Structure and Mechanism of Ferulic Acid Decarboxylase (FDC1) from *Saccharomyces cerevisiae*

Mohammad Wadud Bhuiya,^a Soon Goo Lee,^b Joseph M. Jez,^b Oliver Yu^{a,c}

Conagen, Inc., Bedford, Massachusetts, USA^a; Department of Biology, Washington University in St. Louis, St. Louis, Missouri, USA^b; Wuxi NewWay, Wuxi, Jiangsu, China^c

The nonoxidative decarboxylation of aromatic acids occurs in a range of microbes and is of interest for bioprocessing and metabolic engineering. Although phenolic acid decarboxylases provide useful tools for bioindustrial applications, the molecular bases for how these enzymes function are only beginning to be examined. Here we present the 2.35-Å-resolution X-ray crystal structure of the ferulic acid decarboxylase (FDC1; UbiD) from *Saccharomyces cerevisiae*. FDC1 shares structural similarity with the UbiD family of enzymes that are involved in ubiquinone biosynthesis. The position of 4-vinylphenol, the product of *p*-coumaric acid decarboxylation, in the structure identifies a large hydrophobic cavity as the active site. Differences in the β 2e- α 5 loop of chains in the crystal structure suggest that the conformational flexibility of this loop allows access to the active site. The structure also implicates Glu285 as the general base in the nonoxidative decarboxylation reaction catalyzed by FDC1. Biochemical analysis showed a loss of enzymatic activity in the E285A mutant. Modeling of 3-methoxy-4-hydroxy-5-decaprenylbenzoate, a partial structure of the physiological UbiD substrate, in the binding site suggests that an $\sim$ 30-Å-long pocket adjacent to the catalytic site may accommodate the isoprenoid tail of the substrate needed for ubiquinone biosynthesis in yeast. The three-dimensional structure of yeast FDC1 provides a template for guiding protein engineering studies aimed at optimizing the efficiency of aromatic acid decarboxylation reactions in bioindustrial applications.

The chemical production of benzenoids, such as styrene, from petroleum provides a wide range of building blocks for use in paints, dyes, plastics, and synthetic pharmaceuticals. Current styrene production involves the energy-intensive dehydrogenation of petroleum-derived ethylbenzene and yields more than 30 million tons of material each year (1). As an alternative to petroleum-based synthesis, research into finding renewable sources of benzenoid compounds in nature has focused attention on multiple plant and microbial pathways. Substituted cinnamic acids are abundant molecules in plant lignin polymers and can provide feedstocks for microbial bioprocessing methods aimed at yielding value-added products (2, 3). Degradation of lignin releases ferulic and *p*-coumaric acids that can be converted to 4-vinylguaiacol (4-ethenyl-2-methoxyphenol) and 4-vinylphenol (4-ethenylphenol), respectively, and other hydroxycinnamates can be metabolized to vanillin as a natural flavoring in foods, beverages, and other products (4, 5). Related aromatic compounds are also natural components in wine and other fermented beverages and food (6–8). Similarly, other production routes for catechol and styrene, using microbes that metabolize benzenoid molecules by nonoxidative decarboxylation, have also been explored recently (8–10). For example, overexpression of phenylalanine ammonia lyase from *Arabidopsis thaliana* and of ferulic acid decarboxylase (FDC) from *Saccharomyces cerevisiae* in engineered *Escherichia coli* led to a strain that produced styrene (9, 10). In microbes, a variety of enzymes perform the nonoxidative decarboxylation of aromatic compounds.

Ferulic acid, phenylacrylic acid, and phenolic acid decarboxylases are found in diverse fungi, yeast, and bacteria (4, 6, 7, 11–22). Structural studies have begun to provide insight into the variety of enzymes involved in the decarboxylation of aromatic acids. To date, three distinct types of nonoxidative decarboxylases have been identified by protein crystallography studies. The first X-ray structure of a phenylacrylic acid decarboxylase (i.e., Pad1 from *E. coli*) (23) revealed a dodecameric flavoprotein (monomer molec-

ular mass, $\sim$ 23 to 25 kDa). This structure and a later structural analysis of a putative aromatic acid decarboxylase from *Pseudomonas aeruginosa* (24) showed that each monomer in the dodecamer adopts a Rossman fold motif and contains a noncovalently bound flavin mononucleotide. Genetic studies of *E. coli* suggested that Pad1 (also known as UbiX) functions in ubiquinone biosynthesis to catalyze the decarboxylation of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenylphenol (25, 26). In contrast to the dodecameric UbiX-like proteins, the phenolic acid decarboxylases from *Lactobacillus plantarum*, *Bacillus pumilus*, and *Enterobacter* sp. Px6-4 form a second class of enzymes that catalyze the nonoxidative decarboxylation of aromatic compounds (27–29). These enzymes are dimeric proteins, with each monomer (molecular mass, 19 to 22 kDa) adopting a flattened β -barrel architecture that shares structural homology with the lipocalin fold. A third type of decarboxylase was identified in the UbiD-related putative 3-polyphenyl-4-hydroxybenzoate decarboxylase from *Pseudomonas aeruginosa* PA0254 (30). The UbiD-related proteins from *P. aeruginosa* function as either dimeric or hexameric proteins composed of 50-kDa monomers (30). As with UbiX, UbiD proteins are thought to function in ubiquinone biosynthesis (31); however,

Received 7 March 2015 Accepted 8 April 2015

Accepted manuscript posted online 10 April 2015

Citation Bhuiya MW, Lee SG, Jez JM, Yu O. 2015. Structure and mechanism of ferulic acid decarboxylase (FDC1) from *Saccharomyces cerevisiae*. Appl Environ Microbiol 81:4216–4223. doi:10.1128/AEM.00762-15.

