Structural Basis for the Enzymatic Formation of the Key Strawberry Flavor Compound 4-Hydroxy-2,5-dimethyl-3(2H)-furanone

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Background: Fragaria x ananassa enone oxidoreductase catalyzes the ripening-induced formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone in strawberries.

Results: By determining six x-ray structures of different substrate complexes the enzymatic mechanism was elucidated and experimentally confirmed by deuterium labeling.

Conclusion: The 4R-hydrde of NAD(P)H is transferred to an exo-cyclic carbon double bond.

Significance: Enzymatic 4-hydroxy-2,5-dimethyl-3(2H)-furanone synthesis reveals a new reaction mechanism and advances understanding of a biotechnologically relevant biosynthetic pathway.

The last step in the biosynthetic route to the key strawberry flavor compound 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) is catalyzed by Fragaria x ananassa enone oxidoreductase (FaEO), earlier putatively assigned as quinone oxidoreductase (FaQR). The ripening-induced enzyme catalyzes the reduction of the exocyclic double bond of the highly reactive precursor 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone (HMMF) in a NAD(P)H-dependent manner. To elucidate the molecular mechanism of this peculiar reaction, we determined the crystal structure of FaEO in six different states or complexes at resolutions of ≤1.6 Å, including those with HDMF as well as three distinct substrate analogs. Our crystallographic analysis revealed a monomeric enzyme whose active site is largely determined by the bound NAD(P)H cofactor, which is embedded in a Rossmann-fold. Considering that the quasi-symmetric enolic reaction product HDMF is prone to extensive tautomerization, whereas its precursor HMMF is chemically labile in aqueous solution, we used the asymmetric and more stable surrogate 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (EHMF) and the corresponding substrate (2E)-ethylidene-4-hydroxy-5-methyl-3(2H)-furanone (EDHMF) to study their enzyme complexes as well. Together with deuterium-labeling experiments of EDHMF reduction by [4R-2H]NADH and chiral-phase analysis of the reaction product EHMF, our data show that the 4R-hydrde of NAD(P)H is transferred to the unsaturated exocyclic C6 carbon of HMMF, resulting in a cyclic achiral enolate intermediate that subsequently becomes protonated, eventually leading to HDMF. Apart from elucidating this important reaction of the plant secondary metabolism our study provides a foundation for protein engineering of enone oxidoreductases and their application in biocatalytic processes.

Strawberries emit several hundreds of volatiles of which only a dozen compounds truly contribute to the overall aroma perception of the ripe fruit (1). Among those, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol, HDMF)2 is the most significant component because of its high level (up to 55 mg/kg of fruit fresh weight) and low odor threshold (10 ppb (2)). In strawberry fruit HDMF is further metabolized to its methyl ether 2,5-dimethyl-4-methoxy-3(2H)-furanone, its β-D-glucoside, and subsequently, to the malonylated derivative of the glucoside (3–5).

HDMF was first reported as a product of the Maillard reaction (6) and was subsequently isolated from different fruit including pineapple, strawberry, and tomato (2, 7, 8). It exhibits a caramel-like aroma similar to its structural homologues 4-hydroxy-5-methyl-3(2H)-furanone (HMF, norfuraneol) and 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone or the tautomer 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone (2-EHMF or 5-EHMF, respectively, homofuraneol). All these molecules are based on a cyclic dicarbonyl compound with a planar enol-tautomeric substructure (Fig. 1) capable of forming strong hydrogen bonds (6). Despite its chiral nature and enzymatic origin, HDMF occurs as racemate in different fruit (9), probably due to rapid racemization in aqueous milieu at pH 7.0 involving various tautomeric intermediates (10, 11).

Incorporation experiments with radiolabeled precursors and substrates labeled with stable isotopes indicated D-fructose-1,6-bisphosphate as an effective progenitor of HDMF and provided initial evidence for the enzymatic formation of this important aroma compound in strawberries (12–14). D-Fructose-1,6-bisphosphate is presumably converted, by as yet

The atomic coordinates and structure factors (codes 4IDD, 41DC, 41DI, 41DR, 41DA, and 41DF) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: HDMF, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol); EHMDF, (2E)-ethylidene-4-hydroxy-5-methyl-3(2H)-furanone; 2-EHMF, 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone; 5-EHMF, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone; FaEO, Fragaria x ananassa enone oxidoreductase; HMF, 4-hydroxy-5-methyl-3(2H)-furanone; HMMF, 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone; PDB, Protein Data Bank; FaQR, Fragaria x ananassa quinone oxidoreductase; ESI, electrospray ionization; EO, enone oxidoreductase.
unknown enzymes, into 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone (HMMF), which serves as substrate for an oxidoreductase that catalyzes the final biosynthetic step (15, 16). The corresponding ripening-induced, negatively auxin-regulated enzyme was originally assigned as *Fragaria x ananassa* enone oxidoreductase (FaEO), based on its sequence similarity to known quinone oxidoreductases and catalytic activity on 9, 10-phenanthrenequinone (16), but subsequent enzymatic studies showed that this plant protein efficiently catalyzes the reduction of the exocyclic unsaturated bond of the highly reactive precursor HMMF, as well as derivatives thereof, utilizing NADPH as preferred cofactor (Fig. 1). Consequently, on the basis of this physiological reaction the enzyme was renamed to *Fragaria x ananassa* enone oxidoreductase (FaEO) (15). Notably, the kinetic data of FaEO, and also of the orthologous protein from *Solanum lycopersicon* (SlEO), for the aroma-active substrate HMMF and its chemical homologs resemble those of an earlier characterized enone oxidoreductase from *Arabidopsis thaliana* that was described to catalyze the hydrogenation of 2-alkenals, despite low sequence homology (15).

