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Alternative substrates of 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase

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Abstract

2,4-Dichlorophenoxyacetate/α-ketoglutarate dioxygenase (TfdA), the first enzyme in the catabolic pathway for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), oxidizes α-ketoglutarate (α-kG) to CO_2 and succinate while hydroxylating 2,4-D to yield an unstable hemiacetal that decomposes into 2,4-dichlorophenol and glyoxylate. In an effort to extend the potential biotechnological utility of this enzyme, a variety of non-phenoxyacetate compounds were examined as potential substrates. 2-Naphthoxyacetic acid was the best alternative substrate tested, followed by benzofuran-2-carboxylic acid, 2,4-dichlorocinnamic acid, 2-chlorocinnamic acid, 1-naphthoxyacetic acid, and 4-chlorocinnamic acid. TfdA appeared to oxidize the olefin bond of the cinnamic acids and benzofuran-2-carboxylate to form the corresponding epoxides. Whole cells were observed to also catalyze a TfdA-dependent oxidation of 2,4-dichlorocinnamic acid. Based on the ability of TfdA to metabolize chlorinated cinnamic acids, we speculate that *tfdA*-like sequences present in 2,4-D non-degrading natural isolates may function in metabolism of substituted cinnamic acids. These results support the use of TfdA and related enzymes in the specific oxidation of non-phenoxyacetate substrates.

Keywords: 2,4-Dichlorophenoxyacetic acid; Cinnamic acid; Dioxygenase; α-Ketoglutarate; Epoxide

1. Introduction

,4-Dichlorophenoxyacetic acid (2,4-D), a broad leaf herbicide, is mineralized by a wide variety of environmental isolates [1] including the best-studied example, *Ralstonia eutropha* JMP134 (pJP4) (formerly *Alcaligenes eutrophus*). The *tfdABCDEF* genes, carried on the pJP4 plasmid in this microorganism [2], encode all of the enzymes necessary for the degradation of 2,4-D to chloromaleylacetate. Chromosomally borne genes encode enzymes that carry out the subsequent metabolism of chloromaleylacetate. Since its discovery and characterization, pJP4 has been used to study the evolution and environmental dispersal of the genes encoding a catabolic pathway for xenobiotic degradation [1].

TfdA catalyzes the first step in this 2,4-D degradation pathway. This ferrous ion and α-ketoglutarate (α-kG)-dependent dioxygenase hydroxylates 2,4-D while converting α-kG to succinate plus CO_2 [3] (Scheme 1). Hydroxylation of 2,4-D at the C-2 position yields an unstable hemiacetal that decomposes into 2,4-dichlorophenol and glyoxylate [3]. TfdA is related in sequence (~30% amino acid identity) to a group of sulfonate degrading α-kG dependent dioxygenases [4,5] and an alkyl sulfate ester degrading α-kG

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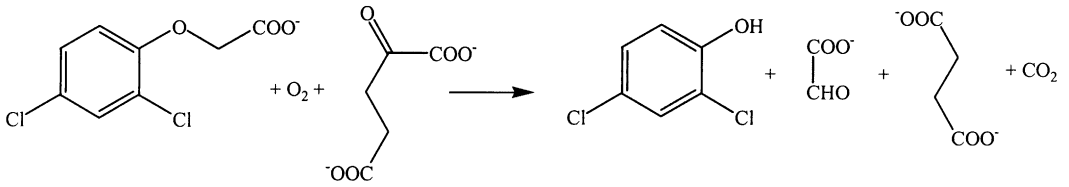


Scheme 1. Reaction catalyzed by TfdA with 2,4-D.

dependent dioxygenase [6]. In addition to these protein homologues, nucleotide sequences closely homologous to *tfdA* are present in approximately 30% of bacterial isolates obtained from agricultural plots. The role of the *tfdA*-like genes containing these sequences is unknown since these soil microorganisms do not degrade 2,4-D [7].

The current study explores alternative substrates for TfdA in order to better define the substrate profile and explore potential biotechnological uses for the enzyme. In particular, we demonstrate that purified enzyme or whole cells can be used to produce synthetically valuable epoxides [8,9] of chlorinated cinnamic acids and benzofuran-2-carboxylic acid. Furthermore, since the 2,4-D-degrading enzyme may retain residual activities for the natural substrate, this work may provide clues about the role of TfdA-like proteins in nature.

