ACS Catalysis

Research Article

pubs.acs.org/acscatalysis

p-Hydroxyphenylacetate 3-Hydroxylase as a Biocatalyst for the Synthesis of Trihydroxyphenolic Acids

Taweesak Dhammaraj,[†] Aisaraphon Phintha,[†] Chatchadaporn Pinthong,[†,‡] Dheeradhach Medhanavyn,[†] Ruchanok Tinikul,[§] Pirom Chenprakhon,[‡] Jeerus Sucharitakul,[∥] Nontima Vardhanabhuti,[⊥] Chutima Jiarpinitnun,[#] and Pimchai Chaiyen*[,†]

[†]Department of Biochemistry and Center of Excellence in Protein Structure and Function, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

[‡]Institute for Innovative Learning, Mahidol University, Nakhon Pathom 73170, Thailand

[§]Mahidol University, Nakhonsawan Campus, Nakhonsawan 60130, Thailand

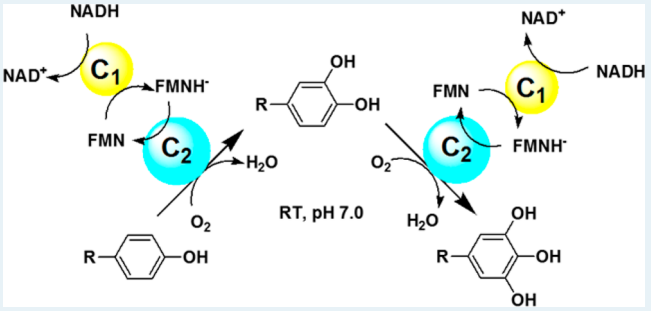
[∥]Department of Biochemistry, Faculty of Dentistry, Chulalongkorn University, Henri-Dunant Road, Patumwan, Bangkok 10300, Thailand

[⊥]Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10300, Thailand

[#]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Supporting Information

ABSTRACT: Trihydroxyphenolic acids such as 3,4,5-trihydroxycinnamic acid (3,4,5-THCA) **4c** and 2-(3,4,5-trihydroxyphenyl)acetic acid (3,4,5-THPA) **2c** are strong antioxidants that are potentially useful as medicinal agents. Our results show that *p*-hydroxyphenylacetate (HPA) 3-hydroxylase (HPAH) from *Acinetobacter baumannii* can catalyze the syntheses of 3,4,5-THPA **2c** and 3,4,5-THCA **4c** from 4-HPA **2a** and *p*-coumaric acid **4a**, respectively. The wild-type HPAH can convert 4-HPA **2a** completely into 3,4,5-THPA **2c** within 100 min (total turnover number (TTN) of 100). However, the wild-type enzyme cannot efficiently synthesize 3,4,5-THCA **4c**. To improve the efficiency, the oxygenase component of HPAH (C_2) was rationally engineered in order to maximize the conversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c**. Results from site-directed mutagenesis studies showed that Y398S is significantly more effective than the wild-type enzyme for the synthesis of 3,4,5-THCA **4c**; it can catalyze the complete bioconversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c** within 180 min (TTN ~ 23 at 180 min). The yield and stability of 3,4,5-THPA **2c** and 3,4,5-THCA **4c** were significantly improved in the presence of ascorbic acid. Thermostability studies showed that the wild-type C_2 was very stable and remained active after incubation at 30, 35, and 40 °C for 24 h. Y398S was moderately stable because its activity was retained for 24 h at 30 °C and for 15 h at 35 °C. Transient kinetic studies using stopped-flow spectrophotometry indicated that the key improvement in the reaction of Y398S with *p*-coumaric acid **4a** lies within the protein−ligand interaction. Y398S binds to *p*-coumaric acid **4a** with higher affinity than the wild-type enzyme, resulting in a shift in equilibrium toward favoring the productive coupling path instead of the path leading to wasteful flavin oxidation.



KEYWORDS: *p-hydroxyphenylacetate hydroxylase, two-component flavin-dependent monooxygenase, biocatalysis, phenol and catechol, phenolic acids*

INTRODUCTION

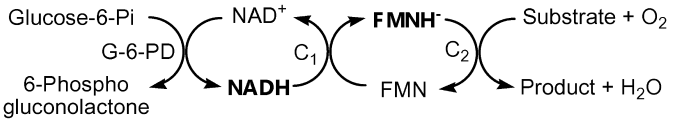
Phenolic acids or simple polyphenols derived from plants are strong antioxidants that are generally considered beneficial to human health due to their ability to scavenge reactive oxygen species.[1] Trihydroxyphenolic acids are high-value compounds that have various biological activities and great potential as therapeutic agents. 3,4,5-Trihydroxycinnamic acid (3,4,5-THCA) **4c** and its ethyl or diethyl ester derivatives exhibit selective inhibitory and cytotoxic effects against cancer cell lines, whereas they do not affect human skin fibroblasts.[2] These compounds also showed anti-inflammatory activities in various lipopolysaccharide (LPS)-stimulated cell lines and mouse models,[3−6] and they could inhibit the growth of *Staphylococcus epidermidis* and *Staphylococcus aureus*.[7] 2-(3,4,5-

Received: March 2, 2015
Revised: May 19, 2015

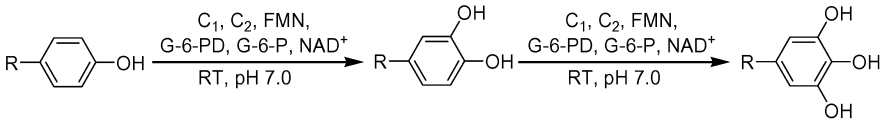
DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

Scheme 1. (A) Multiple turnover reaction of *p*-hydroxyphenylacetate hydroxylase (C_1−C_2) coupled with the reaction of glucose-6-phosphate dehydrogenase (G-6-PD). (B) Bioconversion of 2-(4-Hydroxyphenyl)acetic Acid (4-HPA) 2a and *p*-Coumaric Acid 4a

(A)



(B)



2a : R = CH_2COOH
4a : R = (*trans*-)CH=CHCOOH

2b : R = CH_2COOH
4b : R = (*trans*-)CH=CHCOOH

2c : R = CH_2COOH
4c : R = (*trans*-)CH=CHCOOH

Trihydroxyphenyl)acetic acid (3,4,5-THPA) **2c** displays high free radical scavenging activity,[8] as well as antiproliferative and cytotoxic activities against various types of cancer cells including cervical (Hela) and mammary gland (MDA-MB-231), adenocarcinomas and lymphoblastic leukemia (MOLT-3), while it had insignificant effects on normal cells.[9] Based on antioxidant assays and voltametric studies, trihydroxyphenolic acids generally have higher free radical scavenging and antioxidant activity than their dihydroxyphenolic acid counterparts.[2,8,10,11]

Due to its potential as a therapeutic agent, an efficient and green method for synthesizing trihydroxyphenolic acid is called for. The current methods to prepare catechol from phenol involve strong oxidizing reagents such as transition metals and H_2O_2 under reflux or high temperature conditions.[12,13] Pyrogallol or 1,2,3-trihydroxybenzene can be prepared by reacting phenol with a brominating agent (R_2N−Br) to form 2,6-dibromophenol, followed by alkoxylating with metal alkoxide, and finally dealkylating with HCl or HBr to form 1,2,3-trihydroxybenzene.[14] Several enzymatic systems were explored for their ability to catalyze regio-specific hydroxylation of phenolic acids. P450-dependent monooxygenases such as *p*-coumarate-3-hydroxylase (C3H) from plants[15] and *Rhodopseudomonas palustris*[16] can catalyze *ortho*-hydroxylation of *p*-coumaric acid ester and free *p*-coumaric acid **4a**, respectively. Tyrosine ammonia lyase and 4-coumarate 3-hydroxylase, encoded by *sam8* and *sam5*, from *Saccharothrix espanaensis* were used to convert L-tyrosine caffeic acid **4b**.[17] *p*-Hydroxyphenylacetate (HPA) 3-hydroxylase (HPAH) from *E. coli*[18] and *Pseudomonas aeruginosa*[19] can catalyze the hydroxylation of 2-(4-hydroxyphenyl)acetic acid (4-HPA) **2a** and *p*-coumaric acid **4a** to 2-(3,4-dihydroxyphenyl)acetic acid (3,4-DHPA) **2b** and caffeic acid **4b**, respectively. However, these enzymatic systems cannot efficiently prepare trihydroxyphenolic acids.

HPAH from *Acinetobacter baumannii* is an attractive biocatalyst for synthesizing trihydroxyphenolic acids. The enzyme can use 4-HPA **2a**, one of the major compounds from the degradation of lignin, as a substrate and is a two-component flavin-dependent monooxygenase that consists of reductase (C_1) and oxygenase (C_2) components.[20,21] C_1 is an FMN-bound enzyme that receives a hydride transfer from NADH to generate reduced FMN ($FMNH^-$). The resulting $FMNH^-$ undergoes free-diffusion to the C_2 component which catalyzes hydroxylation of substrate by a C_{4a}-hydroperoxyflavin intermediate.[22−24] The overall catalysis of C_2 is well understood and its X-ray structure with bound substrate is also available.[25−30] The enzyme can also be overexpressed in *E. coli* in large scale;[21] the optimized protocol yields approximately 0.40 g of the purified enzyme per 1 L of cell culture. C_2 can tolerate a wide range of pH conditions and can catalyze the hydroxylation efficiently within a pH range of 6−10.[27] These properties make the enzyme an attractive target for engineering and scale-up production for future applications in biocatalysis.