In the present study, we report the crystallization and x-ray structural analysis of FaEO in complex with different 4-hydroxy-3(2H)-furanone-derived substrates or products, thus providing hints on the catalytic mechanism and how hydride ion transfer from NAD(P)H is initiated. Isotope labeling experiments using stereospecifically deuterated [4R-2H]NADH and chiral-phase analysis of the products served to experimentally confirm the proposed reaction mechanism.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes**—Deuterated formic acid (2HCOO2H, 98% 2H), formate dehydrogenase from *Saccharomyces cerevisiae* (EC 1.2.1.2; 10 units/mg of protein), and β-nicotinamide adenine dinucleotide (NAD+) were purchased from Sigma. β-Nicotinamide adenine dinucleotide reduced disodium salt (NADH) and acetaldehyde were obtained from Fluka (Buchs, Switzerland). 4-Hydroxy-5-methyl-3(2H)-furanone (HMF; 97%) was purchased from SAFC Supply Solutions (St. Louis, MO). Copper(II) acetate and sodium acetate were from Merck (Darmstadt, Germany), whereas all other chemicals and solvents for extraction and HPLC were from Carl Roth (Karlsruhe, Germany).

**Enzymatic Synthesis and Purification of Deuterated NADH**—[4R-2H]NADH was synthesized following a previously described procedure (17). Briefly, a 6-ml solution of 50 mM sodium carbonate, 0.1 m deuterated formic acid, and 15 mM NAD+ was titrated to pH 8.5 with 1 M NaOH. 20 Units of *S. cerevisiae* formate dehydrogenase were then added and the course of NAD+ reduction was spectrophotometrically monitored at 340 nm. After about 3 h at room temperature the reaction was complete. The solution was diluted to 15 ml with water and the product was purified on an ÄKTA purifier system (GE Healthcare, Munich, Germany). A Mono Q 5/50 GL anion exchange column (GE Healthcare) was equilibrated with 10 ml of MilliQ water (Merck Millipore, Billerica, MA). After applying the diluted [4R-2H]NADH solution at 1 ml/min the column was washed with 20 ml of MilliQ water, followed by a linear
(NH$_4$)$_2$HCO$_3$ gradient (0–0.4 M) in 300 ml. Fractions containing 4R-$^2$H]NADH were pooled and lyophilized. Purity and degree of deuteration were determined by LC-UV/ESI-MS$^n$ and NMR spectroscopy (see below).

**Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-UV/ESI-MS$^n$)**—The purified 4R-$^2$H]NADH was dissolved at 2.5 mg/ml in water. For LC-UV/ESI-MS$^n$ analysis 5 μl of the solution was injected into a 1100 HPLC system (Agilent, Waldbronn, Germany) with a Luna 3u C18(2) 100 Å column (15 cm × 2 mm; Phenomenex, Torrance, CA), which was connected to an Agilent 6340 Ion Trap LC/MS mass spectrometer. The LC solvents were 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B). For elution of 4R-$^2$H]NADH a gradient from 100% A/0% B to 0% A/100% B was applied at a flow rate of 0.1 ml/min during 20 min, kept for a further 20 min at the latter conditions, and then reset to 100% A/0% B within 1 min. The voltage of the capillary was set to −4000 V and that of the end plate was −500 V. The dry gas (N$_2$) was heated to 330 °C and applied at a flow rate of 9 liters/min. Full scan mass spectra were measured from m/z 100 to 2200 for up to 200 ms until the ICC target reached 100,000 for positive ions or 70,000 for negative ions, whichever first. Tandem mass spectrometry was performed using helium as the collision gas with the collision energy set to 1.0 V. The target mass for MS$^2$ spectra was set to m/z 400. Mass spectra were acquired in the negative and positive ionization mode. Auto-tandem mass spectrometry was used to break down the most abundant [M + H]$^+$ or [M − H]$^-$ ion. Data analysis was performed using the Jasco ChromPass version 1.9.302.1124 software and the 6300 Series Trap Control Version 6.2 software (Buerk Daltonics).

**Cloning, Expression, and Purification of FaEO**—FaEO was amplified via polymerase chain reaction (PCR) from pET29a-FaQR (16) using *Pfu* DNA polymerase (Fermentas/Thermo Scientific, St. Leon-Rot, Germany) and the phosphorothioate primers (18) 5'-GGG GCA GCT CCA AGT GAG TCC-3' and 5'-GTG GTG CCA TGG TTA GAT GGG ATA CAC AAC CAC C-3', generating a 5' blunt end compatible with EheI and a 3' sticky end compatible with NcoI (both underlined). The PCR product was cut with NcoI (New England Biolabs, Ipswich, MA) and ligated with T4 DNA ligase (Fermentas/Thermo Scientific) with the vector pASK-IBA5plus (IBA, Göttingen, Germany), which had been cut with EheI (Fermentas/Thermo Scientific) and NcoI. The resulting plasmid, pASK-Strep-FaEO was analyzed by restriction digest and DNA sequencing (ABI Prism 310; Applied Biosystems/Invitrogen). A single nucleotide exchange (underlined) was introduced by QuickChange mutagenesis (Stratagene/Agilent Technologies, Santa Clara, CA), using primers 5'-CAG CAT TGG TGA ACC CAA CCA GGT TTG GGT GTG CTT TTG GCC CAC CAC C-3' and 5'-GTC AAA GAC CCA AAC CTG GTT GGG TTC ACC AAT GCT G-3' to substitute Pro-113 by Thr.

Recombinant Strep-FaEO was produced in the *Escherichia coli* K-12 strain MJM33 (19) grown at 22 °C in 2 liters of LB medium (20) supplemented with 100 mg/liter of ampicillin using a 5-liter shake flask. Gene expression was induced at a cell density $A_{550}=0.6$ by adding 0.2 mg/liter of anhydrotetracycline (21). After further shaking overnight the cells were harvested by centrifugation, suspended in 20 ml of lysis buffer (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) supplemented with 0.5 mg/ml of lysozyme, and incubated for 4 h at 4 °C prior to sonication in an S-250D Cell Disrupter (Branson, Danbury, CT). The soluble fraction of the cell extract was prepared by centrifugation (10,000 × g) and sterile filtration (0.45 μm). Strep-FaEO was purified by mutant streptavidin affinity chromatography (22) in lysis buffer. After elution with 5 mM biotin in the lysis buffer, the enzyme was concentrated and subjected to size exclusion chromatography in the same buffer using a Superdex 200 16/60 column (GE Healthcare), eluting with an apparent molecular size of 35.2 kDa. For final polishing by anion exchange chromatography, Strep-FaEO was dialyzed against 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, applied to a
Resource Q column (GE Healthcare), and eluted with a linear concentration gradient of NaCl in the same buffer. Purified Strep-FaEO was finally dialyzed against 10 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.02% (w/v) NaN_{3} and concentrated to 10 mg/ml (283 μM) for further experiments. The yield of the purified enzyme was ~3 mg per liter of E. coli culture.

