobic or anaerobic) was determined at regular time intervals by measuring the rate of decarboxylation at 270 nm using a Cary UV-visible spectrophotometer.

Reconstitution experiments using a filtered UbiX reaction were carried out using 2 mM FMNH₂ anaerobically incubated overnight with 5 mM DMAP and 50 μM UbiX. This reaction mixture was used to reconstitute *A. niger* apo-Fdc1 (supplemented with Mn²⁺) in a 2:1 molar ratio, assuming complete conversion of FMNH₂ to prFMN-red. To obtain filtrate devoid of UbiX, the reaction mixture was anaerobically filtered in a 10 kMWCO centrifugal concentrator (Generon). A negative control with DMAP omitted from the overnight reaction was also performed. Decarboxylase activity was measured using 800 μM aerobic cinnamic acid.

**Crystalization of *P. aeruginosa* UbiX (wild-type and variants).** A total of 7.5 mg ml⁻¹ *P. aeruginosa* UbiX in 20 mM Tris, 200 mM NaCl pH 8.0 was supplemented with 0.5 mM FMNH₂ and mixed with 1 mM DMAP to promote ligand binding in the active site. Initial screening, sitting 0.3 μl protein and 0.3 μl mother liquor next to 50 μl reservoir, yielded a variety of hits after 1–2 days at 25 °C.

The best crystals were obtained after 1–2 days in 12% PEG 3350, 150 mM sodium thiocyanate, and 100 mM Tris pH 7.2 at 25 °C. Crystals of the UbiX variants were obtained in the same conditions.

**Diffraction data collection and structure elucidation.** All crystals were cryoprotected in mother liquor supplemented with 10% PEG 200 and flash-cooled in liquid nitrogen. Reaction intermediates were obtained by flash cooling crystals that had been incubated in cryoprotectant containing sodium dithionite. Where periods of long soaking were necessary, the cryoprotectant was also supplemented with FMN and DMAP to reduce dissociation from the crystals. Diffraction data was collected up to 1.4 Å at Diamond beamlines at 100K and processed using the CCP4 suite. Data was reduced and scaled using XDS. The structure of *P. aeruginosa* UbiX was refined using REFMAC5 as the starting model, and refined by cycles of manual rebuilding in COOT and additional processing in REFMAC5 (ref. 25). 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.
Mass spectrometry. All solvents were of HPLC-MS grade and 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-LTQ 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-workers\(^ {27}\) and performed on a Thermo Hypersil Gold 2.1 μm C\(_{18}\) column at a solvent flow-rate of 0.1 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, the tetrapeptide MRFA and Ultramark 1621 in an acetonitrile/methanol/acetic acid solution. 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 (all systems specific 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. MS source, sample storage/column was kept under identical conditions. Collision induced dissociation (CID) settings were set up to trap target ions 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.

EPR spectroscopy. Continuous wave X-band (~9.4 GHz) EPR spectra were obtained using a Bruker ELEXSYS E500 EPR spectrometer operating at cryogenic temperatures via an Oxford Instruments ESR900 liquid helium cryostat interfaced with an ITC503 temperature controller from the same manufacturer. Samples were 250 μl in Wilmad 4 mm outer diameter quartz tubes. Sample temperatures were as stated, microwave power was 10 μW, modulation amplitude was 1.5 G. Pulsed electron nuclear double resonance (ENDOR) spectra were obtained at 65 K using a Bruker ELEXYS E580 spectrometer equipped with an EN 4118X-MD4 dielectric resonator. Temperature control was effected using an Oxford Instruments CF935 cryostat and ITC503 temperature controller. The Davies pulsed ENDOR sequence was employed at intermediate Q using soft microwave pulses and FID detection (π/2 = 200 ns) with a 9 ms radiofrequency π pulse. DFT calculations. Density function theory (DFT) models of FMN:DMA adducts were optimized in the gas phase using the (U)BP3LYP/6-311+G(d,p) level of theory implemented in Gaussian 09 (ref. 28). The models consist of an FMN truncated after the 2° carbon and 2-methyl-2-butene, that is, the dephosphorylated DMAP (Extended Data Fig. 4). Structural alignments to the crystal coordinates were performed using Swiss-PdbViewer version 4.1 (ref. 29). Harmonic vibrational frequencies calculated using normal mode analysis were used to confirm that optimized geometries were always in local or global minima. 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.
Extended Data Figure 1 | Mass spectrometric analysis of the UbiX product.