2. Experimental

2.1. Purification and assay of TfdA

Escherichia coli DH5α (pUS311) carries *tfdA* on a pUC19-derived plasmid [3]. These cells were grown at 30°C in LB medium for 16–20 h, harvested by centrifugation, and suspended in 30 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.7) containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Crude cell extracts were obtained by passing the cells twice through a pre-cooled French pressure cell (16,000 psi) and clarifying the debris by centrifugation (100,000 × *g* for 45 min) at 4°C. After splitting the sample into two pools, TfdA was enriched by chromatography at 4°C on a column of DEAE-Sepharose (2.5 cm × 19 cm) using TE buffer and a 400 ml linear gradient to 200 mM NaCl. The enzyme eluted at approximately 100 mM NaCl. TfdA-containing fractions were dialyzed in TE buffer and chromatographed on a Mono Q column (HR10/10) (Pharmacia) at room temperature. The enzyme eluted at about 40 mM NaCl when using a 100 ml linear gradient to 200 mM NaCl in TE buffer. When necessary, TfdA was further purified by phenyl-Sepharose chromatography. The sample was adjusted to 1 M in ammonium sulfate, applied to a column (HR10/10) equilibrated with TE buffer containing the same concentration of this salt, and chromatographed with a 100 ml linear gradient from 1 to 0 M ammonium sulfate. The enzyme eluted from this resin at approximately 350 mM salt. TfdA was routinely assayed with 2,4-D as the substrate by using the previously described 4-aminoantipyrene spectrophotometric assay [10]. Protein concentrations were determined by using a commercial protein assay (Bio-Rad) with bovine serum albumin as a standard.

2.2. Kinetics of non-phenoxyacetic acid substrates

The activity of TfdA toward non-phenoxyacetic acid potential substrates was determined at 30°C in 20 mM imidazole buffer (pH 6.8) containing 1 mM α-kG, 50 μM $(NH_4)_2Fe(SO_4)_2$, and 200 μM ascorbic acid by using one or more of four different assays. For all methods, stock solutions of $(NH_4)_2Fe(SO_4)_2$ and ascorbic acid were made fresh prior to each set of experiments.

A YSI model 5300 biological oxygen monitor was used to determine rates of oxygen consumption. The probe was equilibrated using air-saturated MilliQ water, and control runs included samples lacking in ascorbic acid, iron, enzyme, α-kG, or substrate.

Samples analyzed by HPLC (Hewlett-Packard 1050) were resolved by using a Merck Lichrosorb RP-18 column. The peak areas or peak heights for samples absorbing at 230, 254, and/or 280 nm

were determined by using the Hewlett-Packard ChemStation software. Reaction mixtures containing 2,4-dichlorocinnamic acid were quenched with NaOH (0.1 M final concentration), neutralized with HCl (0.1N final concentration), and analyzed by utilizing a mixture of 65:35:0.1 methanol:water:phosphoric acid (effluent A). A standard curve for the reaction product was prepared by complete conversion of selected concentrations of substrate. The area under the product peak was assumed to correspond to the original concentration of substrate. Reaction mixtures that contained naphthoxyacetic acid were quenched with EDTA (5 mM final concentration) and analyzed by using a 50:50:0.1 mixture of methanol:water:phosphoric acid (effluent B) as the mobile phase. Standard curves for the degradation of 1-naphthoxyacetic acid and 2-naphthoxyacetic acid were prepared by using 1-naphthol and 2-naphthol, respectively. All other potential substrates tested were quenched with NaOH (0.1 M final concentration) and neutralized with HCl (0.1N final concentration). Standard curves for the reaction products were created by correlating the loss of substrates with gain of products at various concentrations of substrate. Effluent B was used as the mobile phase, and the kinetics were determined in all cases by analyzing the gain of product, except for the 4-chlorocinnamic acid reaction kinetics, which were estimated from the loss of substrate.