**Crystallization and Structure Determination of FaEO**—Crystals of FaEO in its apo form as well as in complex with the cofactor NADP(H) and substrate/product or analogs were grown by vapor diffusion, initially from combinatorial screens in 400-nl sitting drops using a Freedom Evo robotic system (Tecan, Crailsheim, Germany). Subsequent fine screens were manually performed in hanging drops by mixing 1 μl of protein solution with 1 μl of reservoir solution and equilibrating against a 1-ml reservoir. Crystal manipulation and harvesting was carried out with LithoLoops (Molecular Dimensions, Suffolk, UK).

Diffraction quality crystals of FaEO-apo were obtained from 28–25% (w/v) PEG3350, 0.2 M LiSO_{4}, 0.1 M sodium citrate, pH 5.5–6.5. To obtain crystals of FaEO in complex with NADPH and HDMF or in complex with NADPH and HMF, solutions of the cofactor and substrate/product were added to the protein solution in 10- and 50-fold molar ratio, respectively. The FaEO-NADPH/HDMF complex crystallized from 21–16% (w/v) PEG3350, 0.2 M LiSO_{4}, 0.1 M Tris/HCl, pH 7–8.5, whereas the FaEO-NADPH/HMF complex crystallized from 19% (w/v) PEG3350, 0.2 M LiSO_{4}, 0.1 M Tris/HCl, pH 7.5. The different crystals were cryo-protected in reservoir solution supplemented with 20% (v/v) ethylene glycol prior to freezing in liquid nitrogen. Crystals of FaEO-NADPH/EDHMF, FaEO-NADPH/EHMF, and FaEO-NADP{\*} were obtained by stepwise soaking of FaEO-NADPH/HDMF crystals in 19% (w/v) PEG3350, 0.2 M LiSO_{4}, 0.1 M Tris/HCl, pH 7.5, supplemented with 20% (v/v) ethylene glycol with increasing concentrations of EDHMF, EHMF, and 9,10-phenanthrenequinone, respectively, followed by freezing in liquid nitrogen. Although 9,10-phenanthrenequinone apparently displaced HDMF, it did not bind tightly enough by itself to be observed in the electron density, thus resulting in the FaEO-NADP{\*} complex without bound substrate or product; instead, an ethylene glycol molecule of the cryo-protectant was complexed in the substrate pocket.

X-ray diffraction data were collected on beamlines BL14.1 or BL14.2 operated by the Helmholtz-Zentrum Berlin at the BESSY electron storage ring (Berlin-Adlershof, Germany (23)) and processed with the XDS package (24) (Table 1). The structure of FaEO-NADPH/HDMF was solved by molecular replacement using PHASER (25) with the quinone oxidoreductase HB8 from *Thermus thermophilus* (PDB entry 1IYZ (26)) as the search model. The crystal structure was built and refined in iterative cycles with COOT (27) and REFMAC5 (28). The other five FaEO structures were solved by molecular replacement using the FaEO-NADPH/HDMF complex.

To account for domain motions and flexibilities, Translation, Libration and Screw (TLS) groups were determined with TLSMD (29) and used for a final TLS and restraint refinement with REFMAC5. The six refined FaEO structures were finally validated with COOT and MolProbity (30). Molecular graphics were prepared with PyMOL (Schoedinger, Portland, OR). The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4IDA, 4IDB, 4IDC, 4IDD, 4IDE, and 4IDF.

**FaEO Enzyme Assay**—FaEO assays with EDHMF as substrate and NADH or [4R-2H]NADH as cosubstrate were performed as previously described (15). 24 μg of the purified recombinant enzyme from above was incubated at 30 °C in the presence of 250 μM EDHMF and 350 μM NADH or [4R-2H]NADH in a total volume of 1 ml of 0.1 M K_{2}HPO_{4}/KH_{2}PO_{4}, pH 5.0 or 7.0, for 30 min under agitation. The product EHMF was then extracted with diethyl ether for GC/MS analysis and with ethyl acetate for chiral-phase HPLC/UV analysis. The product solution was dried over sodium sulfate, concentrated under steady nitrogen flow, and was either directly injected into GC/MS or diluted in n-hexane and analyzed by HPLC/UV. Control reactions were performed without the addition of enzyme.

**Gas Chromatography-coupled Mass Spectrometry (GC/MS)**—Isotope-labeled and unlabeled EHMF were analyzed by GC/MS using an Agilent 6890N gas chromatograph equipped with an Agilent 5975 mass selective detector. A 2-μl aliquot of the extract from the FaEO enzyme assay was injected at a port temperature of 250 °C with the purge valve on (split mode), using a split ratio 15:1 and split flow of 16.3 ml/min. Separation of tautomers was achieved on an Agilent VF-5 ms column (10 m, 0.25 mm, 0.25 μm) using helium as carrier gas with a flow of 1.1 ml/min and an average velocity of 38 cm/s. The GC oven temperature was initially 40 °C, then ramped to 200 °C at 5 °C/min, and held there for 10 min. The total run time was 47 min, including a post-run time of 5 min at 320 °C, whereas the GC-mass spectrometer interface was kept at 310 °C. Mass spectra were collected in the scan mode within a m/z 45–350 range using a threshold of 150 and gain factor of 2. Ionization was performed by electron impact at 70 eV with calibration by autotuning. Data were analyzed with the MSD ChemStation E02.00.493 software (Agilent Technologies).

**Chiral-phase High Performance Liquid Chromatography (Chiral-phase HPLC/UV)**—Separation of EHMF isomers and enantiomers was achieved with a HPLC/UV system according to a published procedure (31). The samples, which were EHMF produced from EDHMF by FaEO as described above or synthetic EHMF (Sigma), were applied to a MaxiStar HPLC system (Knauer, Berlin, Germany) connected to a variable wavelength detector set to 288 nm and a Chiralpak IA column (250 × 4.6 mm; Daicel Chemical Industries, Illkirch, France) using n-hexane/ethyl acetate (90:10) as isocratic solvent system at a flow rate of 1 ml/min. Data were analyzed with the EuroChrom 2000 software (Knauer).