In this work, the ability of the oxygenase component (C_2) of HPAH from *A. baumannii* to synthesize 3,4,5-THPA **2c** and 3,4,5-THCA **4c** was investigated. We found that wild-type C_2 can catalyze double hydroxylation of 4-HPA **2a** and *p*-coumaric acid **4a** to yield 3,4,5-THPA **2c** and 3,4,5-THCA **4c**, respectively. However, the synthesis of 3,4,5-THCA **4c** by the wild-type enzyme is inefficient. C_2 was then engineered, and the results showed that the Y398S has higher efficiency than the wild-type enzyme for synthesizing 3,4,5-THCA **4c**. Transient kinetics of the wild-type and the Y398S variant enzymes were investigated using stopped-flow techniques to identify the catalytic feature that makes this variant superior to the wild-type enzyme. Biocatalysis by C_2 can thus contribute to a novel green method for the preparation of trihydroxyphenolic acids.

EXPERIMENTAL SECTION

Products from C_2-Catalyzed Hydroxylation (Single Turnover Reaction). Solutions of FMN and C_2 (or variants) in 50 mM NaH_2PO_4 pH 7.0 were equilibrated inside an anaerobic glovebox to remove oxygen. An FMN solution was gradually titrated with sodium dithionite (5 mg/mL) to obtain $FMNH^-$. The reduction process was monitored by a UV−visible spectrometer inside the anaerobic glovebox. The $FMNH^-$ solution (100 μM) was mixed with C_2 wild-type or variant enzymes (200 μM) to obtain complexes of C_2:$FMNH^-$. This solution (250 μL) was then mixed with air-saturated

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

substrate (400 μM, 250 μL) in 50 mM NaH_2PO_4 pH 7.0 in an enclosed microfuge tube and incubated at room temperature for 15 min. Concentrations are indicated as before substrate and enzyme mixing. This mixing setup allows the reaction to proceed only for one turnover. The reactions were quenched by adding 500 μL of 0.15 M HCl. After quenching, the final concentrations of C_2, $FMNH^-$, and substrate were 50, 25, and 100 μM, respectively. The quenched solution was filtered through a Microcon centrifugal unit (10 kDa cutoff). The filtrate obtained was analyzed by HPLC/diode array detector (DAD)/ mass spectroscopic detector (MSD), of which protocols are described below. For compounds that are commercially available including 3,4-dihydroxybenzoic acid (3,4-DHBA) **1b**, 3,4,5-trihydroxybenzoic acid (3,4,5-THBA or gallic acid) **1c**, 3,4-DHPA **2b**, 3-(3,4-dihydroxyphenyl)-propanoic acid (3,4-DHPPA) **3b**, caffeic acid **4b**, 4-nitrocatechol **5b**, their standard curves were constructed in order to determine the %yield of products. For compounds that are not commercially available, 3,4,5-THPA **2c**, 3-(3,4,5-trihydroxyphenyl)propanoic acid (3,4,5-THPPA) **3c**, and 3,4,5-THCA **4c**, their productions were inferred from substrate consumptions. $FMNH^-$ was the limiting agent in the reaction. Its concentration was used to define the 100% yield of product. All pure substrates were purchased from Sigma-Aldrich except caffeic acid **4b** from Tokyo Chemical Industry. Co., Ltd. (TCI).

Analysis of Products from Multiple Turnover Reactions. Multiple turnover reactions of C_2 were carried out by coupling the reaction with the reactions of C_1 and glucose-6-phosphate dehydrogenase (G-6-PD) to constantly supply $FMNH^-$ and NADH according to the diagram in Scheme 1A. Glucose-6-phosphate (G-6-P) (1 mM), C_1 (100 nM), NAD^+ (10 μM), FMN (1 μM), wild-type or variants of C_2 (2 μM), and 4-HPA **2a** (200 μM) or *p*-coumaric acid **4a** (50 μM) were mixed in 50 mM NaH_2PO_4 pH 7.0 before the reaction was started by adding G-6-PD 0.5 Unit/ml. The reaction mixture was incubated at 25 °C (room temperature) for 2 h or until the substrate was completely consumed. Samples of the reaction were taken at various time points, and quenched by the addition of HCl (final concentration of 0.08 M). The quenched samples were filtered through a Microcon ultrafiltration unit (10 kDa cutoff) and analyzed by HPLC/DAD/MSD to monitor the reaction progress. Peak areas of substrate and the first and second hydroxylated products from individual samples were used for calculating the percentage of products formed during the reaction.

Product Analysis by HPLC/DAD and HPLC/MSD. Products from single or multiple turnover reactions were analyzed and identified using an HPLC (Agilent Technologies 1100 or 1260 Infinity series) equipped with a UV−visible diode array detector (DAD) and an electrospray ionization mass spectroscopic detector (ESI-MSD) (Agilent Technologies 6120 Quadrupole LC/MS). A Novapak (Waters) C18 reverse phase column (Part No.WAT086344), column size 3.9 × 150 mm and particle size 4 μm, was used as the stationary phase for sample analysis. A mixture of H_2O and MeOH with addition of 0.1% formic acid was used as the mobile phase. The column was run at ambient temperature with a flow rate of 0.5 mL/min. MSD was used in negative mode with a scan range of m/z 100−500 or in selected ion monitoring (SIM) mode set at m/z values of the molecular ions of the substrate and the anticipated products in the reaction. The volume of sample injection was 20 μL.

Semilarge Scale Preparation of 2-(3,4,5-Trihydroxyphenyl)acetic Acid (3,4,5-THPA) 2c and 3,4,5-Trihydroxycinnamic Acid (3,4,5-THCA) 4c for NMR Identification. For 3,4,5-THPA **2c** preparation, a mixture of G-6-P (5 mM), C_1 (100 nM), NAD^+ (10 μM), FMN (1 μM), Y398S (10 μM), 3,4-DHPA **2b** (200 μM), with or without ascorbic acid (1 mM), and catalase 1% w/v in 50 mM NaH_2PO_4 pH 7.0 was prepared in a total volume of 280 mL. G-6-PD (0.06 unit/ml) was added to the solution to start the reaction. The reaction was gently stirred and left standing at room temperature for 10 h. Progress of the reaction was monitored by HPLC/MSD, and the results indicated that the reaction was complete at 8 h. The reaction solution was acidified by adding formic acid until the pH reached 4.0 and then filtered through a stirred-cell equipped with a 10 kDa cutoff filter membrane. The filtrate obtained was concentrated and injected into a preparative HPLC Sunfire C18 OBD prep column, 100 Å, particle size 5 μm, 19 × 150 mm equilibrated with the mobile phase, H_2O (90%): MeOH (10%): formic acid (0.1%) with a flow rate of 2.5 mL/min at ambient temperature. Fractions of 3,4,5-THPA **2c** were collected and checked for purity using HPLC/MSD analysis (conditions for analysis as described above). The purified 3,4,5-THPA **2c** was concentrated using a rotatory evaporator and redissolved in DMSO-d_6 for NMR analysis (Bruker Avance 500 MHz).

For 3,4,5-THCA **4c** preparation, the conditions used were similar to those used for 3,4,5-THPA **2c** except that caffeic acid **4b** was used as a substrate. The reaction was complete around 20 h after it was started. The solution was acidified with formic acid until the pH reached ∼4 and was filtered through a stirred-cell with a 10 kDa cutoff filter membrane. The filtrate obtained was purified on a Sep-pak C18 reverse phase column (size 20 mL). The sample was loaded onto a Sep-pak cartridge and the column was washed with 100 mL of 0.1% formic acid. 3,4,5-THCA **4c** was eluted off the column with 200 mL of H_2O (95%):MeOH (5%) with formic acid 0.1%. The eluted 5% MeOH fraction was evaporated until the volume was reduced to ∼1 mL. 3,4,5-THCA **4c** was purified further using a preparative HPLC column. An isocratic mobile phase of H_2O (70%): MeOH (30%): formic acid (0.1%) at a flow rate of 3.0 mL/min was used to separate 3,4,5-THCA **4c**. The purified 3,4,5-THCA **4c** fraction was evaporated and dissolved in DMSO-d_6 for NMR analysis.

Characterization of 2-(3,4,5-Trihydroxyphenyl)acetic Acid (3,4,5-THPA) 2c and 3,4,5-Trihydroxycinnamic Acid (3,4,5-THCA) 4c by NMR. The anticipated 3,4,5-THPA **2c** or 3,4,5-THCA **4c** from the Y398S catalyzed hydroxylation reaction was concentrated under vacuum to remove solvent. The concentrated sample was subsequently dissolved in DMSO-d_6. ^1H and ^{13}C NMR spectra of these compounds were obtained using 500 MHz Bruker Avance NMR spectrometer.