Two spectrophotometric assays also were utilized. Analysis of 1-naphthoxyacetic acid was carried out by using the 4-aminoantipyrene assay with 1-naphthol as the standard. For 2,4-dichlorocinnamic acid, an alternative spectrophotometric assay made use of the 250 nm absorption associated with the conjugated system ($\varepsilon_{250} = 7800\,M^{-1}\,cm^{-1}$). The loss of absorbance at this wavelength was monitored in studies designed to assess the ascorbic acid dependence of the reaction.

As previously documented [10,11], TfdA activity decreased over time under assay conditions, especially when ascorbic acid was absent or at low concentration. To analyze the kinetics of non-phenoxyacetic acid substrates, progress curves were analyzed by fitting the data to the following equation

$$P_t = V_i(1 - e^{-k(\text{inact})t})k(\text{inact})^{-1}$$

where P_t is the accumulated product at time t, V_i the initial velocity, and k(inact) the inactivation rate constant [11].

2.3. Identification of the 2,4-dichlorocinnamic acid and benzofuran-2-carboxylic acid metabolites

Samples for NMR analysis were generated at 30°C in D_2O containing 40 mM phosphate buffer (pH 6.8), 200 μM α-kG, 100 μM substrate, 50 μM $(NH_4)_2Fe(SO_4)_2$, 50 μM ascorbic acid, and 1.6 μM TfdA. The metabolites generated from 2,4-dichlorocinnamic acid and benzofuran-2-carboxylic acid were analyzed by ^{1}H-NMR using a Varian VXR 500 MHz NMR spectrometer.

The 2,4-dichlorocinnamic acid metabolite stability was assessed with a sample prepared by enzymatic conversion of 10 μmol of 2,4-dichlorocinnamic acid. The reaction mixture was applied to a Pharmacia PEP-RPC HR10/10 column, washed with 0.1% trifluoroacetic acid, and eluted by using a 40:60:0.1 mixture of methanol:water:trifluoroacetic acid while monitoring the absorbance at 254 nm. Aliquots of the sample were incubated for varying time periods using specified conditions, and the concentrations of the remaining metabolite and the degradation products were measured by HPLC analysis with effluent A.

2.4. Assessing non-phenoxyacetic acid substrates with whole cells

R. eutropha JMP134 (pJP4) carries the pJP4 plasmid containing *tfdABCDEF* [2]. *R. eutropha* JMP228 (pBH501aE) is a derivative of *R. eutropha* JMP134 (pJP4) where the *tfdA* gene has been interrupted by transposon mutagenesis [12]. *R. eutropha* JMP228 is the strain lacking the plasmid. These cells were grown to late-exponential phase at 30°C in MMO minimal medium [13] amended with the indicated carbon source. Cells were centrifuged and resuspended ($A_{260} = 1.5$) in fresh MMO with no carbon source. The cell suspensions were aerated on a stir plate. Carbon sources were added and samples were removed at various times, centrifuged, diluted, and transferred to HPLC vials. Isocratic HPLC with effluent A was used to analyze the samples for loss of the substrate peak and gain of the product peak.

2.5. Computing curve fits

KaleidaGraph for Windows by Abelbeck Software was used for computing all curve fits.

3. Results

3.1. Identification of non-phenoxyacetic acid substrates

Prior studies have evaluated the ability of purified TfdA to degrade various phenoxyacetic acids, thiophenoxyacetic acids, and phenoxypropionic acids [10,11,14]. The substrate associated with the highest catalytic efficiency was identified as the xenobiotic compound, 2,4-D. Here, we investigated a range of naturally occurring, non-phenoxyacid, aromatic compounds as substrates of the enzyme, along with some related synthetic derivatives of these compounds. In particular, cinnamic acids, auxin-like compounds, and naphthoxyacetic acids were tested by using oxygen electrode, HPLC, and spectrophotometric methods.