**RESULTS**

**Sequence, Bacterial Expression, and Crystallization of FaEO**—For biochemical characterization and protein crystallization FaEO was subcloned from the plasmid pET29a-FaEO (16) onto pASK-IBA5plus (22), thus encoding the gene product MAS-StrepII-G-FaEO(2–321) equipped with an N-terminal affinity tag. DNA sequencing of the resulting expression plasmid and also of the pET29a-FaEO precursor revealed three amino acid exchanges when compared with the published FaEO sequence (16): the substitutions T113P and Y125D and the deletion of...
Ala-210. Except for Pro-113, this FaEO sequence was identical with the one of *Fragaria vesca* (gene 28406) (32).

As all other known enone oxidoreductase (EO) polypeptide sequences from various species, with ≥70% sequence identity as retrieved from UniProt, neither contain an aromatic residue at position 125 nor an additional Ala at position 210 and exhibit either Lys or Thr at position 113, we decided to mutate Pro-113 to Thr and otherwise utilize the sequence as cloned. Thus, except for the N terminally appended Strep-tag II (22), the final FaEO expression construct corresponds to the sequence of the *F. vesca* EO. The recombinant enzyme was produced in the cytoplasm of *E. coli* as soluble monomeric protein and purified to homogeneity from the total cell extract using Strep-Tactin affinity, size exclusion, and anion exchange chromatography.

Crystals of FaEO in the apo-state and in complex with various substrates and analogs grew as tetragonal bipyramids reaching a final size of 300–500 μm in their largest dimension, which corresponded to the 4-fold axis of the crystals. The FaEO-NADPH/HDMF complex was crystallized at pH ~ 7.5 using PEG3350 as precipitant. Crystals appeared overnight and reached their final size within a week. In contrast, FaEO-NADPH/HMF crystals took about 4 weeks to grow to their final size. Crystals of apo-FaEO were obtained around pH 6.0, also with PEG3350 as precipitant, reaching their mature size after 3 weeks. To obtain crystals of the ligand complexes FaEO-NADP*, FaEO-NADP*/EDHMF, and FaEO-NADPH/EHMF, crystals of FaEO-NADPH/HMF were incubated in the presence of an excess of 9,10-phenanthrenequinone (to oxidize NADPH and deplete HDMF), EDHMF, and EHMF, respectively. The x-ray diffraction quality of all FaEO crystals was excellent with a diffraction limit ranging from 1.6 to 1.4 Å resolution (Table 1).

The crystal structure of FaEO was solved by molecular replacement using the published x-ray structure of a quinone oxidoreductase from *T. thermophilus* (PDBe code 1NYZ (26)) as a starting model, having an overall amino acid sequence identity of 30% as calculated by ClustalW2 (33). After refinement, all six FaEO crystal structures showed excellent statistics (Table 1). Except for the N terminally appended Strep-tag II, all residues of FaEO (i.e. amino acids 2–321 encoded on the natural gene), except for the N terminally appended Strep-tag II, all residues from various species, with ≥70% sequence identity as retrieved from UniProt, neither contain an aromatic residue at position 125 nor an additional Ala at position 210 and exhibit either Lys or Thr at position 113, we decided to mutate Pro-113 to Thr and otherwise utilize the sequence as cloned. Thus, except for the N terminally appended Strep-tag II (22), the final FaEO expression construct corresponds to the sequence of the *F. vesca* EO. The recombinant enzyme was produced in the cytoplasm of *E. coli* as soluble monomeric protein and purified to homogeneity from the total cell extract using Strep-Tactin affinity, size exclusion, and anion exchange chromatography.

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Three-dimensional Structure of FaEO—The structure of FaEO (Fig. 2) belongs to the zinc-independent medium chain dehydrogenase/reductase family (34). Its fold comprises two distinct domains, a non-contiguous substrate-binding domain (residues 2–146 and 265–321) with a GroES-like αβ-fold, encompassing a core of antiparallel β-strands, and a nucleotide-binding domain (residues 147–264) with a Rossmann-fold that consists of a 6-stranded parallel β-sheets, and a nucleotide-binding domain (residues 147–264) with a Rossmann-fold that consists of a 6-stranded parallel β-sheet sandwiched by 5 α-helices (35).

FaEO has approximate dimensions of 70 × 40 × 40 Å³ and behaves as a monomeric enzyme in solution as judged by size exclusion chromatography during FaEO purification, in line with previous reports (16). Analysis of all crystal packing contacts with Protein Interfaces Surfaces and Assemblies (PISA) (36) revealed no biologically relevant assemblies within the crystal lattice. Notably, the most prominent crystal contact of APO

| Data collection and refinement statistics | APO | NADP | NADPH | NADPHEDMF | NADPHEDHMF | NADPH/HDMF |
|------------------------------------------|-----|------|--------|-----------|-----------|------------|
| **Data collection**                      |     |      |        |           |           |            |
| Space group                              | P4 321 | P4 321 | P4 321 | P4 321 | P4 321 | P4 321 |
| Unit cell parameters (Å)                 | a = 70.0 | b = 70.0 | c = 70.0 | a = 70.0 | b = 70.0 | c = 70.0 |
| Wavelength (Å)                           | 0.9184 | 0.9184 | 0.9184 | 0.9184 | 0.9814 | 0.9184 |
| Resolution (Å)                           | 30.0-1.60 | 30.0-1.60 | 30.0-1.60 | 30.0-1.60 | 30.0-1.55 | 30.0-1.55 |
| Completeness (%)                         | 100 | 100 | 100 | 100 | 99.9 | 100 |
| Unique reflections (Å)                   | 58,211 | 64,320 | 70,634 | 58,034 | 64,541 | 58,034 |
| Multiplicity                             | 9.6 | 9.7 | 7.9 | 8.1 | 8.1 | 8.1 |
| Mean (I/σ(I))                            | 27.2 | 24.4 | 18.0 | 26.4 | 23.4 | 27.0 |
| **Refinement**                           |     |      |        |           |           |            |
| Reflection (Å)                            |      |      |        |           |           |            |
| R_work (%)                               | 5.0 | 5.8 | 6.6 | 5.4 | 6.8 | 6.5 |
| R_free (%)                               | 17.3 | 17.1 | 16.0 | 17.1 | 18.0 | 16.3 |
| Ramachandran plot: favored/outliers (%)  | 98.7/0.3 | 98.4/0.3 | 98.3/0.3 | 98.4/0.3 | 98.4/0.3 | 98.4/0.3 |
| Root mean square deviation bonds (Å)      | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Root mean square deviation angles (°)     | 1.99 | 1.96 | 2.02 | 1.99 | 2.07 | 1.96 |
| **Values in parentheses refer to the highest resolution shell.** |     |      |        |           |           |            |

* Ramachandran statistics were calculated with MolProbity (36).
FaEO differs significantly between the apo and the complex structures, with buried surface areas of 767 Å² and (on average) 912 Å², respectively.