Sited-Directed Mutagenesis of C_2 and Preparation of Variant Enzymes. Variants of C_2 were constructed on the basis of the general protocol suggested by the QuikChange II site-directed mutagenesis kit instruction manual (Stratagene, La Jolla, CA). The pET-11a plasmid containing the C_2-*hpah* gene was used as a template to prepare single mutant plasmids by PCR. All primers and the PCR methods are described in Supporting Information Table S1. The mutant plasmids were transformed into *E. coli* BL21(DE3) and grown in 3.6 L of autoinduction medium containing 50 μg/mL ampicillin at 37 °C for 2.5−3 h before the incubation temperature was adjusted

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

to 25 °C and maintained for 14 h prior to cell harvest. The variant enzymes were purified using a protocol similar to that used to purify the wild-type enzyme[27] with the exception of the sephacryl S-200 gel chromatography step, which was omitted from the protocol.

Thermostability Assay. C_2 wild-type and Y398S in 50 mM NaH_2PO_4 pH 7.0 were incubated at three temperatures (30, 35, or 40 °C), and enzyme samples were collected at various times over the course of 24 h to assay their activity. The enzyme activity assay contained C_1 1.25 μM (or 1.50 μM for Y398S), C_2 or Y398S (0.3 μM), NADH (150 μM), 4-HPA **2a** (1 mM), DHPAO (2.3 μM) in 50 mM NaH_2PO_4 pH 7.0 at 25 °C. All reagents and enzymes, except NADH, were mixed together before adding NADH to initiate the reaction. Enzyme activities were measured in triplicate and the values were averaged to obtain the data. The activity of enzyme at time zero was designated as 100% activity.

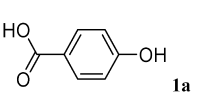
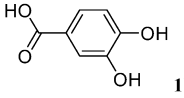
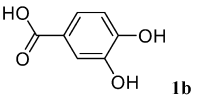
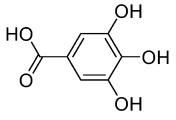
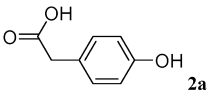
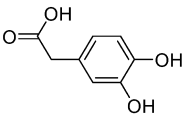
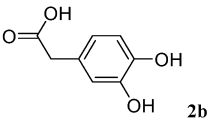
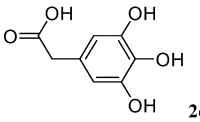
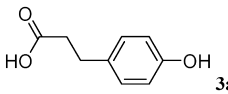
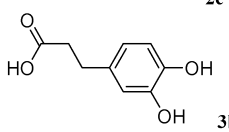
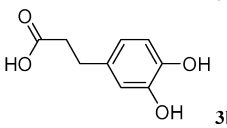
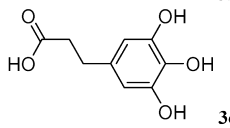
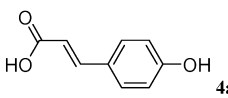
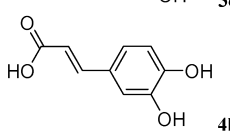
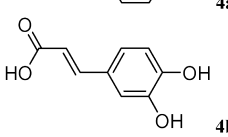
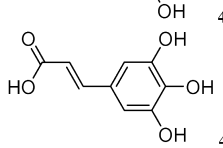
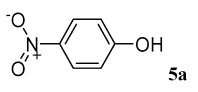
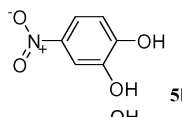
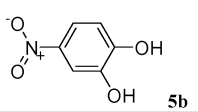
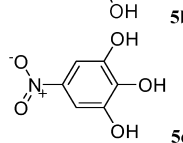
Stopped-Flow Studies of the C_2 Reaction. All stopped-flow experiments were performed at 4 °C in 50 mM NaH_2PO_4 (pH 7.0) using TgK Scientific (Bradford-on-Avon, U.K.) stopped flow spectrophotometers models SHU-61SX2 or SHU-61DX2. The flow paths of the instruments were made anaerobic by leaving an anaerobic solution of dithionite sodium (5 mg/mL) overnight. Excess dithionite was removed by rinsing the flow paths with anaerobic buffers three times before performing the experiments. To study the reactions of C_2 with oxygen, a solution of wild-type C_2 or Y398S (110 μM): FMN (50 μM) was titrated with a dithionite solution (5 mg/mL) inside the ananerobic glovebox to form enzyme:$FMNH^-$ binary complexes. The solution was placed inside an airtight tonometer and loaded onto the stopped-flow instrument. The experiments were performed by mixing the flavin solution with buffers containing various oxygen concentrations similar to the protocol previously described.[25,28,29] Observed rate constants for the kinetic traces were analyzed according to exponential fits using Program A software (developed by C.J. Chiu, R. chang, J. Diverno and D. P. Ballou at the University of Michigan). The rate constants versus oxygen concentrations were plotted and analyzed for their bimolecular rate constants using the KaleidaGraph software (Synergy Software).

RESULTS AND DISCUSSION

Reactions of Wild-Type C_2 with Phenolic Substrates. Twenty-four commercially available 2-(4-hydroxyphenyl)acetic acid (4-HPA) **2a** analogues were subjected to wild-type C_2 catalyzed hydroxylation (single turnover) to examine substrate specificity. Solutions of reduced C_2 were mixed with oxygenated buffers in the presence of phenolic analogue substrates and the resulting products were identified by analysis with an HPLC/DAD/MSD setup as described in the Experimental Section and Supporting Information Figure S1. Results summarized in Table 1 indicated that C_2 could catalyze hydroxylation of phenolic and catecholic compounds containing anionic substituents such as −COOH (**1a, 1b**), $-CH_2COOH$ (**2a, 2b**), $-CH_2CH_2COOH$ (**3a, 3b**), *trans*-CH=CHCOOH (**4a, 4b**), and $-NO_2$ (**5a, 5b**) at the *para*-position. Other compounds which could not be used as substrates for wild-type C_2 are listed in Chart 1 (**6a−20a**).

Based on the *m/z* values, spectroscopic properties and HPLC profiles, the data indicated that products from the single turnover reactions of phenolic substrates **1a−5a** are catecholic compounds **1b−5b** with an −OH group incorporated at the position next (*-ortho*) to the original hydroxyl group, and the

Table 1. Product Formation in Single Turnover Reactions[a] of Wild-type C_2 with Phenolic 1a−5a and Catecholic 1b−5b Substrates

Substrate	Product	% Yield
 **1a**	 **1b**	5 ± 1%[b]
 **1b**	 **1c**	15 ± 1%[b]
 **2a**	 **2b**	91 ± 3%[b]
 **2b**	 **2c**	98 ± 4%[c]
 **3a**	 **3b**	14 ± 1%[b]
 **3b**	 **3c**	85 ± 1%[c]
 **4a**	 **4b**	5 ± 1%[b]
 **4b**	 **4c**	86 ± 6%[c]
 **5a**	 **5b**	61 ± 2%[b]
 **5b**	 **5c**	ND[d]

[a]The reactions were performed by mixing a solution of C_2 (200 μM): $FMNH^-$ (100 μM) 250 μL with a solution of substrates 400 μM, 250 μL (in 50 mM NaH_2PO_4, pH 7.0), left at room temperature for 15 min, and then quenched with 500 μL of 0.15 M HCl. The quenched solutions were filtered through Microcon centrifugal units (10 kDa cutoff), and the filtrates obtained were analyzed using HPLC/DAD/MSD. [b]%Yield of product was calculated from standard curves of pure compounds. [c]%Yield of product was calculated from the amount of substrate consumed compared to the limiting agent (reduced flavin) in the reaction. [d]%Yield of product was not determined. **1a**, 4-Hydroxybenzoic acid (4-HBA); **1b**, 3,4-Dihydroxybenzoic acid (3,4-DHBA); **1c**, 3,4,5-Trihydroxybenzoic acid (3,4,5-THBA or gallic acid); **2a**, 2-(4-Hydroxyphenyl)acetic acid (4-HPA); **2b**, 2-(3,4-Dihyroxyphenyl)acetic acid (3,4-DHPA); **2c**, 2-(3,4,5-Trihydroxyphenyl)acetic acid (3,4,5-THPA); **3a**, 3-(4-Hydroxyphenyl)propanoic acid (4-HPPA); **3b**, 3-(3,4-Dihydroxyphenyl)propanoic acid (3,4-DHPPA); **3c**, 3-(3,4,5-Trihydroxyphenyl)propanoic acid (3,4,5-THPPA); **4a**, *p*-Coumaric acid; **4b**, Caffeic acid; **4c**, 3,4,5-Trihydroxycinnamic acid (3,4,5-THCA), **5a**: 4-Nitrophenol, **5b**: 4-Nitrocatechol, **5c**: 5-Nitropyrogallol