**a** Structural elucidation of the reduced UbiX product. From an initial full scan TIC on UbiX extract, a 525 m/z ion extracted chromatogram was created under a gradient elution using H₂O/acetonitrile both containing 0.1% formic acid indicating a major peak apex at 9.57 min with a 54/46 solvent elution composition (not shown). Subsequent data dependant TIC and 525 m/z scan extracted chromatograms were created under 55% A/45% B isocratic solvent elution and ion extraction between 524.5–525.5 m/z produced a singular peak at 2.28 min displaying an associated full-scan molecular ion peak with m/z 5525.1726 (M⁺ = C22H30N4O9P) at a resolution of 58,500 with a mass accuracy of 3.59 p.p.m. Fragmentation of the 5525.1726 m/z molecular ion peak 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 newly formed, more labile, tertiary ring at the MS² level. Subsequent removal of the phosphate head group at the MS³ level was achieved using CID 35 on the 456.23 m/z molecular species with a final MS⁴ step using CID 35 on 358.18 m/z completely removing the tail group from the central three-ring isoalloxazine system.

**b** Structural elucidation of the re-oxidized UbiX/Fdc1 cofactor. From an initial full scan TIC on UbiX extract (i), a 524 m/z ion extracted chromatogram was created under a gradient elution using H₂O/acetonitrile both containing 0.1% formic acid indicating a major peak apex at 9.24 min with a 48/52 solvent elution composition (not shown). Subsequent data dependant TIC and 524 m/z scan extracted chromatograms (ii) were created under 50% A/50% B isocratic solvent elution and ion extraction between 523.5–524.5 m/z produced a singular peak at 2.08 min displaying an associated full-scan molecular ion peak with m/z 5524.1656 (M⁺ = C22H29N4O9P) at a resolution of 58,500 with a mass accuracy of 2.78 p.p.m. Fragmentation of the 5524.1656 m/z molecular ion peak in an automated data dependent manner using helium-based chemical-induced dissociation (CID level 35) generated a spectral tree (iii) that indicates the removal of the newly formed, more labile, tertiary ring at the MS² level. Subsequent removal of the phosphate head group at the MS³ level was achieved using CID 35 on the 498.31 m/z molecular species to create 327.18 (A) alongside a sister fragment 455.31 (B) that represents the full removal of the tertiary ring but retaining the phosphate head group.
**Extended Data Figure 2 | EPR spectroscopic analysis of the UbiX radical product.**

**a,** X-band continuous wave EPR spectra of UbiX in frozen solution: (i) wild type (WT) as isolated; (ii) WT plus DMAP; (iii) WT reduced with dithionite; (iv) WT + DMAP reduced with dithionite; (v) WT + DMAP reduced with dithionite and reoxidized with oxygen; (vi) Y169F mutant + DMAP reduced with dithionite and reoxidized with oxygen; (vii) W200F mutant + DMAP reduced with dithionite and reoxidized with oxygen. The FMN–DMAP adduct radical is only formed when UbiX is reoxidized in the presence of DMAP and this formation is not affected by mutation of those aromatic residues forming the π-cage that could give rise to Y or W radical species.