A structural similarity search of FaEO/H18528NADPH/HDMF with the protein structure comparison service "Fold at European Bioinformatics Institute" (ebi.ac.uk/msd-srv/ssm) revealed a putative quinone oxidoreductase from *Coxiella burnetii* with bound NADPH (PDB code 3TQH) as the most similar structure with a root mean square deviation of 1.6 Å for 290 aligned Cα positions, despite merely 36% amino acid sequence identity.

As mentioned above, FaEO was also initially annotated as a quinone oxidoreductase and renamed after identification of its natural substrate. Thr-113, which has been mutated (see above), mediates two hydrogen bonds, one with its side chain hydroxyl group from the amide nitrogen of Phe-115 and one with its amide nitrogen to the side chain carboxamide of Asn-105. Both of these hydrogen bonds cannot be formed by a Pro residue at position 113. In fact, the two hydrogen bonds apparently restrain the conformation of the 104–114 loop, which participates in the substrate-binding pocket (see below).

The apo-structure of FaEO shows an open active-site cleft (Fig. 2B) that is largely filled with water molecules. A sulfate ion from the crystallization buffer is bound in the cofactor-binding site via residues Ser-197, Lys-200, and Tyr-215 as part of the Rossmann-fold. Furthermore, an ethylene glycol molecule of the cryo-protectant is bound in the substrate pocket. The crystal structure of the FaEO/NADP⁺ complex shows NADP⁺ tightly bound in the cofactor-binding site, which is located between the two domains described above and stretches across the small dimension of FaEO (Fig. 2B). Again, an ethylene glycol molecule is bound in the substrate pocket.

Compared with the apoenzyme, binding of NADP⁺ causes a small conformational change, resulting in a 5° rotation between the two domains of FaEO. NADP⁺ forms a total number of 168 contacts (within 4 Å) with FaEO, including 16 hydrogen bonds, 12 water-mediated hydrogen bonds, and 2 salt bridges. The salt bridges are formed between the 2’-phosphate group and resi-

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**FIGURE 2. Three-dimensional structure of FaEO and its ligand complexes.** A, schematic representation of FaEO in complex with NADP⁺ and EDHMF. The two domains of FaEO are depicted in green and orange; the cosubstrate NADP⁺ and the surrogate substrate EDHMF are shown as sticks and spheres, respectively. B, the shape of the FaEO substrate/cosubstrate pocket in different complex structures. Bound ethylene glycol (EG), sulfate (SO₄²⁻), and NADP⁺ are shown as sticks, whereas EDHMF is depicted as spheres.

**FIGURE 3. Substrate binding to FaEO.** A, stereo representation of the FaEO-NADP⁺/EDHMF complex. FaEO is depicted as ribbon diagram and side chains that form substrate contacts are shown as sticks. The shape of the substrate pocket is illustrated by a translucent surface. NADP⁺ and EDHMF are highlighted as stick models in light blue and salmon, respectively. Water molecules are shown as red spheres, and hydrogen bonds are indicated as black dashed lines. B, the Fₒ/Fc omit electron density map for EDHMF, NADP⁺, Lys-59, and Wat-1 contoured at 3σ (blue mesh) illustrates the high definition of the FaEO-ligand complex.

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3 M. C. Franklin, J. Cheung, M. Rudolph, M. Cassidy, E. Gary, F. Burshteyn, and J. Love, unpublished data.
FIGURE 4. Structural comparison of different FaEO-NADP(H)/ligand complexes. A, chemical configuration of products/substrates or analogs bound in the different FaEO complex structures. B, illustration of the polar interactions in the substrate pocket of FaEO. The side chain of Lys-59, the cofactor NADP(H), and the bound ligands are shown as green, gray, and salmon stick models, respectively. Hydrogen atoms at the asymmetric C2 carbon atom of R-HDMF and R-2-EHMF, at the C5 carbon atom of HMF, and also at the C4 carbon atom of the NADP(H) nicotinamide ring are depicted also. Distances are given in Å. C, the structural fragments illustrated in B rotated by 90° around the x axis.

...dyes Lys-200 and Arg-311. This position is occupied by the sulfate ion in the apo-structure described above. The large number of contacts seen between both domains of FaEO and NADP* suggests that binding of NAD(H), which can replace NADP(H) as redox cofactor (16), might induce a similar conformational change despite lacking the 2'-phosphate group.

Complexes of FaEO with Substrates/Products and/or Analogs and Implications for the Catalytic Mechanism—The substrate pocket of FaEO is lined by the side chains of amino acids Pro-55, Val-56, Lys-59, Phe-65, Ala-108, Leu-109, Leu-146, Val-265, and Leu-266 (Fig. 3). These residues are part of the substrate-binding domain and, with the exception of Lys-59, exclusively mediate hydrophobic contacts. Phe-65 adopts two distinct conformations of which only either one is observed in each of the complexes. However, there is no structural evidence that these different conformations play a role during catalysis. Apart from the protein residues, a significant portion of the substrate pocket is formed by residues Lys-59, Phe-65, Ala-108, Leu-109, Leu-146, Val-265, and the noncovalently bound redox cofactor NADP(H).

Also, two structurally conserved water molecules are bound within the substrate pocket. Water molecule 1 (Wat-1) is held in place by the main chain nitrogen of Val-56 (Fig. 3) and a second water molecule (Wat-2). Wat-2 is fixed by the side chain of Asn-54 and the 5'-phosphate of the nicotinamide nucleotide. The substrate EDHMF, the products HDMF and EHMF as well as the product analog HMF are all tightly bound within the substrate pocket; they are involved in numerous Van der Waals contacts and four hydrogen bonds, namely with the side chain of Lys-59, with Wat-1, and twice with the 2'-OH of the ribose moiety that carries the nicotinamide group (Figs. 3 and 4).