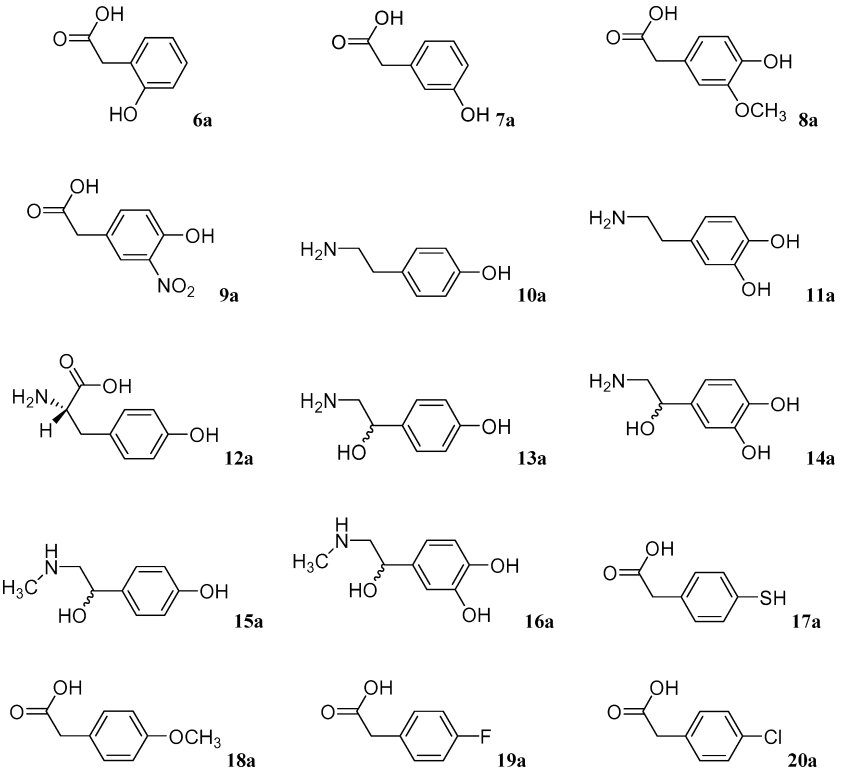
DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

reaction generally produces 3,4-dihydroxyphenolic acids **1b**−**5b** (Table 1). For catecholic substrates **1b**−**5b**, the reaction also incorporates an −OH group to form trihydroxyphenolic products **1c**−**5c** (Table 1). As examples, typical chromatograms from the reaction of wild-type C_2 with 3,4-DHPA **2b** are shown in the Supporting Information data (Supporting Information Figure S1). The substrate and product were eluted with retention times of 10.55 and 8.21 min, respectively (Supporting Information Figure S1A). The 3,4-DHPA **2b** peak has a molecular mass of 167 (Supporting Information Figure S1C), while the product peak has a mass of 183 (Supporting Information Figure S1D), indicating that the reaction incorporated a single atom of oxygen (a hydroxyl group) into the starting substrate. For the reaction of 3,4-DHBA **1b**, the product had the same retention time and *m*/*z* profiles as those of 3,4,5-THBA (or gallic acid) **1c** but not of 2,3,4-THBA (Data not shown).

Identification of Trihydroxyphenolic Acid Products 2c and 4c from the Reactions of C_2 (Y398S Variant) with 2-(3,4-Dihydroxyphenyl)acetic Acid (3,4-DHPA) 2b and Caffeic Acid 4b. As the genuine compounds of 3,4,5-THPA **2c** or 3,4,5-THCA **4c** that are the putative products from the reactions of C_2 with 3,4-DHPA **2b** and caffeic acid **4b** are not available, scale-up preparation and purification of these compounds via C_2 (Y398S variant)-catalyzed hydroxylation were performed (see Experimental Section). The structures of these compounds were elucidated on the basis of NMR and mass spectrometry analysis results (see Supporting Information). The ^{1}H NMR spectrum of 3,4,5-THPA **2c** obtained from C_2 hydroxylation of 3,4-DHPA **2b** (Supporting Information Figure S2) showed the presence of two equivalent aromatic protons (singlet) at the 2′ and 6′-positions (at 6.21 ppm) indicating that the hydroxylation occurred at the *ortho*-position to 4-phenolic group of 3,4-DHPA **2b**. Its ^{13}C NMR and DEPT-135 spectra (Supporting Information Figure S3) showed that there are two pairs of equivalent aromatic carbons at the 2′,6′- and 3′,5′ position at the chemical shifts of 108.6 and 146.2 ppm. These data are consistent with the ^{1}H NMR results that the product is 3,4,5-THPA **2c**. ^{1}H NMR and ^{13}C NMR spectra of the trihydroxycinnamic acid product (Supporting Information Figure S4 and Figure S5) also identified the product obtained from the C_2 (Y398S variant) hydroxylation of caffeic acid **4b** as 3,4,5-THCA **4c** (see Experimental Section). The ^{1}H NMR spectra showed a singlet peak equivalent to the 2′,6′-aromatic protons at the chemical shift of 6.55 ppm (Supporting Information Figure S4), while the ^{13}C NMR and DEPT-135 showed two pairs of equivalent 2′,6′ and 3′,5′ aromatic carbons at 107.5 and 146.2 ppm, respectively (Supporting Information Figure S5).

Biotransformation of Phenolic Compounds by C_2 to Synthesize 2-(3,4,5-Trihydroxyphenyl)acetic Acid 2c and 3,4,5-Trihydroxycinnamic Acid 4c. Results in Table 1 suggest that C_2 may potentially be used as a biocatalyst to synthesize trihydroxyphenolic acids such as 3,4,5-THPA **2c** and 3,4,5-THCA **4c**. Since 4-HPA **2a** and *p*-coumaric acid **4a** are major compounds that can be derived from lignin degrada-

Chart 1. Structures of Compounds That Are Not Substrates for Wild-Type C_2 Reaction[a]



[a]**6a**, 2-(2-Hydroxyphenyl)acetic acid; **7a**, 2-(3-Hydroxyphenyl)acetic acid; **8a**, 2-(4-Hydroxy-3-methoxyphenyl)acetic acid; **9a**, 2-(4-Hydroxy-3-nitrophenyl)acetic acid; **10a**, Tyramine; **11a**, Dopamine; **12a**, L-Tyrosine; **13a**, Octopamine; **14a**, Norepinephrine; **15a**, Synephrine; **16a**, Epinephrine; **17a**, 2-(4-Mercaptophenyl)acetic acid; **18a**, 2-(4-Methoxyphenyl)acetic acid; **19a**, 2-(4-Fluorophenyl)acetic acid; **20a**, 2-(4-Chlorophenyl)acetic acid.

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

tion,[31] the C_2-catalyzed hydroxylation reaction would provide a green synthetic route to transform degraded lignin products to value-added 3,4,5-THPA **2c** and 3,4,5-THCA **4c**. Therefore, multiple turnover reactions of C_2 were set up by coupling the reactions to the reactions of C_1 and an NADH-regenerating system of glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), and NAD^+ (Scheme 1A) (Experimental Section). G-6-PD catalyzes the oxidation of G-6-P by NAD^+ and thus constantly generates NADH for the C_1−C_2 reaction. C_1 catalyzes the reduction of FMN by NADH to generate $FMNH^-$, which is transferred to C_2 via free diffusion.[22−24] Some phenolic acids such as 4-HPA **2a** can also bind to C_1 and enhance the rate of flavin reduction and the rate of $FMNH^-$ release.[22,24] C_2 binds $FMNH^-$ to form a binary complex of C_2:$FMNH^-$ before reacting with oxygen to form C_{4a}-hydroperoxy-FMN which hydroxylates phenolic substrates to form products.[25,27,29] The results in Figure 1A indicate that the multiple turnover reactions of 4-HPA **2a** with C_2 first transformed 4-HPA **2a** (filled black circles) into 3,4-DHPA **2b** (filled blue squares), which was then converted to 3,4,5-THPA **2c** (filled red diamonds) as the final product (Scheme 1B). The reaction could completely consume 3,4-DHPA **2b** within 100 min with a total turnover number (TTN) of 75 (Table 2). TTN was calculated using eq 1.

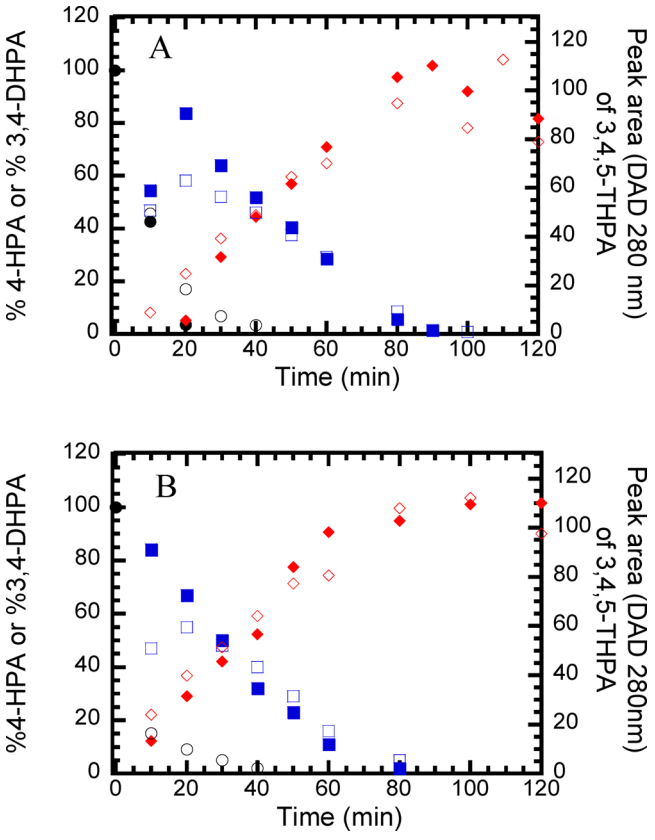


Figure 1. (A) Multiple turnover reactions of wild-type C_2 and Y398S with 4-HPA **2a**. Percentages of 2-(4-hydroxyphenyl)acetic acid (4-HPA) **2a** consumption (filled black circle (WT) and empty black circle (Y398S)) and 2-(3,4-dihydroxyphenyl)acetic acid (3,4-DHPA) **2b** formation and consumption (filled blue square (WT) and empty blue square (Y398S)). Peak area (DAD 280 nm) of 2-(3,4,5-trihydroxyphenyl)acetic acid (3,4,5-THPA) **2c** (filled red diamond (WT) and empty red diamond (Y398S)) are plotted against reaction time. (B) Multiple turnover reactions of wild-type C_2 and Y398S with 4-HPA **2a** in the presence of ascorbic acid. Same symbols are used as in part A.