Editor: A. A. Brakhage

Address correspondence to Joseph M. Jez, jjez@wustl.edu, or Oliver Yu, oliver.yu@conagen-inc.com.

M.W.B. and S.G.L. contributed equally to this article.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.00762-15

the metabolic relationship between these two proteins is unclear. In *S. cerevisiae*, both ferulic acid decarboxylase (FDC1) and phenylacrylic acid decarboxylase (PAD1) are required for *in vivo* decarboxylation of substituted cinnamic acids (21). In other microbes, the biochemical functions of UbiX and UbiD may be redundant (29, 32).

Given the interest in using aromatic acid decarboxylases in bioprocessing and metabolic engineering applications, we present the three-dimensional structure of FDC1 from *S. cerevisiae*. The overall structure establishes FDC1 as a UbiD-like enzyme. Crystallization with a decarboxylated reaction product identifies a large apolar cavity as the active site of the enzyme and suggests a catalytic mechanism for the nonoxidative decarboxylation reaction of aromatic substrates.

MATERIALS AND METHODS

Isolation of yeast FDC1 clone and generation of bacterial expression vector. Genomic DNA was prepared from *S. cerevisiae* YJM993 (33). Yeast cells (1.5 ml) were grown for ~24 h at 30°C in yeast extract-peptone-dextrose (YPD) medium. Cells were pelleted by centrifugation (20,000 × g; 5 min). Following addition of resuspension buffer (2% [vol/vol] Triton X-100, 1% [wt/vol] SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), the tube was immersed in a dry ice-ethanol bath (2 min) and then transferred to a 95°C water bath (1 min). Following chloroform extraction and centrifugation (20,000 × g; 5 min), the upper aqueous phase was transferred to a microcentrifuge tube containing ice-cold ethanol. After incubation at room temperature (5 min) and centrifugation (20,000 × g; 5 min), the supernatant was removed by vacuum aspiration, and the pellet was washed with 70% (vol/vol) ethanol. After a second wash, the final pellet was air dried and resuspended in Tris-EDTA (TE; pH 8.0) buffer.

For PCR amplification of FDC1 (YDR539W), which does not contain an intron, from the genomic DNA, the following primers were used: YFDC-F, 5'-ATGAGGAAGCTAAATCCAGCTTTAGA-3'; and YFDC-R, 5'-TTATTTATATCCGTACCTTTTCCAA-3'. The resulting 1,512-bp PCR fragment was gel purified and subcloned into the PCR8/GWff OPO vector (Invitrogen). The resulting plasmid was fully sequenced by Genewiz (South Plainfield, NJ), using standard M13 forward and M13 reverse primers. For expression of FDC1 in *E. coli*, the coding region was cloned into the Gateway vector pDEST17 (Invitrogen), in which expression of a hexahistidine-tagged protein is driven by a T7 promoter, using LR Clonase (Life Technologies) for *in vitro* recombination of the entry clone and the destination vector to yield the pDEST17-FDC1 vector, according to the manufacturers' protocols. Site-directed mutagenesis of Glu285 to alanine (E285A) in yeast FDC1 was performed by QuikChange PCR mutagenesis using the pDEST17-FDC1 vector as the template, with appropriate mutant oligonucleotides.

Protein expression and purification. The pDEST17-FDC1 plasmid was transformed into *E. coli* BL21(DE3). Cells were grown at 37°C in Terrific broth supplemented with ampicillin (100 µg ml⁻¹) to an A₆₀₀ of ~0.8 to 1.0. Protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and the cells were grown for 16 h at 16°C. Cells were pelleted by centrifugation (10,000 × g; 10 min; 4°C), washed with 1× phosphate-buffered saline (PBS), and then resuspended in buffer A [50 mM Tris-HCl, pH 8.0; 50 mM Na₂S₂O₃; 25 mM Tris(2-carboxyethyl-phosphine), 500 mM NaCl, 0.5 mM phenylmethanesulfonyl fluoride, 20 mM β-mercaptoethanol, 10% glycerol, and 10 mM imidazole] containing 10 mM MgCl₂, 0.2% (vol/vol) Triton X-100, 2 µg ml⁻¹ DNase, 2 µg ml⁻¹ RNase, and 4 µg ml⁻¹ lysozyme. The cells were disrupted by sonication, and cell debris was removed by centrifugation (15,000 × g; 30 min; 4°C). The resultant supernatant was filtered through a 0.45-µm polyethersulfone filter and then applied to a Ni²⁺-nitrilotriacetic acid-agarose affinity purification column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A and the protein eluted with buffer A containing 250 mM

TABLE 1 Crystallographic statistics

Parameter	Value or description ^d
Crystal statistics	
Space group	C2
Cell dimensions (Å) (<i>a</i> , <i>b</i> , <i>c</i>)	252.0, 121.0, 159.6
Data collection statistics	
Wavelength (Å)	0.979
Resolution range (Å)	47.1–2.35 (2.40–2.35)
No. of reflections (total/unique)	596,697/162,297
% completeness	96.1 (70.3)
<I/σ>	17.2 (2.0)
R _{sym} ^a	8.2 (39.1)
Model and refinement statistics	
R _{cryst} ^b /R _{free} ^c (%)	17.8/22.7
No. of protein atoms	30,457
No. of water molecules	1,381
No. of ligand atoms	45
RMSD for bond lengths (Å)	0.009
RMSD for bond angles (°)	1.182
Avg B factor (Å ²) (protein, water, ligand)	41.8, 37.8, 49.7
Stereochemistry (% most favored, % allowed, % outliers)	96.5, 3.3, 0.2

^a R_{sym} = Σ|I_h - <I_h>|/ΣI_h, where <I_h> is the average intensity over symmetry.