**b,** X-band continuous wave EPR spectra of frozen solutions of WT UbiX + DMAP and reduced with dithionite with the addition of potassium ferricyanide to the following concentrations: (i) 260 μM; (ii) 160 μM; (iii) 50 μM; (iv) 40 μM; (v) 30 μM; (vi) 20 μM; (vii) 0 μM. Experimental conditions: microwave power 10 μW, modulation amplitude 1.5 G, temperature 20 K. Showing the radical can also be formed using chemical oxidation in the absence of oxygen and thus does not arise from a peroxide species generated by the reaction of reduced oxygen species formed when the dithionite sample is exposed to oxygen. An initial radical is formed under these conditions that exhibits a considerably broader EPR signal than the prFMNradical and is as yet unidentified.
Extended Data Figure 3 | Pulsed Davies ENDOR spectra of the prFMNredox–UbiX complex. The spectrum was measured at a field equivalent to \( g_{av} = 2.0033 \). Although a complete assignment of the spectrum requires specific deuteration of FMN and DMAP, the ENDOR spectrum is dominated by two large hyperfine couplings to \( \beta \)-protons indicated as \( H_A \) and \( H_B \). Using the Heller-McConnell equation the values of the dihedral angles, \( \theta \), can be determined as shown and are consistent with the orientation of the C19-protons of the DMAP-derived fragment of the radical observed crystallographically, as shown in the figure above. The unpaired electron spin density, \( \rho \), at N5 of the FMN-derived fragment of the radical can also be estimated from the Heller-McConnell equation. \( B'' \) is negligible, whereas \( B''' \) is thought to have a value of \(-160\), although studies of \( \beta \)-protons coupled to unpaired electron spin density at a nitrogen atom are rare, giving an unpaired spin density at N5 of \(-0.3\), consistent with calculations and considerably smaller than the unpaired electron spin density of 0.4 or greater expected for C1 of an aromatic amino acid radical.
Extended Data Figure 4 | DFT modelling of the UbiX product. Top, DFT model of the purple radical species showing the location of significant atomic spin densities ($\geq 0.02$) to the right. The optimized structure (blue carbons) overlaid with the crystal coordinates (green carbons) is shown below. The model was geometry optimized in the gas phase using the UB3LYP/6-311++G(d,p) level of theory. Cartesian coordinates of the optimized structure are given in the Supplementary Information.
Extended Data Figure 5 | Additional UbiX solution studies.
a, Reconstitution of \textit{A. niger} Fdc1 activity with UbiX–prFMN\textsuperscript{reduced} and prFMN\textsuperscript{reduced} obtained through filtration of a UbiX–prFMN\textsuperscript{reduced} reaction. Control reactions are devoid of any DMAP substrate. b, Rate of formation of spectral species 2 (see Fig. 1f) in function of DMAP concentration. c, Rate of decay of spectral species 2 (see Fig. 1f) in function of DMAP concentration. d, Spectral species obtained from singular value decomposition of rapid-scan stopped-flow spectrophotometric data following mixing of UbiX–prFMN\textsuperscript{reduced} with oxygenated buffer. e, The rate of purple radical (species B in panel d of this figure) formation as obtained from singular value decomposition of rapid-scan stopped-flow spectrophotometric data following mixing of UbiX–prFMN\textsuperscript{reduced} with oxygenated buffer has a linear dependence on oxygen concentration. Error bars are s.e.m., \( n = 3 \).
Extended Data Figure 6 | Multiple sequence alignment of UbiX/Pad1 enzymes from selected bacterial or fungal species. *Pseudomonas aeruginosa* UbiX (NP_252708), *Escherichia coli* O157:H7 EcdB (NP_311620), *Escherichia coli* UbiX (YP_490553), *Bacillus subtilis* BsdB (WP_009966530), *Saccharomyces cerevisiae* Pad1 (AAB64980), *Aspergillus niger* PadA1 (ABN13117), and orf8 from the *Thauera aromatica* phenylphosphate carboxylase gene cluster (PAAD_THAAR). Conserved residues involved in phosphate binding, N5 polar network or formation of the substrate binding π-cage are indicated by labelled arrows. Secondary structure elements of *P. aeruginosa* UbiX crystal structure are shown. Alpha helices and 3_10 helices (denoted as η) are shown as squiggles, β-strands as arrows and β-turns as TT.
Extended Data Figure 7 | Crystal structure of *P. aeruginosa* UbiX–FMN–DMAP flash cooled to 100 K at 30 s following complete reduction by sodium dithionite. Two orientations are displayed as in Fig. 2. The omit map for the prFMN<sup>reduced</sup> product is shown as a green mesh, contoured at 4σ.
Extended Data Figure 8 | Crystal structures of *P. aeruginosa* UbiX<sup>V169F</sup>

a, Detailed view of the UbiX<sup>V169F</sup>–FMN–DMAP complex with individual amino acids contributing to active site structure shown in atom-coloured sticks (carbons colour coded as in Fig. 2a). Two orientations are displayed as in Fig. 2.

b, Detailed view of the UbiX<sup>V169F</sup> N5–C1′ adduct species obtained through flash-cooling following reduction. The omit map for the N5–C1′ adduct is shown as a green mesh, contoured at 4σ.
Extended Data Figure 9 | DFT models of proposed intermediate species in the UbiX reaction. a, DFT models of species II and IVa (as defined in Fig. 4). Conversion from II to IVa is achieved by −180° rotation about C1’–C2’ (blue arrow) and the N5–H and methanol species (red) are only found in species IVa models. b, Overlay of the species II DFT model (green carbons) with the crystal coordinates of species II and S15 (teal carbons). c, Three DFT models of IVa were examined and two orthogonal projections are shown overlaid with the crystal coordinates (teal carbons): (Vi, yellow carbons) with a methanol analogue of S15 (a, in red) with the C–N5 distance fixed to the crystallographic distance of 4.0 Å; (Vii, magenta carbons) with N5 protonated (no methanol), and (Viii, light pink carbons) with N5 deprotonated and no methanol. DFT model of species V and VI are shown in d and e, respectively, and are overlaid in f (V green carbons, VI magenta carbons). g, Overlay of the species VI DFT model (magenta carbons) with the crystal coordinates (teal carbons). Models were geometry optimized in the gas phase using the B3LYP/6-311+G(d,p) level of theory. Harmonic vibrational frequencies calculated using normal mode analysis were used to confirm that optimized geometries of all species were in local or global minima. In the case of species Vi, ‘ModRedundant’ optimisation was performed to fix the C–N5 distance and one imaginary frequency of 67.60 cm⁻¹ was observed. Cartesian coordinates of the optimized structures are given in the Supplementary Information.
## Extended Data Table 1 | Data collection and refinement statistics