$$\text{TTN} = \frac{\text{mol product formed}}{\text{mol of wild-type } C_2 \text{ or mutant present in reaction}} \quad (1)$$

Table 2. Total Turnover Numbers (TTN) of Multiple Turnover Reactions[a] of Wild-type C_2 and Y398S with 4-HPA 2a and *p*-Coumaric Acid 4a in the Absence and in the Presence of Ascorbic Acid

enzyme	substrate	time (min)	product	TTN[c]
wild-type	**2a**	100	**2c**	75
	2a(ascorbic acid)[b]	100	**2c**	100
	4a	240	**4c**	8.8[d]
	4a (ascorbic acid)[b]	240	**4c**	7.5[d]
Y398S	**2a**	120	**2c**	62
	2a (ascorbic acid)[b]	100	**2c**	100
	4a	180	**4c**	12.8
	4a (ascorbic acid)[b]	180	**4c**	23.2

[a]The reactions were performed by preparing a solution of G-6-P (1 mM), C_1 (100 nM), NAD^+ (10 μM), FMN (1 μM), wild-type or variant C_2 (2 μM) and 4-HPA **2a** (200 μM) or *p*-coumaric acid **4a** (50 μM) in 50 mM NaH_2PO_4 pH 7.0. G-6-PD 0.5 U/ml was then added to start the reactions. The reactions were collected at the given times and quenched with an equal volume of 0.15 M HCl. The quenched solutions were filtered through Microcon centrifugal units (10 kDa cutoff). The filtrates obtained were analyzed using HPLC/DAD/MSD. [b]Ascorbic acid 1.4 mM was also added into the reaction mixture before starting the reaction with G-6-PD. [c]Total turnover numbers (TTN) were calculated using eq 1; TTN = μmol of product/ μmol of C_2 or variant. [d]The reactions were not complete at 240 min and contained a mixture of **4a** and **4b**.

In contrast, the kinetics of the bioconversion of *p*-coumaric acid **4a** by C_2 to generate 3,4,5-THCA **4c** (Scheme 1B) was significantly less efficient (Figure 2A). Consumption of the starting substrate, *p*-coumaric acid **4a**, was not complete even after 180 min; a substantial amount of *p*-coumaric acid **4a** (filled black circles) remained in the reaction. Even after 660 min of reaction time, *p*-coumaric acid **4a** was still not completely converted (Supporting Information Table S4). These results indicated that the wild-type enzyme cannot efficiently convert *p*-coumaric acid **4a** into 3,4,5-THCA **4c**.

Engineering of the C_2 Active Site for Synthesis of 2-(3,4,5-Trihydroxyphenyl)acetic Acid 4c. As the results obtained from the multiple turnover reactions of wild-type C_2 and *p*-coumaric acid **4a** indicated that the wild-type enzyme is not efficient in converting *p*-coumaric acid **4a** into 3,4,5-THCA **4c**, we subsequently engineered the active site of C_2 to obtain variants in order to enhance the bioconversion process to yield 3,4,5-THCA **4c**. On the basis of the X-ray structure of C_2 in complex with $FMNH^-$ and 4-HPA **2a** (PDB code 2JBT),[26] we targeted residues located within 4 Å of the 4-HPA **2a** binding site for site-directed mutagenesis. These residues included H120 (2.8 Å), S146 (2.8 Å), I148 (3.5 Å), R263 (2.8−3.1 Å), F266 (2.9 Å), and Y398 (3.5 Å) (Figure 3). Our previous work revealed that H120 and S146 are crucial for efficient electrophilic phenolic substitution of 4-HPA **2a**.[29] Previous investigation using molecular dynamics simulations, site-directed mutagenesis, and transient kinetics indicate that F266 is a gating residue that controls oxygen diffusion into the active site. Mutation of F266 resulted in variants with

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

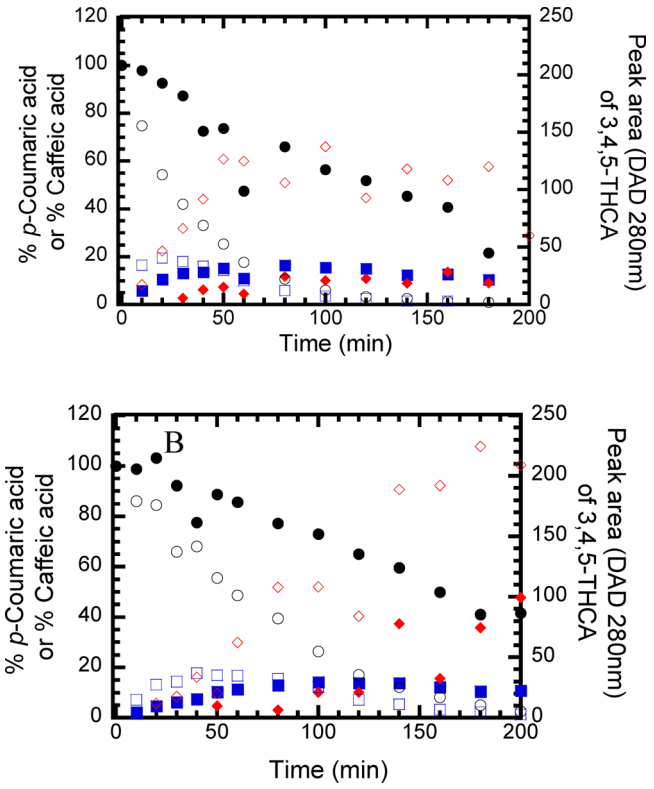


Figure 2. (A) Multiple turnover reactions of wild-type C_2 and Y398S with *p*-coumaric acid **4a**. Percentages of *p*-coumaric acid **4a** consumption (filled black circle (WT) and empty black circle (Y398S)) and caffeic acid **4b** formation and consumption (filled blue square (WT) and empty blue square (Y398S)). Peak area (DAD 280 nm) of 3,4,5-trihydroxycinnamic acid (3,4,5-THCA) **4c** (filled red diamond (WT) and empty red diamond (Y398S)) are plotted against reaction time. (B) Multiple turnover reactions of wild-type C_2 and Y398S with *p*-coumaric acid **4a** in the presence of ascorbic acid; Same symbols are used as in (A).

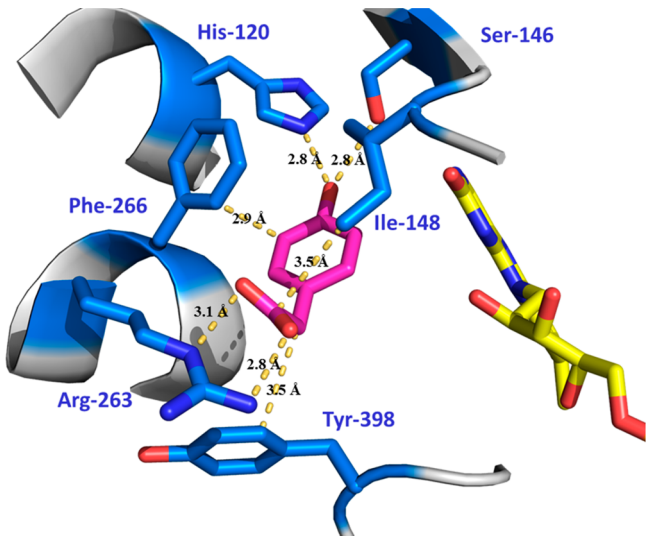


Figure 3. Active site structure of the C_2:$FMNH^-$:4-HPA (**2a**) complex (PDB code 2JBT).[26] Amino acid residues located near 4-HPA **2a** are shown in blue. H120 (2.8 Å), S146 (2.8 Å), I148 (3.5 Å), R263 (2.8–3.1 Å), F266 (2.9 Å), and Y398 (3.5 Å). $FMNH^-$ and 4-HPA **2a** are shown in yellow and pink, respectively.

significantly slow rate of C_{4a}-hydroperoxyflavin formation.[32] Therefore, these residues are not good targets for enzyme engineering. We thus only focused on the ability of I148, R263, and Y398 variants to perform the conversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c**. As *p*-coumaric acid **4a** has a larger aliphatic chain compared to the native substrate 4-HPA **2a** and these three residues are located close to this side, I148, R263 and Y398 were mainly mutated into smaller side chains to create more space for accommodating *p*-coumaric acid **4a**. Product yields from single and multiple turnover reactions of I148A, I148S, R263A, R263D, R263E, R263K, and Y398S, Y398T, and Y398A variants are in Supporting Information Table S2–S4.