^b R_{cryst} = Σ|F_o - <F_c>|/ΣF_o, where summation is over the data used for refinement.

^c R_{free} is defined the same as R_{cryst} but was calculated with 5% of the data excluded from refinement.

^d Data in parentheses are for the highest shell.

imidazole. Size-exclusion chromatography was performed using a Superdex-200 26/60 fast-performance liquid chromatography (FPLC) column equilibrated in buffer A. The final protein concentration was determined spectrophotometrically (Σ₂₈₀ = 64,455 M⁻¹ cm⁻¹).

Protein crystallization and structure determination. Crystals of yeast FDC1 were obtained by the hanging-drop vapor diffusion method, using a 2-µl drop of protein (6.6 mg ml⁻¹) and 5 mM 4-hydroxycinnamic acid (*p*-coumaric acid) mixed with a 2-µl drop of reservoir buffer (100 mM HEPES, pH 7.5; 10% [wt/vol] polyethylene glycol 6000 [PEG 6000]; and 5% [vol/vol] 2-methyl-2,4-pentandiol [MPD]) over a 0.5-ml crystallization reservoir. Single crystals grew within 7 days at 4°C. For X-ray data collection, a crystal was harvested from the mother liquor with a nylon loop, mounted on a goniometer, and directly frozen in a liquid N₂ vapor stream at 100 K. Diffraction data were collected using beamline 19ID at the Advanced Photon Source of the Argonne National Laboratory, with a charge-coupled device (CCD) detector (ADSC Quantum 315r) at a distance of 378.9 mm. Images (180 frames) were collected at 0.979 Å in 1.0° oscillations (a 180° hemisphere), indexed, and scaled using HKL3000 (34). Unit cell parameters and data reduction statistics are summarized in Table 1. The three-dimensional structure of yeast FDC was phased by molecular replacement implemented with PHASER (35). The search model for molecular replacement was a homology model of yeast FDC1 generated in I-TASSER (36), using the *P. aeruginosa* UbiD-like protein (PDB entry 4IWS) (30) as the template. Based on the Matthews coefficient, eight protein monomers were expected in the asymmetric unit. The solution from PHASER had a top log likelihood gain (LLG) of 498, a top translation function Z-score (TFZ) of 28.7, and an initial R_{cryst} value of 55.9%. Refinement of the model was performed using PHENIX (37). Simulated annealing and B-factor refinement of the initial solution produced a model with an R_{cryst} value of 36.3% and an R_{free} value of 46.4%. At this stage, manual fitting was initiated using COOT (38). Iterative rounds of model building and refinement, which included TLS refinement, led to a final model with the statistics summarized in Table 1. The final model includes residues 3 to 165, 169 to 188, 208 to 228, and 236

FIG 1 Monomeric structure and domain architecture of yeast FDC1. (a) Ribbon diagram of a yeast FDC1 monomer, with the α -helices (blue) and β -strands labeled. Portions of the monomer corresponding to domains 1 to 3 are noted. The N and C termini are also indicated. (b) Schematic showing the topology of yeast FDC1, with the three domains of the monomer indicated. Coloring of α -helices and β -strands is the same as for panel a. The positions of 4-vinylphenol and the active site are noted.

to 503 of chain A, residues 3 to 187, 208 to 231, 234 to 293, and 297 to 503 of chain B, residues 3 to 503 of chain C, residues 3 to 188, 208 to 293, and 296 to 503 of chain D, residues 3 to 228, 235 to 293, and 297 to 503 of chain E, residues 3 to 503 of chain F, residues 4 to 187, 208 to 229, 235 to 294, and 298 to 503 of chain G, residues 4 to 187 and 207 to 503 of chain H, and 1,381 waters. 4-Vinylphenol, the product of decarboxylation of *p*-coumaric acid, was modeled into the active sites of chains A, C, E, F, and G.

Docking. Molecular docking of 3-methoxy-4-hydroxy-5-decaprenylbenzoate, a partial substrate analog, into the yeast FDC1 active site was performed using AutoDock Vina (ver. 1.1.2) (39). The ligand was generated by editing the structure of amofruitin B {3-[(2*E*)-3,7-dimethylocta-2,6-dien-1-yl]-2-hydroxy-4-methoxy-6-(2-phenylethyl)benzoic acid}, which was obtained from PDB entry 4A4W (40). The ligand was manually placed into the active site by using the position of 4-vinylphenol as a guide, with docking using a grid box of 30 by 30 by 30 Å and the level of exhaustiveness set to 8.

Assay of phenolic acid decarboxylase activity. Decarboxylation of ferulic acid and *p*-coumaric acid catalyzed by yeast FDC1 was assayed by high-pressure liquid chromatography (HPLC) (12). The standard reaction mixture (0.5 ml; 30°C) consisted of 25 mM potassium phosphate buffer (pH 6.5), 5 mM dithiothreitol, 0 to 3 mM substrate, and enzyme (0.1 mg). The reaction was started by the addition of the enzyme and quenched by the addition of glacial acetic acid. After quenching, 2-propanol was added in equal volume to the reaction mixture to solubilize the product. An Agilent 1100 series HPLC system using a SymmetryShield C₁₈ column (150 × 3.9 mm; Waters) was used for separation of the substrate and product. Samples (30 µl) were injected for analyses at a flow rate of 1.0 ml min⁻¹. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). The elution program was as follows: 0 to 5 min, 0% B; 5 to 35 min, a linear gradient from 0 to 90% B; 35 to 38 min, 90% B; and 38 to 40 min, 0% B. Conversion of substrate to product was determined using a standard curve of either ferulic acid or *p*-coumaric acid. Steady-state kinetic parameters were determined under standard assay conditions with varied substrate concentrations (0 to 3

mM) by fitting the untransformed data to the equation $v = k_{\text{cat}}[S]/(K_m + [S])$, using Kaleidagraph (Synergy Software).