| UbiX  | WT FNNDMAP | WT N5 adduct 5 s following reduction | WT NSC6 adduct, 30 s following reduction | WT NSC6 adduct, co-crystallised (radical) | E4QK FNNDMAP | E4QK FNNDMAP | E4QK N5 adduct >15 s following reduction | Y169F FNNDMAP | Y169F N5 adduct >15 s following reduction |
|-------|------------|-------------------------------------|----------------------------------------|------------------------------------------|--------------|--------------|------------------------------------|--------------|------------------------------------------|
| PDB code | 4ZAI | 4ZAV | 4ZAW | 4ZAX | 4ZAG | 4ZAL | 4ZAY | 4ZAN | 4ZAZ |
| **Data collection** | | | | | | | | | |
| Space group | F 2 3 | F 2 3 | F 2 3 | F 2 3 | F 2 3 | F 2 3 | F 2 3 | F 2 3 | F 2 3 |
| α, β, γ (°) | 141.9 | 142.18 | 142.74 | 141.79 | 142.31 | 142.07 | 142.02 | 141.73 | 142.26 |
| Resolution (Å) | 70.95-1.71 | 50.27-1.40 | 43.04-1.89 | 42.75-1.61 | 32.65-1.68 | 42.84-1.62 | 32.58-1.54 | 70.86-1.76 | 50.30-1.45 |
| R_{max} | 2.7 (29.8) | 2.7 (29.7) | 4.3 (33.0) | 2.4 (33.5) | 3.5 (35.6) | 2.4 (29.2) | 2.1 (26.6) | 2.6 (36.0) | 2.8 (55.6) |
| E/σI | 18.7 (2.8) | 16 (2.7) | 13.4 (2.7) | 20.1 (2.5) | 13.5 (2.2) | 16.9 (2.8) | 20.3 (3.0) | 19.3 (2.4) | 15.7 (2.4) |
| Completeness (%) | 100 (100) | 99.6 (100) | 99.9 (99.9) | 99.9 (100) | 99.9 (99.9) | 99.8 (100) | 100 (100) | 99.9 (100) | 99.9 (99.9) |
| Redundancy | 6.7 (6.8) | 6.8 (6.8) | 6.7 (6.7) | 5.6 (5.4) | 5.6 (5.5) | 6.6 (6.7) | 6.7 (6.7) | 6.7 (6.4) | 5.5 (5.4) |
| **Refinement** | | | | | | | | | |
| Resolution (Å) | 70.95-1.71 | 50.27-1.40 | 43.04-1.89 | 42.75-1.61 | 32.65-1.68 | 42.84-1.62 | 32.58-1.54 | 70.86-1.76 | 50.30-1.45 |
| No. reflections | 24314 | 44084 | 18350 | 29027 | 25816 | 28593 | 33354 | 22555 | 40068 |
| R_{work}, R_{free} | 13.74/17.23 | 9.77/12.90 | 14.76/19.16 | 14.36/17.10 | 15.15/17.97 | 13.97/16.34 | 14.54/17.60 | 14.24/18.20 | 10.26/14.72 |
| No. atoms | 1621 | 1627 | 1554 | 1620 | 1641 | 1664 | 1727 | 1593 | 1658 |
| Protein | 41 | 36 | 36 | 41 | 41 | 41 | 36 | 36 | 36 |
| Ligand | 13 | 18 | 13 | 14 | 4 | 6 | 15 | 7 | 20 |
| Ion | 139 | 194 | 100 | 127 | 141 | 139 | 144 | 116 | 156 |
| Water | 2322 | 17.562 | 24.13 | 22.685 | 23.492 | 26.251 | 22.928 | 26.577 | 20.892 |
| R.m.s deviations | 0.0253 | 0.0258 | 0.0209 | 0.0260 | 0.0229 | 0.0262 | 0.0262 | 0.0222 | 0.0250 |
| Bond lengths (Å) | 2.1189 | 1.9826 | 1.9719 | 2.4312 | 2.1375 | 2.3693 | 2.3773 | 2.4068 | 2.2280 |

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