The results indicated that the regio-specificity and reaction efficiency of these C_2 variants were different from the wild-type enzyme. I148S variant could not catalyze the hydroxylation of *p*-coumaric acid **4a** (Supporting Information Table S4), while the R263K variant could catalyze the conversion of *p*-coumaric **4a** to caffeic acid **4b**. However, the bioconversion rate by R263K was considerably slow and the enzyme was unstable. The reaction of Y398T with *p*-coumaric acid **4a** yielded a mixture of caffeic acid **4b** and 3,4,5-THCA **4c** after 2 h and was inefficient in producing 3,4,5-THCA **4c** similar to the wild-type enzyme reaction. Among the C_2 variants investigated, Y398S was the most efficient enzyme for preparation of 3,4,5-THCA **4c** because it can complete double hydroxylation of 4-HPA **2a** within 120 min which is comparable to the wild-type reaction (Figure 1A and Supporting Information Table S3). Notably, Y398S can catalyze the complete conversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c** within ~180 min, which is significantly improved from the reaction of wild-type C_2 (Figure 2A and Supporting Information Table S4). Y398A can also generate 3,4,5-THCA **4c** with a similar yield to Y398S (Supporting Information Table S4). However, the enzyme is less stable than the Y398S variant (see results later, Supporting Information, Figure S7). Therefore, we only carried out further studies with the Y398S variant. The total turnover numbers (TTN) for the conversion of 4-HPA **2a** to 3,4,5-THPA **2c** by wild-type C_2 and Y398S were calculated, using eq 1, to be 75 per 100 min and 62 per 120 min, respectively (Table 2). The TTN of Y398S and *p*-coumaric **4a** was 12.8 in 180 min (Table 2). The TTN of the wild-type C_2 and *p*-coumaric acid **4a** for formation of 3,4,5-THCA **4c** was 8.8 within 240 min (Table 2). However, the wild-type reaction was not complete even at 660 min (Supporting Information Table S4).

Stabilization of 3,4,5-Trihydroxyphenolic Products by Ascorbic Acid. From multiple turnover reactions of wild-type and Y398S enzymes with 4-HPA **2a** (Figure 1A), the yield of 3,4,5-THPA **2c** significantly decreased when the reactions proceeded longer than 80 min. The loss could be due to the auto-oxidation of 3,4,5-THPA **2c**. Therefore, ascorbic acid was added in order to increase the compound stability. Multiple turnover reactions of wild-type C_2 and Y398S with 4-HPA **2a** and *p*-coumaric acid **4a** were then carried out in the presence of ascorbic acid (1.4 mM). The results indicated that 3,4,5-THPA **2c** reached the highest yield ~80 min and remained stable, enhancing the overall yield of 3,4,5-THPA **2c** (Figure 1B and Supporting Information Table S3). The reaction of Y398S and *p*-coumaric acid **4a** with the addition of ascorbic acid (1.4 mM) also resulted in a higher yield of 3,4,5-THCA **4c** than the reaction in the absence of ascorbic acid (Figure 2A,B and Supporting Information Table S4). In the presence of ascorbic acid, the TTN of the reactions of wild-type and Y398S with 4-

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492–4502

HPA **2a** and Y398S with *p*-coumaric acid **4a** were determined as 100 in 100 min, 100 in 100 min and 23.2 in 180 min, respectively (Table 2). The TTN value of the wild-type C_2 and *p*-coumaric acid **4a** in the presence of ascorbic at 240 min was 7.5; however, the reaction was not complete even at 240 min (Table 2 and Supporting Information Table S4).

Preparation of 3,4,5-Trihydroxyphenylacetic Acid 2c and 3,4,5-Trihydroxycinnamic Acid 4c in a Semilarge Scale Preparation. In order to validate that the reactions of C_2 can be used to synthesize 3,4,5-THPA **2c** and 3,4,5-THCA **4c** on a preparative scale and to obtain the purified product for NMR analysis. Multiple turnover reactions of 3,4-DHPA **2b** and caffeic acid **4b** by Y398S variant were carried out (see Experimental Section). Y398S can completely convert 3,4-DHPA **2b** and caffeic acid **4b** to 3,4,5-THPA **2c** and 3,4,5-THCA **4c** (Supporting Information Figure S6) within 8 and 20 h, respectively.

To evaluate the amount of trihydroxyphenolic products synthesized from 1 L of culture, results from the bioconversion of wild-type C_2 and Y398S with 4-HPA **2a** and *p*-coumaric acid **4a** substrates (Supporting Information Table S3 and S4, and Table 2) were calculated for μmol of purified enzymes obtained from 1 L of culture and μmol of trihydroxyphenolic products **2c** and **4c** that can be produced from 1 L of culture (Table 3). The

Table 3. Trihydroxyphenolic Products 3,4,5-THPA 2c and 3,4,5-THCA 4c That Can Be Obtained from 1 L Culture of Enzyme

enzyme	substrate	product	purified enzyme obtained from 1 L of culture (μmol)	product that can be produced from 1 L of culture (μmol)
wild-type	**2a**	**2c**	8.5	850[a]
	4a	**4c**		67.2[b]
Y398S	**2a**	**2c**	5.1	510[a]
	4a	**4c**		118[b]

[a]3,4,5-THPA **2c** that can be obtained from 1 L culture of wild-type C_2 and Y398S in mg unit are 156.4 and 93.8, respectively. [b]3,4,5-THCA **4c** that can be obtained from 1 L culture of wild-type C_2 and Y398S in mg unit are 13.2 and 23.2, respectively.

wild-type enzyme, a suitable biocatalyst for synthesis of 3,4,5-THPA **2c**, can produce ~850 μmol or 156 mg of 3,4,5-THPA **2c** from 1 L of culture of C_2. Y398S, a suitable biocatalyst for production of 3,4,5-THCA **4c** can synthesize ~118 μmol or 23.2 mg of 3,4,5-THCA **4c** from 1 L of culture of Y398S.

Thermostability of C_2 Wild-Type and Y398S Enzymes. In order to explore whether C_2 wild-type and Y398S enzymes can be practically used as biocatalysts, we explored the thermostability of these enzymes at 30, 35, and 40 °C. The activity of C_2 measured by the dihydroxyphenylacetate oxygenase (DHPAO) coupled assay[20] was used for measuring the amount of active enzyme and its stability after incubation at various temperatures over the course of 24 h as described in the Experimental Section. DHPAO converts 3,4-DHPA **2b**, the product from the C_2 reaction, into 5-carboxymethyl-2-hydroxymuconate semialdehyde (CHS), a yellow compound that can be monitored at 400 nm (ε_{400} = 21.3 mM^{-1} cm^{-1}).[20] The results of the thermostability studies of C_2 wild-type and Y398S enzymes at 30, 35, 40 °C are shown in Figure 4. The data indicate that the wild-type enzyme is very stable. The activities remained almost unchanged after incubation at 30, 35, and 40 °C for 24 h. Y398S is less stable than the wild-type enzyme at high temperature. At 30 °C, its activity remained

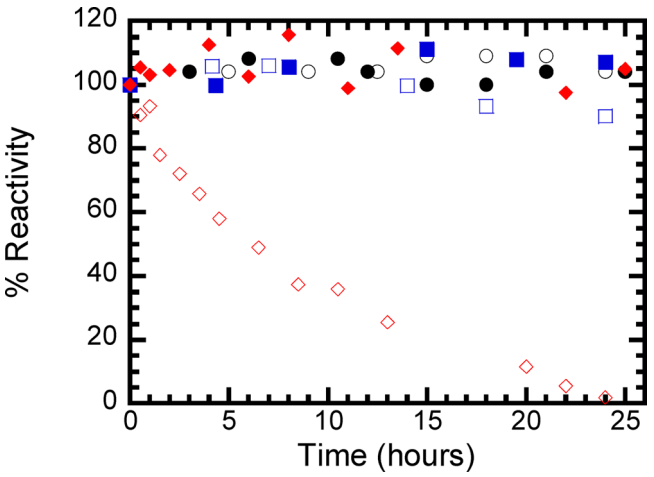


Figure 4. Thermostability of the wild-type C_2 and Y398S variant at 30, 35, and 40 °C. The plot shows activities of wild-type C_2 and Y398S remaining after incubation at 30 °C (filled and empty black circles), 35 °C (filled and empty blue squares) and 40 °C (filled and empty red diamonds) for various periods of time. Values for the wild-type enzyme are shown in filled symbols, while those for Y398S are in empty symbols.

unchanged for 24 h. At 35 °C, Y398S activity was unchanged for 15 h, but decreased to 93% and 90% after 18 and 24 h, respectively. At 40 °C, the activity of Y398S decreased significantly with a $t_{1/2}$ of ~5.8 h. Thermostability of Y398A was also investigated in comparison with Y398S and wild-type C_2 at 40 °C, 8 h (Supporting Information Figure S7), the results showed that among the three enzymes, Y398A is the least stable one because its activity decreased the fastest ($t_{1/2}$ of ~2 h). Y398S has a $t_{1/2}$ of ~3 h while activity of the wild-type enzyme remained unchanged until 8 h.