Protein structure accession number. Coordinates and structure factors were deposited in the Protein Data Bank (PDB) under accession number 4S13.

RESULTS

Overall structure of yeast FDC1. For crystallographic analysis of yeast FDC1, the full-length protein (residues Met1 to Lys503) was expressed in *E. coli* as an N-terminally His₆-tagged protein (monomer molecular mass, ~56.1 kDa) and purified by Ni²⁺-affinity and size-exclusion chromatographies. Yeast FDC1 eluted from the gel filtration column as a dimeric species (molecular mass, ~100 kDa). The three-dimensional structure of yeast FDC1 was determined at a 2.35-Å resolution by molecular replacement with eight molecules (chains A to H) in the asymmetric unit (Table 1).

Yeast FDC1 folds into a three-domain structure (Fig. 1). Domains 1 and 2 form the N-terminal portion of the monomer and are linked to the C-terminal domain 3 by α 8. The first domain consists of a central, four-stranded β -sheet (β 1a to -d) flanked by two α -helices (α 1 and α 2). Three of the β -strands are from N-terminal residues (β 1a to -c; residues 23 to 69), and one is from C-terminal residues (β 1d; residues 312 to 317). Two α -helices (α 3 and α 4) connect the first domain to the second domain. Domain 2, the largest of the three domains, predominantly contains multiple β -structural features, including a six-stranded antiparallel β -sheet. One side of the β -sheet is capped by α 7 and a two-stranded β -sheet (β 3a and -b). The third domain also contains a core β -sheet (β 4a to -e) that is capped by multiple α -helices (α 8 to

FIG 2 Dimeric structure of yeast FDC1. Top and side views of the yeast FDC1 dimer are shown. In one monomer, α -helices (blue) and β -strands (gold) are colored as in Fig. 1. In the second monomer, α -helices and β -strands are colored rose and green, respectively. Domains 1 to 3 and the β 2e- α 5 loop are indicated on the monomer at left. The reaction product 4-vinylphenol is shown as a space-filling model in each monomer.

α 14). An extended flexible loop leads to the C-terminal helix (α 15) and terminus.

Dimerization of yeast FDC1 is mediated through domain 3 of each monomer. Domain 3 of one monomer packs against domain

3 and α 8 of domain 2 of the adjacent monomer (Fig. 2). The overall shape of the yeast FDC1 dimer resembles a “U,” with domains 1 and 2 of each monomer extending from the domain 3 dimerization region. The C-terminal loop after α 14, which extends to α 15 of one monomer, exclusively interacts with the α 1 and α 2 helices of domain 1 in the second monomer.

Comparison of yeast FDC1 with structures in the Protein Data Bank by using the DALI server (41) reveals the highest structural similarity with the UbiD-like PA0254 protein from *P. aeruginosa* (39% amino acid sequence identity; $Z = 52.3$; 2.6-Å root mean square deviation [RMSD] for 492 C α atoms; PDB entry 4IWS) and with *E. coli* UbiD (25% amino acid sequence identity; $Z = 39.4$; 2.9-Å RMSD for 452 C α atoms; PDB entry 4IDB). The *P. aeruginosa* protein (30) adopts a dimeric structure similar to that of yeast FDC1. Crystallographic analysis of the oligomeric state of the *E. coli* protein suggests a hexamer (PDB entry 2IDB); however, the solution data remain to be reported. Amino acid sequence comparison of yeast FDC1 and the UbiD-related proteins from *P. aeruginosa* and *E. coli* indicates that conserved amino acids are distributed throughout the polypeptide (Fig. 3).

Identification of the yeast FDC1 active site. Within the eight molecules of the asymmetric unit, differences in the β 2e- α 5 loop (Fig. 2) and the presence of electron density near Glu285 were observed (Fig. 4a). In chains C and F, a contiguous electron density for residues 3 to 503 of yeast FDC1 allowed for unambiguous tracing of the β 2e- α 5 loop (Fig. 2). In other molecules of the asymmetric unit, this loop was disordered. The additional elec-

FIG 3 Multiple-sequence alignment of UbiD proteins. Amino acid sequences of yeast FDC1 (ScFDC1; accession no. [AHY75481.1](#)), the UbiD-like PA0254 protein from *P. aeruginosa* (PaUbiD; accession no. [WP_023115699.1](#)), and *E. coli* UbiD (EcUbiD; accession no. [YP_491601.1](#)) were aligned using the MultAlin webpage. The α -helices (blue rectangles) and β -strands (gold rectangles) of yeast FDC1 are depicted above the alignment. Conserved residues are highlighted in orange, with residues in white indicating a variation from the conserved sequence. Residues in the catalytic site (red) and substrate binding site (blue) are indicated. Residues corresponding to the metal binding site of the *P. aeruginosa* protein are highlighted in green (30).

FIG 4 Yeast FDC1 active site. (a) Binding of 4-vinylphenol (4VP; gold) in the yeast FDC1 active site. Side chains of active site residues are shown as stick models and are labeled. The initial $F_o - F_c$ omit map (2.0σ) for 4-vinylphenol is shown. (b) Surface view of the open and closed active site forms of yeast FDC1. The view of the active site in chain A of the crystal structure is shown as the open active site, in which the $\beta 2e - \alpha 5$ loop is disordered. The view of the active site in chain C of the crystal structure shows how the ordered $\beta 2e - \alpha 5$ loop caps the active site. The surface corresponding to Glu285 is colored rose, with 4VP shown as a stick model in gold.

tron density near Glu285 observed in chains A, C, E, F, and G was modeled as 4-vinylphenol, the decarboxylation product of *p*-coumaric acid included in the crystallization conditions.