The oxygen electrode assay showed clearly enhanced levels of oxygen consumption over background for TfdA (1.25 μM) assay mixtures containing 2-chloro, 4-chloro, and 2,4-dichlorocinnamic acids, 1- and 2-naphthoxyacetic acids, and benzofuran-2-carboxylic acid (each at 300 μM). When each enzyme-containing sample was adjusted to 200 μM 2,4-D, oxygen consumption immediately increased to match that observed for samples containing only 2,4-D. Thus, none of these compounds exhibited significant inhibition or inactivation of 2,4-D hydroxylation activity by TfdA. For other compounds, oxygen consumption rates were close to background levels. This assay did not directly measure substrate conversion (e.g. some substrates may have uncoupled oxygen consumption from substrate oxidation), and spurious results were observed for at least one sample: 3,4-hydroxycinnamic acid. This compound formed a blue color when mixed with ferrous ion (consistent with metal chelation by the catechol group) and consumed oxygen in the absence of enzyme when ascorbic acid was present, presumably similar to the non-enzymatic oxygen consumption observed for EDTA (not shown) [15]. Given these concerns, the potential substrates were quantitatively studied by using HPLC and spectrophotometric methods, which provided similar results for conversion of the potential substrates.

Under conditions equivalent to those used with the oxygen electrode, 2-chloro, 4-chloro, and 2,4-dichlorocinnamic acids, 1- and 2-naphthoxyacetic acids, and benzofuran-2-carboxylic acid were converted to products. In addition, small and variable amounts of 3,4-dihydroxycinnamic acid (caffeic acid), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 2,4-dimethoxycinnamic acid, 3,5-dimethoxycinnamic acid, unsubstituted cinnamic acid, 4-methoxycinnamic acid, and 3-methoxycinnamic acid were converted to products. No indication of substrate loss or product formation was observed for 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid (coumaric acid), or 2-methoxycinnamic acid. Similarly, chromone-2-carboxylic acid, indole-2-carboxylic acid, indole-3-carboxylic acid, indole-3-acetic acid (auxin), indole-3-acrylic acid, hippuric acid, and phenylpropionic acid were not substrates of TfdA.

3.2. Kinetic analysis of non-phenoxyacetic acid substrates

Detailed studies of the enzyme kinetics were carried out with the test compounds that were convincingly shown to be substrates. Characterization of the kinetic parameters was complicated due to an irreversible inactivation of the ferrous containing enzyme that occurs during exposure to oxygen [11]. An average $k(\text{inact}) = 0.49 \pm 0.26\,\text{min}^{-1}$ was determined for all substrates, regardless of the substrate concentration. Initial rates were obtained by fitting the progress curves for substrate conversion, as described in the Materials and Methods. The enzyme kinetics were further complicated by the dependence of the reaction on ascorbic acid. As illustrated in Fig. 1, the concentration of this reductant had a large effect on the initial rate of 2,4-dichlorocinnamic acid utilization. Similar results were previously reported for conversion of the poor substrate, thiophenoxyacetic acid [11]. To overcome this requirement, 200 μM ascorbic acid was used for all kinetics studies.

The results of the kinetic investigations are provided in Table 1. For comparison, data also were obtained for TfdA metabolism of 2,4-D. The values of K_m, k_{cat}, and k_{cat}/K_m (the catalytic efficiency) associated with 2,4-D were similar to, and more accurate than, previous reports ($17.5 \pm 1.0\,\mu\text{M}$, $529 \pm 16\,\text{min}^{-1}$, and $30{,}200\,\text{min}^{-1}\,\mu\text{M}^{-1}$, respectively) [10]. Each of the non-phenoxyacetic acid substrates was associated with a higher K_m, a lower k_{cat}, and a lower catalytic efficiency than was observed for 2,4-D.