Catalytic Superiority of Y398S Is Due to the Improvement of Interaction between the Protein and *p*-Coumaric Acid 4a. Transient kinetic experiments of Y398S and wild-type enzymes were carried out in order to shed light on the source of the differences in their catalytic properties as observed in the conversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c**. Previous studies of wild-type enzyme with 4-HPA **2a** using stopped-flow and rapid-quench experiments elucidated the reaction mechanism of C_2 as summarized in Supporting Information (Supporting Information Scheme S2).[25,27,29] In this report, we investigated the transient kinetics of the reaction of Y398S:$FMNH^-$ with oxygen. The results showed that Y398S also reacted with oxygen to form Y398S:C_{4a}-hydroperoxyflavin intermediate in the first step. The intermediate subsequently decayed to eliminate H_2O_2 (Supporting Information Figure S8A and Scheme S1). Rate constants of both reaction steps for both the wild-type and Y398S enzymes are in the same range (Supporting Information Scheme S1); both enzymes rapidly form C_{4a}-hydroperoxyflavin and eliminate H_2O_2 slowly via the uncoupling path in the absence of phenolic acid.

The reaction of wild-type C_2:$FMNH^-$:*p*-coumaric acid (**2a**) (2 or 10 mM) with oxygen was monitored using stopped-flow spectrophotometry at 390 and 446 nm (Figure 5). The absorbance changes at 390 nm reflected formation and decay of the C_{4a}-flavin adduct (C_{4a}-hydroperoxyflavin or C_{4a}-hydroxyflavin intermediates), while absorbance changes at 446 nm reflected the formation of the oxidized flavin. The results in Figure 5 showed that the presence of *p*-coumaric acid **4a** affected the second kinetic phase after the C_{4a}-hydroperoxy-

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

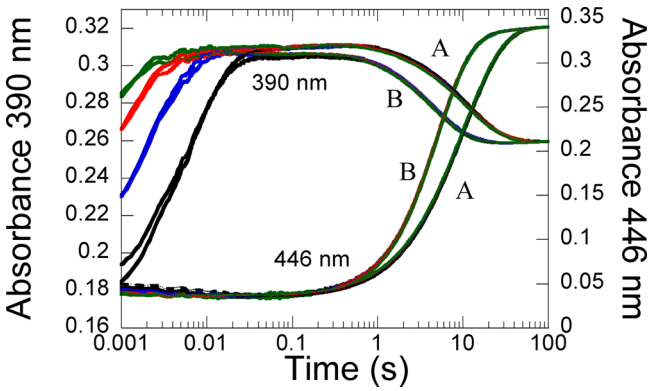


Figure 5. Comparison of kinetics monitored at 390 and 446 nm of the wild-type C_2 reactions in the presence of 2 and 10 mM *p*-coumaric acid **4a**. (A) Reaction of wild-type C_2 with 2 mM *p*-coumaric acid **4a**. A solution of C_2 (110 μM): $FMNH^-$ (50 μM): *p*-coumaric acid **4a** (2 mM) was mixed with solutions of 0.26, 0.62, 1.22, and 2.06 mM oxygen and 2 mM *p*-coumaric acid **4a** in 50 mM NaH_2PO_4 pH 7.0 at 4 °C. Concentrations of oxygen after mixing were 0.13, 0.31, 0.61, and 1.03 mM oxygen and are indicated in black, blue, red, and green lines, respectively. (B) Reaction with 10 mM *p*-coumaric acid **4a**. The experiment was performed similar to (A) except that 10 mM *p*-coumaric acid **4a** was used instead.

flavin adduct was formed, indicating that the first step of the reaction is formation of C_{4a}-hydroperoxyflavin even *p*-coumaric acid **4a** was preincubated with the enzyme (Scheme 2). The second phase of the reaction was 0.1−80 s for reaction with 2 mM *p*-coumaric acid **4a**, and 0.1−30 s for reaction with 10 mM of *p*-coumaric acid **4a**. The spectroscopic changes observed in the second phase of the reaction were the combined results of hydroxylation and dehydration steps (Scheme 2). As C_{4a}-hydroperoxyflavin and C_{4a}-hydroxyflavin in the C_2 reaction have similar spectroscopic properties, individual rate constants for the steps following the formation of C_{4a}-hydroperoxyflavin could not be determined.[27] However, the results indicated that the final species (oxidized flavin) was formed during the second kinetic phase. The results showed that, in the presence of 10 mM *p*-coumaric acid **4a**, the oxidized enzyme forms faster than in the reaction performed in the presence of 2 mM of *p*-coumaric acid **4a**. This is probably due to a larger fraction of the enzyme forming a binary complex of C_2:*p*-coumaric acid at the higher *p*-coumaric acid **4a** concentration, pushing the reaction toward the hydroxylation path in the presence of 10 mM *p*-coumaric acid **4a**. The path in which the C_2:*p*-coumaric acid complex is formed returns to the final oxidized flavin state much faster than in the uncoupling path. In order to validate our proposed explanation, similar reactions were carried out with various concentrations of *p*-coumaric acid **4a**. The results shown in Table 4 and Supporting Information Table S5 confirmed that more of the hydroxylation product was obtained and the kinetics of flavin oxidation was faster as the concentration of *p*-coumaric acid **4a** was increased.

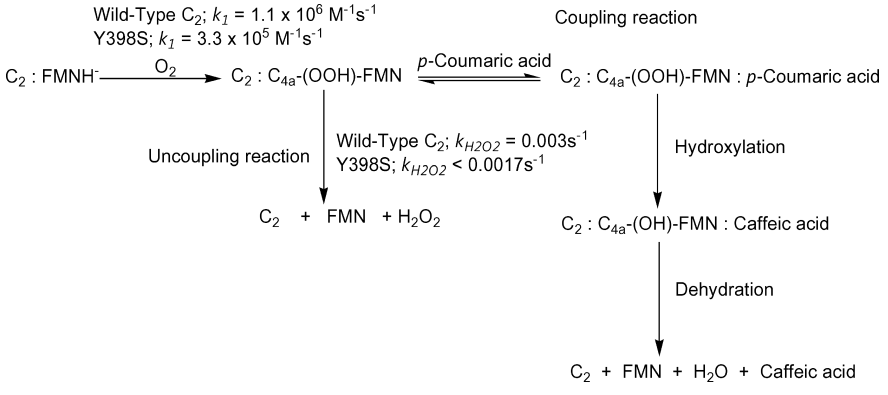
Table 4. Product Analysis (%Yield of Caffeic Acid 4b or % Hydroxylation) of Single Turnover Reactions[a] of Wild-type C_2 and Y398S in the Presence of *p*-Coumaric Acid 4a (0.5−8 mM)

	% yield of caffeic acid **4b**[b]	
[*p*-coumaric acid **4a**] (mM)	wild-type C_2	Y398S
0.5	5 ± 0	12 ± 2
1	7 ± 3	20 ± 4
2	11 ± 5	21 ± 5
4	13 ± 1	37 ± 5
8	26 ± 5	50 ± 4

[a]The reactions were performed by mixing solutions of C_2 or Y398S (200 μM): $FMNH^-$ (100 μM) 250 μL with *p*-coumaric acid **4a** 1, 2, 4, 8, 16 mM (250 μL) in 50 mM NaH_2PO_4 pH 7.0 at room temperature for 15 min. The reactions were then quenched with an equal volume of 0.15 M HCl. The quenched solutions were filtered through Microcon centrifugal units (10 kDa cutoff), and the filtrates obtained were analyzed using HPLC/DAD/MSD. [b]%Yield of caffeic acid **4b** or % hydroxylation was calculated from the peak area of the product using a standard curve derived from the pure compound.

Using a similar analysis as for the wild-type C_2 and *p*-coumaric acid **4a** reaction described above, experiments of Y398S:$FMNH^-$ with oxygen and various concentrations of *p*-coumaric acid **4a** were carried out and monitored using stopped-flow spectrophotometry (see details in Supporting Information Figures S10−12). The results in Table 4 indicate that at all of the concentrations of *p*-coumaric acid **4a** employed, product formation levels from the single turnover reactions of Y398S were all higher than those obtained from the reaction of the wild-type enzyme. The dependency of the observed rate constants of the flavin oxidation on *p*-coumaric acid **4a** reached saturation at a much lower concentration of *p*-coumaric acid **4a** (Supporting Information Table S5). These data imply that for the Y398S variant, the binding between enzyme and *p*-coumaric acid **4a** is better than the wild-type enzyme. This allows more of the enzyme to partition into the

Scheme 2. Reactions of Wild-Type C_2 and Y398S Variant with *p*-Coumaric Acid 4a



DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

productive path with lower *p*-coumaric acid **4a** concentrations and result in much improved coupling efficiency in the bioconversion of *p*-coumaric acid **4a** to 3,4,5-THCA **4c**.