The position of 4-vinylphenol in a large hydrophobic pocket between $\alpha 8$ and the $\beta 2e - \alpha 5$ loop (Fig. 4a) identifies a potential active site in domain 2 of yeast FDC1. The hydroxyl group of the reaction product interacts with Glu285, which is positioned by a charge-charge interaction with Arg175. Multiple apolar residues (Met228, Met286, Thr326, Ile330, Phe397, Ile398, Phe440, Pro441, and Leu442) form the ligand binding site. Differences in the $\beta 2e - \alpha 5$ loop (i.e., ordered in chains C and F and disordered in other chains) suggest a conformational flexibility of this region of the protein (Fig. 4b). In chains C and F, the $\beta 2e - \alpha 5$ loop encloses the binding site and residues Val188, Ile189, and Lys190 along one side of the hydrophobic pocket to enclose the bound ligand. Because of how the $\beta 2e - \alpha 5$ loop caps the active site, movement of this loop seems to be necessary for substrate entrance to the catalytic site.

In comparison to 4-vinylphenol, 3-methoxy-4-hydroxy-5-hexaprenylbenzoate, the proposed physiological substrate of UbiD in yeast (26), contains an extended polyprenyl group. The apolar binding pocket readily accommodates 4-vinylphenol, but it also appears to be large enough to fit the physiological substrate of yeast FDC1. Docking of a partial substrate analog containing a shorter, two-isoprene extension from the 4-hydroxybenzoate moiety (i.e., 3-methoxy-4-hydroxy-5-decaprenylbenzoate) suggests how the physiological substrate may fit into the active site of yeast FDC1 (Fig. 5). In this model, the 3-methoxy-4-hydroxybenzoate portion of the substrate approximates the binding mode of 4-vinylphenol in the X-ray crystal structure. Binding of this mol-

FIG 5 Docking of 3-methoxy-4-hydroxy-5-decaprenylbenzoate into the yeast FDC1 active site. The surface of the active site cavity is shown in white, with colors corresponding to the indicated residues. The ligand is shown as a stick model (green).

ecule orients the hydroxyl group toward Glu285, with the isoprenoid tail fitting into a pocket that extends to Trp168 (22 Å from Glu285 to Trp168) and then bends toward Trp171 and Met258 (11 Å from Trp168 to Met258). The 33-Å-long tunnel can readily accommodate the 30-carbon side chain of the physiological substrate from yeast ubiquinone synthesis.

Biochemical analysis of yeast FDC1. To test the enzymatic activity of yeast FDC1, ferulic acid (4-hydroxy-3-methoxycinnamic acid), *p*-coumaric acid (4-hydroxycinnamic acid), 3-hydroxycinnamic acid, 2-hydroxycinnamic acid, 3,4-dimethoxycinnamic acid, and 2,5-dimethoxycinnamic acid were screened as substrates. Yeast FDC1 catalyzed the decarboxylation of ferulic acid and *p*-coumaric acid, with comparable steady-state kinetic parameters for both substrates (Table 2).

To test the potential role of Glu285 as the catalytic residue for yeast FDC1, the E285A mutant of yeast FDC1 was generated by PCR-based site-directed mutagenesis. The E285A protein was expressed and purified as described for the wild-type enzyme. In contrast to the wild-type enzyme, the yeast FDC1 E285A mutant displayed no catalytic activity with any of the substituted cinnamic acids tested.

DISCUSSION

The potential uses of aromatic acid decarboxylases in bioindustrial applications drive the interest in understanding how these diverse enzymes function at the molecular level (3–10). The diverse ferulic acid, phenylacrylic acid, and phenolic acid decarboxylases in fungi, yeast, and bacteria provide a wide range of proteins for tailoring physiochemical properties and/or enzymatic activities of these useful biocatalysts (4, 6, 7, 11–22). To aid in the development of these enzymes as tools for biotransformations, we determined the X-ray crystal structure of yeast FDC1.

Structural studies of phenol acid decarboxylases define the following three general types of this enzyme: (i) dodecameric, UbiX-type flavoproteins (23, 24); (ii) the microbial lipocalin-related

TABLE 2 Kinetic analysis of yeast FDC1^a

Substrate	$V_{\max}$ (nmol min ⁻¹ mg protein ⁻¹)	K_m (mM)
Ferulic acid	6.8 ± 0.4	0.79 ± 0.11
<i>p</i> -Coumaric acid	7.2 ± 0.5	0.92 ± 0.17

^a Values are the means $\pm$ standard deviations for three experiments.

FIG 6 Proposed reaction mechanism for the nonoxidative decarboxylation of aromatic acids catalyzed by yeast FDC1.

phenylacrylic acid decarboxylases (27–29); and (iii) the UbiD-like enzymes (30). The X-ray crystal structure of yeast FDC1 clearly establishes this enzyme as a member of the UbiD family of decarboxylases (Fig. 1 to 3). Although three-dimensional structures for each type of phenolic acid decarboxylase have been determined, few insights into the active sites and/or substrate binding sites of these enzymes are currently available. For example, the UbiX-type flavoproteins and the *P. aeruginosa* UbiD-like protein PA0254 display no detectable activity with phenolic acid substrates (23, 24, 30); however, yeast FDC1 does show decarboxylation activity with both ferulic and *p*-coumaric acids (Table 2). Screening of a range of substituted cinnamoyl-derived substrates indicates the importance of the 4-hydroxyl group for turnover. In comparison to the structurally and biochemically characterized lipocalin-related phenylacrylic acid decarboxylases from *L. plantarum*, *B. pumilus*, *Bacillus subtilis*, and *Enterobacter* (15, 16, 19, 22), the specific activities of yeast FDC1 for ferulic acid and *p*-coumaric acid are approximately 1,000-fold lower than those of the microbial enzymes, but with comparable K_m values. This may suggest a lack of optimized fit in the yeast FDC1 active site for these nonphysiological substrates.