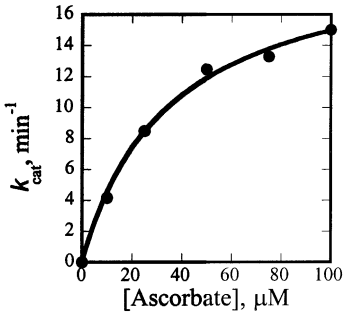


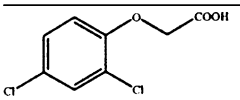
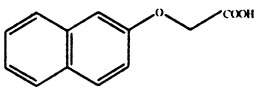
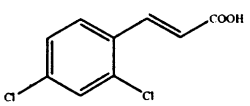
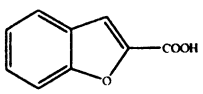
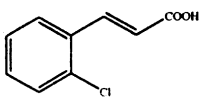
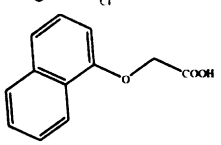
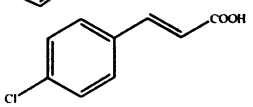
Fig. 1. Ascorbic acid dependence of 2,4-dichlorocinnamic acid utilization by TfdA. The effect of ascorbic acid concentration on the initial rate of 2,4-dichlorocinnamic acid loss was monitored by using a continuous spectrophotometric assay. The standard assay buffer contained 391 nM TfdA dimer and 100 μM 2,4-dichlorocinnamic acid.

3.3. Metabolite analysis

Similar to the conversion of substituted phenoxyacetic acids to their corresponding phenols, 1-naphthoxyacetic acid and 2-naphthoxyacetic acid were shown by co-elution from an HPLC column to be converted to 1- and 2-naphthol (respectively).

^{1}H-NMR analysis of the sample mixture after complete transformation of 2,4-dichlorocinnamic acid by TfdA revealed (a) the disappearance of two doublets at δ 6.4 ppm and δ 7.6 ppm associated with protons bound to carbon atoms participating in the olefin bond and (b) the appearance of two new doublets at δ 3.5 ppm and δ 4.2 ppm (data not shown). The epoxide protons of phenylglycidic acid (the epoxide of cinnamic acid) exhibit identical resonances [16]; thus, we conclude that TfdA oxidized the side chain double bond of 2,4-dichlorocinnamic acid to produce 2,4-dichlorophenylglycidic acid. In the case of benzofuran-2-carboxylic acid, NMR evidence revealed an analogous change in chemical shift of the proton on carbon-3 from δ 7.2 ppm to δ 3.6 ppm (data not shown). This result was consistent with similar formation of an epoxide during substrate conversion.

Table 1
Kinetics of alternative substrates of TfdA

Structure	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
	2,4-D	20.0 ± 5.4	1020 ± 90	51000
	2-Naphthoxyacetic acid	134 ± 32	263 ± 5	1960
	2,4-Dichlorocinnamic acid	190 ± 56	52.5 ± 5.1	276
	Benzofuran-2-carboxylic acid	254 ± 120	105 ± 16	413
	2-Chlorocinnamic acid	264 ± 52	22.1 ± 1.3	83.9
	1-Naphthoxyacetic acid	622 ± 310	15.7 ± 2.9	25.2
	4-Chlorocinnamic acid	>900	>38	

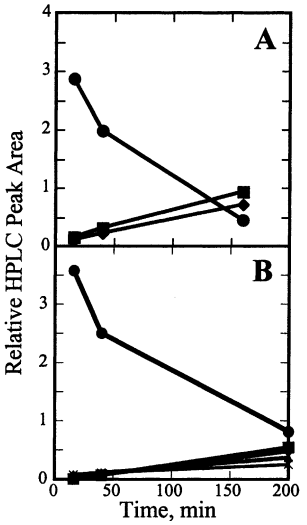


Fig. 2. pH stability and nucleophilic reactivity of the TfdA reaction product derived from 2,4-dichlorocinnamic acid. The peak areas at 230 nm were determined for the 2,4-dichlorocinnamic acid reaction product (●) and degradation products associated with retention times of 2.5 (◆), 4 (■), 6 (▲), and 7 min (X) of samples incubated at pH 3 (A) and pH 4 with 2 mM hydroxylamine (B).