In order to confirm whether the binding of *p*-coumaric acid **4a** is better accommodated in the active site pocket of Y398S than in the wild-type enzyme, *p*-coumaric acid **4a** was docked into the wild-type enzyme structures (Figure 6). The analysis

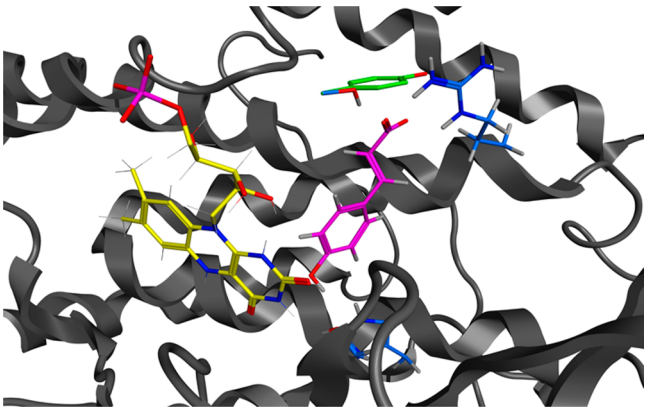


Figure 6. Wild-type C_2 with *p*-coumaric acid **4a** modeled into the enzyme active site.

indicates that the residue Y398 in the wild-type enzyme is located very close to the aliphatic chain of *p*-coumaric acid. This may possibly create steric hindrance for *p*-coumaric acid **4a** binding in the wild-type pocket. For Y398S variant, significant reduction of the side chain creates more space in the area and may allow better binding of *p*-coumaric acid **4a** in the Y398S variant.

CONCLUSIONS

Biocatalysis by the HPAH system can efficiently synthesize the antioxidants 3,4,5-THPA **2c** and 3,4,5-THCA **4c** from 4-HPA **2a** and *p*-coumaric acid **4a**. Rational engineering of C_2 resulted in the Y398S variant that is more effective than the wild-type enzyme for preparation of 3,4,5-THCA **4c**. Transient kinetic studies indicated that the Y398S variant has more favorable interaction with *p*-coumaric acid **4a** and can form more enzyme:*p*-coumaric acid complexes. The reaction of Y398S thus occurs through a productive oxygenation path more so than the wild-type enzyme. The wild-type and Y398S variant enzymes are stable and can be overexpressed in large amounts suitable for scale-up preparation in future studies. Therefore, C_2 wild-type and Y398S enzymes are green and efficient biocatalysts that are useful for the bioconversion of low value lignin degradation products such as 4-HPA **2a** and *p*-coumaric acid **4a** to valuable antioxidants such as 3,4,5-THPA **2c** and 3,4,5-THCA **4c**.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00439.

Primers for variants and PCR methods, HPLC chromatograms, ^{1}H and ^{13}C NMR spectra of 3,4,5-trihydroxyphenolic products, single turnover reactions of variants, multiple turnover reactions of C_2 variants, thermostability of Y398A, presteady state kinetic studies using stopped-flow experiments (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: pimchai.cha@mahidol.ac.th. Phone: +66-2201 5596. Fax: +66-2354 7174.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was financially supported by The Thailand Research Fund through grants RTA5680001 (to P.C.) and Royal Golden Jubilee Ph.D. Program Grant PHD/0335/2551 (to T.D.) and from a grant from Mahidol University.

REFERENCES

(1) Lin, Y.; Jain, R.; Yan, Y. *Curr. Opin. Biotechnol.* **2014**, *26*, 71−78.
(2) Esteves, M.; Siquet, C.; Gaspar, A.; Rio, V.; Sousa, J. B.; Reis, S.; Marques, M. P. M.; Borges, F. *Arch. Pharm.* **2008**, *341*, 164−173.
(3) Lee, J. W.; Bae, C. J.; Choi, Y. J.; Kim, S. I.; Kim, N. H.; Lee, H. J.; Kim, S. S.; Kwon, Y. S.; Chun, W. *Korean J. Physiol. Pharmacol.* **2012**, *16*, 107−112.
(4) Lee, J. W.; Choi, Y. J.; Park, J. H.; Sim, J. Y.; Kwon, Y. S.; Lee, H. J.; Kim, S. S.; Chun, W. *Biomol. Ther.* **2013**, *21* (1), 60−65.
(5) Lee, J. W.; Bae, C. J.; Choi, Y. J.; Kim, S. I.; Kwon, Y. S.; Lee, H. J.; Kim, S. S.; Chun, W. *Mol. Cell. Biochem.* **2014**, *390*, 143−153.
(6) Lee, J. W.; Kwon, J. H.; Lim, M. S.; Lee, H. J.; Kim, S. S.; Lim, S. Y.; Chun, W. *Mol. Cell. Biochem.* **2014**, *397*, 109−116.
(7) Srivastava, V.; Darokar, M. P.; Fatima, A.; Kumar, J. K.; Chowdhury, C.; Saxena, H. O.; Dwivedi, G. R.; Shrivastava, K.; Gupta, V.; Chattopadhyay, S. K.; Luqman, S.; Gupta, M. M.; Negi, A. S.; Khanuja, S. P. S. *Bioorg. Med. Chem.* **2007**, *15*, 518−525.
(8) Siquet, C.; Paiva-Martins, F.; Lima, J. L. F. C.; Reis, S.; Borges, F. *Free Radical Res.* **2006**, *40* (4), 433−442.
(9) Gomes, C. A.; Girao da Cruz, T.; Andrade, J. L.; Milhazes, N.; Borges, F.; Marques, M. P. M. *J. Med. Chem.* **2003**, *46*, 5395−5401.
(10) Teixeira, J.; Gaspar, A.; Garrido, E. M.; Garrido, J.; Borges, F. *BioMed Res. Int.* **2013**, *2013*, 2013.
(11) Teixeira, J.; Silva, T.; Benfeito, S.; Gaspar, A.; Garrido, E. M.; Garrido, J.; Borges, F. *Eur. J. Med. Chem.* **2013**, *62*, 289−296.
(12) Bansal, V. K.; Kumar, R.; Prasad, R.; Prasad, S.; Niraj, S. *J. Mol. Catal. A: Chem.* **2008**, *284*, 69−76.
(13) Jin, M.; Yang, R.; Zhao, M.; Li, G.; Hu, C. *Ind. Eng. Chem. Res.* **2014**, *53*, 2932−2939.
(14) Shinichi, S.; Mitsuaki, Y.; Shinichi, I.; Hisao, E. Japan Kokai Tokkyo Koho; Patent No. JP 09291053, November 11, 1997.
(15) Bourgaud, F.; Hehn, A.; Larbat, R.; Doerper, S.; Gontier, E.; Kellner, S.; Matern, U. *Phytochem. Rev.* **2006**, *5*, 293−308.
(16) Furuya, T.; Arai, Y.; Kino, K. *Appl. Environ. Microbiol.* **2012**, *78* (17), 6087−94.
(17) Berner, M.; Krug, D.; Bihlmaier, C.; Vente, A.; Müller, R.; Bechthold, A. *J. Bacteriol.* **2006**, *188* (7), 2666−2673.
(18) Lin, Y.; Yan, Y. *Microb. Cell Fact.* **2012**, *11*, 42.
(19) Furuya, T.; Kino, K. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1145−1154.
(20) Chaiyen, P.; Suadee, C.; Wilairat, P. *Eur. J. Biochem.* **2001**, *268*, 5550−5561.
(21) Thotsaporn, K.; Sucharitakul, J.; Wongratana, J.; Suadee, C.; Chaiyen, P. *Biochim. Biophys. Acta, Gene Struct. Expression* **2004**, *1680* (1), 60−66.
(22) Sucharitakul, K.; Phongsak, T.; Entsch, B.; Svasti, J.; Chaiyen, P.; Ballou, D. P. *Biochemistry* **2007**, *46* (29), 8611−8623.
(23) Phongsak, T.; Sucharitakul, J.; Thotsaporn, K.; Oonanant, W.; Yuvaniyama, J.; Svasti, J.; Ballou, D. P.; Chaiyen, P. *J. Biol. Chem.* **2012**, *287* (31), 26213−26222.
(24) Sucharitakul, J.; Tinikul, R.; Chaiyen, P. *Arch. Biochem. Biophys.* **2014**, *555*−*556*, 33−46.
(25) Sucharitakul, J.; Chaiyen, P.; Entsch, B.; Ballou, D. P. *J. Biol. Chem.* **2006**, *281* (25), 17044−17053.

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502

(26) Alfieri, A.; Fersini, F.; Ruangchan, N.; Prongjit, M.; Chaiyen, P.; Mattevi, A. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (4), 1177−1182.
(27) Ruangchan, N.; Tongsook, C.; Sucharitakul, J.; Chaiyen, P. *J. Biol. Chem.* **2011**, *286* (1), 223−233.
(28) Thotsaporn, K.; Chenprakhon, P.; Sucharitakul, J.; Mattevi, A.; Chaiyen, P. *J. Biol. Chem.* **2011**, *286* (32), 28170−28180.
(29) Tongsook, C.; Sucharitakul, J.; Thotsaporn, K.; Chaiyen, P. *J. Biol. Chem.* **2011**, *286* (52), 44491−44502.
(30) Chenprakhon, P.; Trisrivirat, D.; Thotsaporn, K.; Sucharitakul, J.; Chaiyen, P. *Biochemistry* **2014**, *53* (25), 4084−4086.
(31) Jeffries, T. W. Biodegradable of Lignin and Hemicelluloses. In *Biochemical of Microbial Degradation, 1*; Ratledge, C., Ed.; Kluwer Academic Publishers: Netherlands, 1994; pp 233−277.
(32) Baron, R.; Riley, C.; Chenprakhon, P.; Thotsaporn, K.; Winter, R. T.; Alfieri, A.; Forneris, F.; van Berkel, W. J. H.; Chaiyen, P.; Fraaije, M. W.; Mattevi, A.; McCammon, J. A. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (26), 10603−10608.

DOI: 10.1021/acscatal.5b00439
ACS Catal. 2015, 5, 4492−4502