According to chemical principles, reduction of α,β-unsaturated ketones such as in HMMF by way of hydride ion transfer from NAD(P)H, in contrast with FADH2, can either occur at the polarized carbonyl group (here at C3; Fig. 1) in a 1,2-hydrogen addition reaction or at the exocyclic double bond (here at C6) via a 1,4 addition. The reaction itself suggests a 1,4 addition mechanism as no alkene intermediates have been identified. Comparison of the different FaEO ligand complexes crystallized in this study provides insight into how the latter reaction is catalyzed.

The FaEO-NADPH/HDMF complex was obtained by co-crystallization of FaEO in the presence of 10- and 50-fold molar ratios of NADPH and HDMF, respectively. Due to the large excess of HDMF over NADPH it has to be assumed that the NADP+ cofactor became reduced upon mixing with the enzyme, with the excess HDMF remaining in its reduced state. Although hydrogen atoms cannot be resolved in the crystal structure, despite the high resolution obtained, we conclude that the complex represents an unproductive assembly of FaEO with bound NADPH and HDMF (Fig. 4). Although HDMF exists as a racemic mixture in aqueous solution, the clearly defined electron density shows that only its 2R-4,5-enolic-3-keto form is bound in the substrate pocket. There, HDMF forms hydrophobic contacts with the side chains of Val-56, Lys-59, Phe-65, Leu-109, and Val-265, whereas Lys-59 is also involved in a hydrogen bond with the carbonyl oxygen of HDMF (at C3). Another hydrogen bond to the hydroxyl group of HDMF (at C4) is mediated via the tightly bound water molecule Wat-1. Furthermore, HDMF forms contacts with the nicotinamide group and the adjacent ribose of the cofactor NADPH, including two hydrogen bonds between the 2'-OH of the ribose and the hydroxyl as well as carbonyl groups of HDMF. Otherwise, the ring atoms O1, C2, C5, and the two...
methyl substituents of HDMF exclusively form apolar contacts in the substrate pocket. In this way, the methyl group at C5 of HDMF points into the substrate pocket and is positioned in close proximity, with a distance of 3.4 Å, to the C4 atom of the nicotinamide ring of NADPH (Fig. 4). On the other hand, the methyl group at the chiral C2 of HDMF points out of the substrate pocket into solvent.

The FaEO-NADPH/EHMF complex was obtained by soaking crystals of FaEO-NADPH/HDMF (see above) with EHMF, hence leaving the bound NADPH presumably reduced. Due to its extended keto-enol tautomerism, EHMF can occur as several structural isomers in aqueous solution that show distinct stereochemistries at C2 and C5 (Fig. 1). The clearly defined electron density indicates that only the R-configuration at the chiral, ethyl-substituted C2 of EHMF is complexed by the enzyme (Fig. 4). The overall binding mode of EHMF is very similar to that of HDMF, again resembling an unproductive, doubly reduced complex. Similarly, EHMF forms contacts with FaEO side chains Val-56, Lys-59, Phe-65, and Leu-109 as well as with the nicotinamide and the adjacent ribose of the cofactor NADPH. Also, EHMF participates in four hydrogen bonds equivalent to the hydrogen bond pattern of HDMF. EHMF binds with the methyl group at C5 into the hydrophobic subpocket such that the methyl group is within 3.5 Å distance to the C4 atom of NADPH, whereas the ethyl group at C2 points out of the substrate pocket into solvent (Fig. 4). Thus, EHMF is clearly bound in an opposite orientation as would be necessary for the hydride ion transfer in the backward reaction leading to EDHMF (cf. Fig. 1 and the next panel in Fig. 4).

Likewise, the FaEO-NADP$^+$/EDHMF complex was obtained by soaking crystals of FaEO-NADP$^+$/HDMF with EDHMF to replace the bound HDMF. Due to the large excess of EDHMF, the enzyme-bound NADPH probably became oxidized, resulting in the unproductive, this time double oxidized FaEO-NADP$^+$/EDHMF complex (Fig. 4). Indeed, the (fully) unsaturated EDHMF clearly binds as a planar molecule to the substrate pocket and forms contacts with the side chains of Val-56, Lys-59, Ala-108, Leu-146, and Val-265 as well as with the nicotinamide and ribose of NADP$^+$. Compared with EHMF, the ring plane of EDHMF is flipped by 180° but still forms similar hydrogen bonds within the substrate pocket. The EDHMF hydroxyl group is hydrogen-bonded by both the side chain of Lys-59 and the 2‘-OH of the NADP$^+$ ribose, whereas its carbonyl group forms hydrogen bonds with the 2‘-OH of the NADP$^+$ ribose and Wat-1. In this way, the EDHMF ethylidene group at C2 points into the hydrophobic environment of the substrate pocket, resulting in a short distance of 3.4 Å between C6 of EDHMF and the C4 atom of NADP$^+$ . In fact, this would be an almost ideal arrangement for the reduction reaction, provided that NADP$^+$ was replaced by NADPH (Figs. 3 and 4).

Finally, the FaEO-NADPH/HMF complex was prepared by co-crystallization of FaEO with a 10- and 50-fold molar ratio of NADPH and HMF, respectively. HMF is a substrate analog of HDMF (in the backward reaction) that lacks the methyl substituent at C5. HMF binds in a very similar manner to FaEO as HDMF and EHMF via contacts with the side chains of Val-56, Lys-59, Leu-109, and Val-265. Again, HMF participates in four hydrogen bonds. The hydroxyl group of HMF forms hydrogen bonds with the 2‘-OH of the NADPH ribose and with Wat-1, whereas the carbonyl group forms hydrogen bonds with the same 2‘-OH of the NADPH and the side chain of Lys-59. Due to the missing methyl substituent at C5, HMF can form even tighter contacts with the nicotinamide ring of the NADPH coenzyme. The methyl group of HMF at C2 shows, with 3.4 Å, the same close distance to the C4 atom of NADPH as HDMF and EDHMF in their respective complexes (Fig. 4). Indeed, this arrangement corresponds to the quasi “productive” orientation observed for EDHMF above; however, in this case both the substrate/product ligand HMF and the cofactor are present in the reduced state.