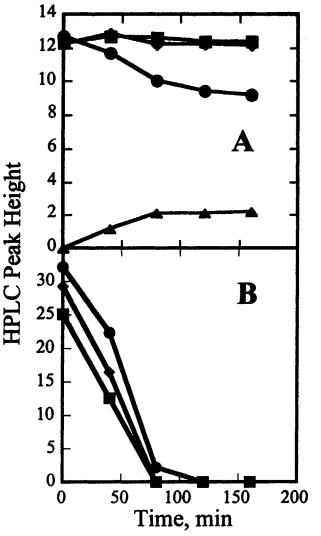


Fig. 3. Degradation of cinnamic acids by intact cells. The time dependence was examined for decomposition of 2,4-dichlorocinnamic acid (panel A) and cinnamic acid (panel B) by *R. eutropha* JMP134 (pJP4) (●), *R. eutropha* JMP228 (■), and *R. eutropha* JMP228 (pBH501aE) (◆) by using HPLC methods and analysis of the resulting absorbances at 230 nm. In addition, the time dependence was determined for production of the metabolite derived from 2,4-dichlorocinnamic acid by *R. eutropha* JMP134 (pJP4) (▲) in panel A.

To further test whether the product of the 2,4-dichlorocinnamic acid is 2,4-dichlorophenylglycidic acid, we examined its pH stability and nucleophilic reactivity. Acidic conditions (pH 3) led to the rapid decomposition of the sample as two degradation products were formed (Fig. 2, panel A). In contrast, the reaction product was stable for at least 8 days at pH 13 (data not shown). Inclusion of the strong nucleophile hydroxylamine at low pH led to the formation of two new degradation products (Fig. 2, panel B). The low pH reactivity and distinct product profile generated in the presence of hydroxylamine are consistent with an epoxide being the initial product of 2,4-dichlorocinnamic acid metabolism by TfdA.

3.4. Whole cell studies

The potential of using intact bacterial cells to metabolize 2,4-dichlorocinnamic acid was examined using *R. eutropha* JMP134 (pJP4), *R. eutropha* JMP228 (pBH501aE), and *R. eutropha* JMP228 (Fig. 3). The first strain encodes the entire 2,4-D pathway, whereas the second and third cultures lack *tfdA* and the pJP4 plasmid, respectively. Degradation of 2,4-dichlorocinnamic acid was observed only in the first isolate, consistent with a requirement for *tfdA* (panel A). Notably, product accumulation mirrored substrate disappearance in agreement with the inability of this strain to grow on 2,4-dichlorocinnamic acid as a sole carbon source. In contrast to the 2,4-dichlorocinnamic acid results, all three strains decomposed unsubstituted cinnamic acid (panel B). These results demonstrate that a cinnamic acid degrading system exists in the cells and does not involve *tfdA* or other loci on the pJP4 plasmid.

4. Discussion

4.1. Expansion of the substrate range of TfdA

We demonstrated that several substituted cinnamic acids are utilized (albeit poorly) as substrates by TfdA. The best of this group of substrates is 2,4-dichlorocinnamic acid, whereas the 2- or 4-monochloro

derivatives are used with less efficiency and the unsubstituted cinnamic acid is metabolized even more poorly. This pattern of substrate preference parallels the situation for phenoxyacetic acids, where the 2,4-dichloro species is preferred over the monochlorinated derivatives, which are more readily metabolized than the unsubstituted species [10]. Based on NMR evidence and stability/reactivity studies of the 2,4-dichlorocinnamic acid metabolite, we propose that the TfdA-generated products arising from the cinnamic acids are the side chain epoxides. Thus, TfdA is not limited to inserting oxygen into unactivated C–H bonds — it also can catalyze the easier oxidation of a C–C double bond.

Because 2,4-D chemically mimics auxin in its action as an herbicide [17], we tested whether the converse situation may occur; i.e. can auxin or auxin-like compounds chemically mimic 2,4-D and serve as a substrate for TfdA? We showed that the natural plant hormone, indole-3-acetic acid, is not a substrate of the enzyme. Furthermore, we demonstrated that indole-3-carboxylate, indole-3-acrylate, and indole-2-carboxylate also are not transformed by the enzyme. Interestingly, however, benzofuran-2-carboxylate (the analogue of indole-2-carboxylate in which the indole N is replaced by O) does serve as a substrate of TfdA. Perhaps related to this reaction, benzofuran-2-carboxylate mimics phenoxyacetic acid with the side chain linked to the aromatic ring via a methenyl carbon. NMR evidence suggests that the double bond of this substrate is converted to an epoxide by TfdA. The ability of TfdA to selectively catalyze the transformation of benzofuran-2-carboxylate while not oxidizing indole-2-carboxylate may relate to the differences in resonance energies between the pyrrole (22 kcal/mol) and furan (16 kcal/mol) rings [18]. Thus, oxygen insertion into the double bond of benzofuran-2-carboxylate is more favorable than for the indole-2-carboxylate. Another compound related in structure to both benzofuran-2-carboxylate and phenoxyacetic acid is chromone-2-carboxylate, which is not a substrate of TfdA.