Crystallization of yeast FDC1 with a bound reaction product (i.e., 4-vinylphenol) defined the location of the active site for the UbiD-related decarboxylases. In yeast FDC1, the large apolar pocket (Fig. 4a) that forms the binding site of 4-vinylphenol can easily accommodate other aromatic acids, such as ferulic acid, for decarboxylation and is consistent with earlier biochemical studies of the enzyme (21). Amino acid sequence comparison of yeast FDC1 with the UbiD-like PA0254 protein from *P. aeruginosa* and *E. coli* UbiD revealed that the substrate binding pockets of these enzymes vary, although the apolar nature of the site is generally conserved (Fig. 3). Structural studies of the UbiD-like PA0254 protein from *P. aeruginosa* identified a metal binding site consisting of a His and a Glu residue (Fig. 3, green shading) near the proposed active site cleft (30); however, no evidence for metal binding at the corresponding residues of yeast FDC1 was observed. Moreover, computational docking of 3-diprenyl-4-hydroxybenzoate, an analog of the physiological substrate, into the crystal structure of yeast FDC1 (Fig. 5) suggested a possible model for binding of the isoprene-derived tail of the substrate from ubiquinone synthesis. The long (~33 Å) pocket extending from the active site provides sufficient space for the 30-carbon-long isoprenoid tail of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate. Comparison of the open and closed active site forms of yeast FDC1 (Fig. 4b) suggests that movement of the $\beta 2e$ - $\alpha 5$ loop serves

to enclose substrates in the hydrophobic active site cavity. In addition to insights into natural and nonnatural substrate binding, the yeast FDC1 structure and subsequent functional analysis suggest an important role for Glu285 in the reaction mechanism of the enzyme.

The position of 4-vinylphenol bound to yeast FDC1 (Fig. 4a) places the substrate hydroxyl group in proximity to the carboxylate side chain of Glu285. Based on the structure, a plausible reaction mechanism for decarboxylation of aromatic acids by FDC1 can be proposed (Fig. 6). The first step of the reaction involves conversion of the phenolic acid into a quinoid. Glu285 functions as a catalytic base to abstract a proton from the substrate hydroxyl group, which begins an electron relay to yield a *para*-quinone intermediate. Spontaneous decarboxylation of the reaction intermediate leads to release of CO_2 and formation of the final reaction product. Mutation of Glu285 to an alanine disrupts the decarboxylation of *p*-coumaric acid catalyzed by yeast FDC1. The overall reaction is consistent with earlier biochemical studies examining proton transfer during the decarboxylation of aromatic acid substrates (12, 17).

Sequence comparison shows that the acidic nature of the residue corresponding to Glu285 is conserved in both the *P. aeruginosa* and *E. coli* UbiD-like proteins, although an aspartate substitution occurs in the *E. coli* protein (Fig. 3). Similarly, the residue corresponding to Arg175, which positions Glu285 in the yeast FDC1 active site, is invariant in each of these proteins. This conservation suggests a common reaction mechanism for the UbiD family of enzymes. Interestingly, the reaction mechanism of yeast FDC1 also provides an example of convergent evolution of chemistry in enzyme families with distinct structural folds. Structural and biochemical studies of the *p*-coumaric acid decarboxylase from *L. plantarum* and the ferulic acid decarboxylase from *Enterobacter* sp. Px6-4, both of which contain a lipocalin fold, suggest that a catalytic glutamate is required for catalysis (22, 26).

Ultimately, structural information on the various aromatic acid decarboxylases should prove useful in the engineering of modified versions of these enzymes (42–44). Initial studies of bacterial phenolic acid decarboxylases with different substrate specificities showed that the generation of chimeric enzymes could be used to alter product profiles (44). Now, similar studies guided by three-dimensional structures become a logical next step. For example, the K_m values of many aromatic acid decarboxylases fall in the millimolar range (4, 6, 7, 11–22). This may reflect the use of phenolic acid substrates that are significantly smaller than the physiological one (i.e., 3-methoxy-4-hydroxy-5-hexaprenylbenzoate) found in ubiquinone biosynthesis of yeast. Protein engineering to optimize the active site structure for binding and de-

carboxylation of hydroxycinnamates may lead to improved steady-state kinetic properties for bioindustrial applications.

ACKNOWLEDGMENTS

This work was supported by the Chinese National Science Foundation 863 Project (grant 2013AA102801 to O.Y.). Portions of this research were carried out at the Argonne National Laboratory Structural Biology Center of the Advanced Photon Source, a national user facility operated by the University of Chicago for the Department of Energy Office of Biological and Environmental Research (DE-AC02-06CH11357).

M.W.B. was an employee of Conagen, and O.Y. cofounded Conagen and Wuxi NewWay.