Taken together, in all four FaEO complex structures with bound substrate, product, or substrate analog, the reactive exo carbon atoms are within close distance (3.4–3.5 Å) to the C4 atom of the NADP(H) cofactor. This strongly suggests that transfer of the 4R-hydride from NADP to the substrates HMMF or EDHMF occurs at the outward carbon atom of the exo-double bond, thus leading to the formal 1,4-hydrogen addition reaction as postulated above.

Regiospecificity of Hydride Ion Transfer to a Surrogate Substrate—To experimentally confirm the atomic position of hydride ion transfer from NAD(P)H to the substrate, the enzyme was stereospecifically deuterated and subsequently used to enzymatically synthesize 2H-labeled EHMF from EDHMF. Because NADH has a very similar $K_m$ value (361 µM) as NADPH ($K_m = 325$ µM), despite a lower specificity constant $k_{cat}/K_m = 0.02$ s$^{-1}$ µM$^{-1}$ versus 0.15 s$^{-1}$ µM$^{-1}$, respectively, 2H-labeled NADH was employed for this experiment, which was accessible by means of an established synthesis procedure (37). To this end, enzymatic synthesis of [4R-2H]NADH from NAD$^+$ with $[^1H]$formic acid catalyzed by formate dehydrogenase was monitored spectrophotometrically at 340 nm until completion. Single deuteration in the anion exchange-purified [4R-2H]NADH was confirmed by LC-UV/ESI-MS$^+$ (Fig. 5). This cofsubstrate, regioselective deuteration of EHMF in the presence of FaEO was investigated via GC-MS analysis (Fig. 5). Comparison of the mass spectrometric fragmentation pattern of the enzymatic reaction product either in the presence of [4R-2H]NADH or of unlabeled NADH (Fig. 5) with published data (38) clearly revealed that the deuterium was transferred to the exo-carbon of the ethylidene moiety (attached to C2 of the furanone ring; cf. Fig. 1).

Considering the prochiral nature of EDHMF, one would expect the preferential enzymatic synthesis of one stereoisomer of EHMF by FaEO. However, in solution EHMF comprises a mixture of tautomers with distinct keto-enol structures similar to HDMF (Fig. 1), including four prevailing diastereomers that can be chromatographically resolved by chiral-phase HPLC (31). Commercially available EHMF is a synthetic racemic mixture of the constitutional isomers 5-EHMF and 2-EHMF in a ratio of about 1:3 to 1:5 (Fig. 6) (31, 39). In contrast, EHMF freshly produced by FaEO comprises a mixture of 5-EHMF and 2-EHMF in a significantly lower ratio of 1:1.75 (Fig. 6). This indicates that the enzymatically catalyzed reaction has regiospecific preference, which will be discussed in the light of the proposed mechanism below.
DISCUSSION

The elucidation of all six different crystal structures of FaEO in complex with various ligands has provided detailed insight into the catalytic mechanism of this novel kind of enzyme.

Binding of NADP(H) to FaEO leads to conformational changes that result in its tight encapsulation and proper positioning of the nicotinamide ring within the cofactor-binding site, also shaping the adjacent substrate pocket. The substrate pocket is

**FIGURE 5.** Mass spectrometric analysis of enzymatically synthesized [4R-2H]-NADH and GC-MS analysis of FaEO catalyzed EHMF formation. A, LC-UV/ESI-MS of NADH and [4R-2H]-NADH. Left spectra show the full scan (+MS) and product ion spectrum (+MS2 of m/z 666) of an NADH reference and the right spectra show the full scan (+MS) and product ion spectrum (+MS2 of m/z 667) of the enzymatically synthesized [4R-2H]-NADH. B, gas chromatographic separation (left, top and bottom) and corresponding mass spectra (middle and right, respectively, for each of the two product peaks) of the EHMF isomers that were enzymatically synthesized by recombinant FaEO from EDHMF in the presence of NADH (top row) or [4R-2H]-NADH (bottom row). The previously elucidated fragmentation pattern of 3(2H)-furanones (38) and the deduced molecular fragments are indicated.

**FIGURE 6.** Chiral analysis of enzymatically produced EHMF stereoisomers. The distribution of EHMF configurational isomers (tautomers) and stereoisomers enzymatically produced by FaEO (continuous line) was compared with a synthetic reference sample (dashed line). The thermodynamically more stable racemate of R(+)-2-EHMF and S(-)-2-EHMF clearly prevails over the racemate of the 5-EHMF configurational isomers in the reference sample, whereas a higher level of the latter two compounds (R,S-5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone racemate) results immediately after enzymatic synthesis, notably without apparent stereospecificity. Numbers inside the ring refer to the numbering scheme applied to all furanone compounds in this article (cf. Fig. 1), whereas IUPAC numbering is used for the chemical names.
lined by hydrophobic side chains on one side and provides the hydrogen-bonding partners Lys-59, Wat-1, and the 2'-OH of NADPH as well as its nicotinamide ring that acts as hydride ion donor on the other side. However, due to their high pK values none of the former groups can act as a general acid/base during catalysis. There is also no other distant amino acid side chain that might assist proton transfer, e.g. via the structural water molecule Wat-1, suggesting that the catalytic mechanism of the redox reaction does not depend on enzyme-mediated proton transfer.

All four substrate-product complexes have in common that both the carbonyl group and the hydroxyl group of the hydroxyfuranone ring system are engaged in two hydrogen bonds, each with the 2'-OH of the NADP(H) ribose and either one with Lys-59 or Wat-1 (Fig. 4). All four ligands form similar Van der Waals contacts with FaEO and the nicotinamide group of NADP(H). Notably, the C6 exo-carbon atom of the furaneone derivatives EDHMF and HMF is in Van der Waals distance (3.4 Å) to the C4 position of NADP(H), which clearly suggests that the hydride ion transfer occurs to the methylidene (or ethylidene) group of the oxidized substrate HMMF (or the surrogate substrate EDHMF) rather than to the neighboring carbonyl C atom.

The complex structures with the corresponding reduced compounds HDMF and EHMF clearly reveal that the reaction product cannot enter the substrate pocket with its sp3-hybridized C2 carbon ahead. In both complexes the chiral C2 ring carbon points out of the substrate pocket. By contrast, the oxidized surrogate substrate EDHMF, which adopts a planar sp2 configuration at the unsaturated C2 carbon, can enter the substrate pocket with its ethylidene group ahead. Similarly, the product analog HMF in its enol form, which lacks the methyl group of HDMF at C5, can even better enter the substrate pocket and form closer contacts with NADPH than HDMF. This suggests that a planar configuration at the hydride acceptor site of the substrate plays a role during enzymatic catalysis.

Due to their dynamic keto/enol tautomerism both products, HDMF and EHMF, can also adopt a fully planar di-enol configuration (Fig. 1). However, in aqueous solution only mixed keto/enol tautomers of HDMF and EHMF are observed in significant quantities by NMR, which also seems to be the case if bound at the substrate pocket of FaEO in the crystals. As clearly visible in the electron density, FaEO binds the chiral compounds only in the R-configuration. Notably, the observed binding mode of the reduced entities HDMF and 2-EHMF represents an unproductive assembly that would not allow hydride abstraction via backward reaction in the presence of the oxidized cofactor NADP+. In fact, this orientation suggests a possible mode of product inhibition of FaEO by HDMF and EHMF.

In contrast, the fully planar surrogate substrate EDHMF in principle can enter the substrate pocket of FaEO with either its ethylidene group at C2 or its methyl group at C5 ahead. However, only the orientation with C2 pointing into the substrate pocket was observed in the electron density (Fig. 3B). Presumably, the ethylidene group can form better hydrophobic contacts among the FaEO side chains that line the substrate pocket. Consequently, EDHMF binds in an orientation in which it could accept a hydride ion from NADPH at its C6 carbon of the exo-double bond. Despite being crystallized in the presence of NADP+, this orientation should represent the substrate-binding mode during hydride transfer. This interpretation is supported by deuteration experiments that unambiguously identified the C6 carbon of EDHMF as the hydride ion acceptor (Fig. 5). Based on this data, we conclude that the chemically more labile natural furaneol precursor HMMF during catalysis most likely binds in the same orientation as EDHMF to the FaEO substrate pocket, such that it can accept the hydride ion at its C6 carbon in the same way.

The keto/enol forms of the reduced products HDMF and 2-EHMF that are populated in aqueous solution each have a C-H acidic sp3-hybridized C2 carbon with theoretical pK values of 7.1 and 7.5, respectively (Fig. 1). This group should be predominantly deprotonated at the slightly alkaline physiological pH of the plant cytoplasm. However, as outlined above, even in a deprotonated state the sp3-hybridized C2 carbon is likely not able to enter the substrate pocket of FaEO in a productive manner, due to its non-planar geometry. In contrast, the planar di-enol(ate) forms of HDMF and EHMF, which show theoretical pK values of 7.1 and 7.0 for their enolic groups, respectively (Fig. 1), can easily enter, or leave, the substrate pocket. According to the principle of microscopic reversibility, this means that the enzyme actually prefers a less populated tautomer considering the backward reaction. Indeed, the negative charge of the enolate anion of the reduced product can be
compensated by the positive charges of both the basic Lys-59 side chain and the pyridinium ring of the oxidized NADP⁺ cofactor. Thus, in the backward reaction hydride ion transfer can occur from the C6 carbon of HDMF or EHMF to the cofactor, resulting in the unsaturated and fully planar FaEO substrates HMMF and EDHMF.

Taken together, the in planta substrate HMMF, as well as the surrogate substrate EHMF, is a planar molecule that can enter the substrate pocket of FaEO with its methylidene, or ethyldiene, moiety, ahead. The substrate forms several contacts with side chains in the active site of FaEO and, importantly, also with the NADPH cofactor, including four hydrogen bonds with Lys-59, Wat-1, and twice the 2'-OH of the NADPH ribose. In this way the unsaturated exo-carbon is oriented in optimal position to the C4 atom of the NADPH nicotinamide ring (Fig. 7). Transfer of the hydride ion leads to reduction via a formal 1,4-hydrogen addition. This initially results in an enol/enolate product that corresponds to the aromatic furan structure of HDMF (or EHMF). The emerging positively charged oxidized nicotinamide, together with Lys-59, should favor this hydride transfer by electrostatically stabilizing the negative charge of the enolate anion (Fig. 7).

Subsequently, the proton of the enol group on the other side of the product molecule may be transferred onto the enolate, possibly mediated by the 2'-OH of the NADPH ribose. In this way the unsaturated exo-carbon is oriented in optimal position to the C4 atom of the NADPH nicotinamide ring (Fig. 7). Transfer of the hydride ion leads to reduction via a formal 1,4-hydrogen addition. This initially results in an enol/enolate product that corresponds to the aromatic furan structure of HDMF (or EHMF). The emerging positively charged oxidized nicotinamide, together with Lys-59, should favor this hydride transfer by electrostatically stabilizing the negative charge of the enolate anion (Fig. 7).

In principle, protonation of the product can occur by two different mechanisms: (i) the planar mixed enolate/enol form of HDMF (or EHMF) (Fig. 1) directly leaves the substrate pocket or (ii) it first tautomerizes to the corresponding keto/enol form with an sp³-hybridized deprotonated C5 carbon (Fig. 1), which can point out of the pocket in a sterically favorable manner, prior to dissociating from the substrate pocket. In the case of HDMF these alternative protonation scenarios cannot be distinguished due to the pseudo-symmetry of the molecule (cf. Fig. 1). However, the second scenario appears to be of importance for protonation of the surrogate product EHMF as the enzymatically synthesized compound shows an increased ratio between the 5-EHMF and 2-EHMF tautomers compared with a synthetic racemic mixture (Fig. 6; note that the 2-EHMF stereoisomers are only formed after keto/enol tautomerization of the primary reaction product).

Only recently, NAD(P)H-dependent, non-flavin ene reductases have been investigated for their ability to reduce C = C double bonds in a number of structurally diverse substrates (40 – 43). In this regard, FaEO exhibits a narrow substrate spectrum and, beside its natural substrate HMMF, predominantly reduces α,β-unsaturated diesters and nitroalkanes, apart from the earlier described quinones. This is in line with the reaction mechanism deduced here and the structural finding that only planar enones can enter the active site of FaEO. Therefore, our results not only provide insight into the peculiar catalytic cycle of this novel enone oxidoreductase but also should facilitate protein-engineering efforts for the development of improved biocatalysts for biotechnological processes.

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