REFERENCES

- Mimura N, Saito M. 2000. Dehydrogenation of ethylbenzene to styrene over $\text{Fe}_2\text{O}_3/\text{Al}_2\text{O}_3$ catalysts in the presence of carbon dioxide. *Catal Today* 55:173–178. [http://dx.doi.org/10.1016/S0920-5861\(99\)00236-9](http://dx.doi.org/10.1016/S0920-5861(99)00236-9).
- Yu O, Jez JM. 2008. Nature's assembly line: biosynthesis of simple phenylpropanoids and plant polyketides. *Plant J* 54:750–762. <http://dx.doi.org/10.1111/j.1365-3113X.2008.03436.x>.
- Schroeder AC, Kumaran S, Hicks LM, Cahoon RE, Halls C, Yu O, Jez JM. 2008. Contributions of conserved serine and tyrosine residues to catalysis, ligand binding, and cofactor processing in the active site of tyrosine ammonia lyase. *Phytochemistry* 69:1496–1506. <http://dx.doi.org/10.1016/j.phytochem.2008.02.007>.
- Chow KT, Pope MK, Davies J. 1999. Characterization of a vanillic acid non-oxidative decarboxylation gene cluster from *Streptomyces* sp. D7. *Microbiology* 145:2393–2403.
- Furuya T, Miura M, Kino K. 2014. A coenzyme-independent decarboxylase/oxygenase cascade for the efficient synthesis of vanillin. *ChemBiochem* 15:2248–2254. <http://dx.doi.org/10.1002/cbic.201402215>.
- Huang Z, Dostal L, Rosazza JP. 1993. Mechanisms of ferulic acid conversions to vanillic acid and guaicol by *Rhodotorula rubra*. *J Biol Chem* 268:23954–23958.
- Smit A, Cordero Otero RR, Lambrechts MG, Pretorius IS, Van Rensburg P. 2003. Enhancing volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*. *J Agric Food Chem* 51:4909–4915. <http://dx.doi.org/10.1021/jf026224d>.
- Frost JW, Draths KM. 1995. Biocatalytic syntheses of aromatics from D-glucose: renewable microbial sources of aromatic compounds. *Annu Rev Microbiol* 49:557–579. <http://dx.doi.org/10.1146/annurev.mi.49.100195.003013>.
- McKenna R, Nielsen DR. 2011. Styrene biosynthesis from glucose by engineered *E. coli*. *Metab Eng* 13:544–554. <http://dx.doi.org/10.1016/j.ymben.2011.06.005>.
- McKenna R, Thompson B, Pugh S, Nielsen DR. 2014. Rational and combinatorial approaches to engineering styrene production by *Saccharomyces cerevisiae*. *Microb Cell Fact* 13:123. <http://dx.doi.org/10.1186/s12934-014-0123-2>.
- Rahouti M, Seigle-Murandi F, Steiman R, Eriksson K-E. 1989. Metabolism of ferulic acid by *Paecilomyces variotti* and *Pestalotia palmarum*. *Appl Environ Microbiol* 55:2391–2398.
- Huang Z, Dostal L, Rosazza JP. 1994. Purification and characterization of a ferulic acid decarboxylase from *Pseudomonas fluorescens*. *J Bacteriol* 176:5912–5918.
- Clausen M, Lamb CJ, Megnet R, Doerner PW. 1994. PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* 142:107–112. [http://dx.doi.org/10.1016/0378-1119\(94\)90363-8](http://dx.doi.org/10.1016/0378-1119(94)90363-8).
- Zago A, Degrassi G, Bruschi CV. 1995. Cloning, sequencing, and expression in *Escherichia coli* of the *Bacillus pumilus* gene for ferulic acid decarboxylase. *Appl Environ Microbiol* 61:4484–4486.
- Degrassi G, DeLaureto PP, Bruschi CV. 1995. Purification and characterization of ferulate and *p*-coumarate decarboxylase from *Bacillus pumilus*. *Appl Environ Microbiol* 61:326–332.
- Cavin JF, Dartois V, Divies C. 1998. Gene cloning, transcriptional analysis, purification, and characterization of phenolic acid decarboxylase from *Bacillus subtilis*. *Appl Environ Microbiol* 64:1466–1471.
- Hashidoko Y, Tahara S. 1998. Stereochemically specific proton transfer in decarboxylation of 4-hydroxycinnamic acids by 4-hydroxycinnamate decarboxylase from *Klebsiella oxytoca*. *Arch Biochem Biophys* 359:225–233. <http://dx.doi.org/10.1006/abbi.1998.0911>.
- Prim N, Pastor FI, Diaz P. 2003. Biochemical studies on cloned *Bacillus* sp. BP-7 phenolic acid decarboxylase PadA. *Appl Microbiol Biotechnol* 63:51–56. <http://dx.doi.org/10.1007/s00253-003-1371-y>.
- Rodríguez H, Landete JM, Curiel JA, de las Rivas B, Mancheño JM, Muñoz R. 2008. Characterization of the *p*-coumaric acid decarboxylase from *Lactobacillus plantarum* CECT748^T. *J Agric Food Chem* 56:3068–3072. <http://dx.doi.org/10.1021/jf703779s>.
- Landete JM, Rodríguez H, Curiel JA, de las Rivas B, Mancheño JM, Muñoz R. 2010. Gene cloning, expression, and characterization of phenolic acid decarboxylase from *Lactobacillus brevis* RM84. *J Ind Microbiol Biotechnol* 37:617–624. <http://dx.doi.org/10.1007/s10295-010-0709-6>.
- Mukai N, Masaki K, Fujii T, Kawamukai M, Iefuji H. 2010. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 109:564–569. <http://dx.doi.org/10.1016/j.jbiosc.2009.11.011>.
- Gu W, Li X, Huang J, Duan Y, Meng Z, Zhang KQ, Yang J. 2011. Cloning, sequencing, and overexpression in *Escherichia coli* of the *Enterobacter* sp. Px6-4 gene for ferulic acid decarboxylase. *Appl Microbiol Biotechnol* 89:1797–1805. <http://dx.doi.org/10.1007/s00253-010-2978-4>.
- Rangarajan ES, Li Y, Iannuzzi P, Tocilj A, Hung LW, Matte A, Cygler M. 2004. Crystal structure of a dodecameric FMN-dependent UbiX-like decarboxylase (Pad1) from *Escherichia coli* O157:H7. *Protein Sci* 13:3006–3016. <http://dx.doi.org/10.1110/ps.04953004>.
- Kopeck J, Schnell R, Schneider G. 2011. Structure of PA4019, a putative aromatic acid decarboxylase from *Pseudomonas aeruginosa*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 67:1184–1188. <http://dx.doi.org/10.1107/S174430911102923X>.
- Gulmezian M, Hyman KR, Marbois BN, Clarke CF, Javor GT. 2007. The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Arch Biochem Biophys* 467:144–153. <http://dx.doi.org/10.1016/j.abb.2007.08.009>.
- Clarke CE. 2000. New advances in coenzyme Q biosynthesis. *Protoplasma* 213:134–147. <http://dx.doi.org/10.1007/BF01282151>.
- Rodríguez H, Angulo I, de las Rivas B, Campillo N, Páez JA, Muñoz R, Mancheño JM. 2010. *p*-Coumaric acid decarboxylase from *Lactobacillus plantarum*: structural insights into the active site and decarboxylation catalytic mechanism. *Proteins* 78:1662–1676. <http://dx.doi.org/10.1002/prot.22684>.
- Matte A, Grosse S, Bergeron H, Abokitse K, Lau PC. 2010. Structural analysis of *Bacillus pumilus* phenolic acid decarboxylase, a lipocalin-fold enzyme. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 66:1407–1414. <http://dx.doi.org/10.1107/S174430911003246X>.
- Gu W, Yang J, Lou Z, Liang L, Sun Y, Huang J, Li X, Cao Y, Meng Z, Zhang KQ. 2011. Structural basis of enzymatic activity for the ferulic acid decarboxylase (FADase) from *Enterobacter* sp. Px6-4. *PLoS One* 6:e16262. <http://dx.doi.org/10.1371/journal.pone.0016262>.
- Jacewicz A, Izumi A, Brunner K, Schnell R, Schneider G. 2013. Structural insights into the UbiD protein family from the crystal structure of PA0254 from *Pseudomonas aeruginosa*. *PLoS One* 8:e63161. <http://dx.doi.org/10.1371/journal.pone.0063161>.
- Zhang H, Javor GT. 2000. Identification of the *ubiD* gene on the *Escherichia coli* chromosome. *J Bacteriol* 182:6243–6246. <http://dx.doi.org/10.1128/JB.182.21.6243-6246.2000>.
- Lupa B, Lyon D, Gibbs MD, Reeves RA, Wiegel J. 2005. Distribution of genes encoding the microbial non-oxidative reversible hydroxyarlic acid decarboxylases/phenol decarboxylases. *Genomics* 86:342–351. <http://dx.doi.org/10.1016/j.ygeno.2005.05.002>.
- Harju S, Fedosyuk H, Peterson KR. 2004. Rapid isolation of yeast genomic DNA: bust n' grab. *BMC Biotechnol* 4:8. <http://dx.doi.org/10.1186/1472-6750-4-8>.
- Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. 2006. HKL-3000: the integration of data reduction and structure solution—from diffraction images to an initial model in minutes. *Acta Crystallogr Sect D Biol Crystallogr* 62:859–866. <http://dx.doi.org/10.1107/S0907444906019949>.
- McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007. Phaser crystallographic software. *J Appl Crystallogr* 40:658–674. <http://dx.doi.org/10.1107/S0021889807021206>.
- Roy A, Kucukural A, Zhang Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5:725–738. <http://dx.doi.org/10.1038/nprot.2010.5>.
- Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Mo-

- riarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**: 213–221. <http://dx.doi.org/10.1107/S0907444909052925>.
38. Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**:486–501. <http://dx.doi.org/10.1107/S0907444910007493>.
39. Trott O, Olson AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multi-threading. *J Comp Chem* **31**:455–461. <http://dx.doi.org/10.1002/jcc.21334>.
40. de Groot JC, Weidner C, Krausze J, Kawamoto K, Schroeder FC, Sauer S, Büssow K. 2013. Structural characterization of amorfrutins bound to the peroxisome proliferator-activated receptor γ . *J Med Chem* **56**:1535–1543. <http://dx.doi.org/10.1021/jm3013272>.
41. Holm L, Rosenström P. 2010. Dali server: conservation mapping in 3D. *Nucleic Acids Res* **38**:W545–W549. <http://dx.doi.org/10.1093/nar/gkq366>.
42. Bhan N, Xu P, Koffas MA. 2013. Pathway and protein engineering approaches to produce novel and commodity small molecules. *Curr Opin Biotechnol* **24**:1137–1143. <http://dx.doi.org/10.1016/j.copbio.2013.02.019>.
43. Yang H, Li J, Shin HD, Du G, Liu L, Chen J. 2014. Molecular engineering of industrial enzymes: recent advances and future prospects. *Appl Microbiol Biotechnol* **98**:23–29. <http://dx.doi.org/10.1007/s00253-013-5370-3>.
44. Barthelmebs L, Diviès C, Cavin JF. 2001. Expression in *Escherichia coli* of native and chimeric phenolic acid decarboxylases with modified enzymatic activities and method for screening recombinant *E. coli* strains expressing these enzymes. *Appl Environ Microbiol* **67**:1063–1069. <http://dx.doi.org/10.1128/AEM.67.3.1063-1069.2001>.