1- and 2-naphthoxyacetic acids, a third class of test compounds, were shown to be substrates of the enzyme. These compounds closely resemble phenoxyacetic acid, but contain a larger aromatic ring. The fact that TfdA is capable of transforming these compounds demonstrates that the active site is sufficiently large to allow entry of these species. Of interest, 2-naphthoxyacetic acid was the herbicidal predecessor to 2,4-D and was replaced because smaller amounts of 2,4-D could be applied to achieve the same benefits [19].

4.2. The ancestral role of TfdA?

Soon after 2,4-D was introduced into the environment in the early 1940s, reports suggested that 2,4-D was rapidly decomposed in soil (e.g. [20]). To account for decomposition of this xenobiotic compound, we earlier proposed the existence of an ancestral gene encoding a degradative enzyme with greatest specificity toward a natural product, but capable of utilizing 2,4-D with low efficiency [1]. According to this scenario, mutations arose over time to enhance the specificity of the ancestral enzyme toward 2,4-D and the new gene recombined with genes for 2,4-dichlorophenol degradation to create a 2,4-D degradation pathway. In support of this hypothesis, approximately a third of soil bacterial isolates possess a *tfdA*-like gene, but do not measurably degrade 2,4-D [7]. Thus, environmental isolates possess *tfdA*-like genes that appear to play another, yet unidentified, role besides 2,4-D catabolism. Based on the studies reported above, we propose that the ancestral role of TfdA may have involved degradation of cinnamic acids. Ring-substituted cinnamic acids are widely distributed in nature and are present in sizable amounts. For example, 4-hydroxycinnamic acid is found at a concentration of 8.7 mg carbon per gram carbon in the top meter of peat soil [21]. Plants synthesize large amounts of cinnamic acids by phenylalanine deamination, and they catalyze a variety of ring substitution, condensation, degradation, reduction, or conjugation reactions to produce a host of cinnamic acid-related compounds including flavonoids, hydroxybenzoates, and lignin [22]. Degradation of substituted cinnamic acids occurs by several pathways (for a review, see [23]), but it would not be surprising to learn that another pathway remains to be described. Future studies will test the cinnamic acid degrading abilities of the enzyme products from *tfdA*-like genes in environmental isolates.

4.3. Biocatalysis

Because of the wide range of enantiospecific chemical reactions in which epoxides can participate, these

compounds serve as intermediates in a variety of medically important chemical syntheses [24]. For example, phenylglycidic acid, the epoxide of unsubstituted cinnamic acid, has been used as an intermediate in the production of taxol [8] and (2*S*,3*S*)-diltiazem [9]. In order to further develop TfdA for biocatalytic production of epoxides from substituted cinnamic acids, three issues must be addressed: chemical yield, cost, and enantiospecificity. Because of the low k_{cat} values observed for *R. eutropha* enzyme, it may be necessary to isolate mutants or obtain isozymes from other environmental sources to increase the chemical yield. With regard to cost, we note that pyruvic acid can replace α-kG for in vitro assays [10]; hence, this less expensive α-ketoacid potentially could be used in immobilized enzyme reactors. Alternatively, our demonstration that whole cells containing this enzyme can convert chlorinated cinnamic acids to the corresponding epoxides offers the possibility that cellular TfdA can be used to expand the repertoire of available synthetic building blocks. Whole cells can be grown in an inexpensive minimal media and used to carry out the desired reaction. Since whole cells do not utilize either 2,4-dichlorocinnamic acid or 2,4-dichlorophenylglycidic acid, substrate is directly converted to product stoichiometrically. We have not characterized the stereospecificity of the TfdA oxygenase reaction for substituted cinnamic acids. However, we expect enantiopure epoxidation. TfdA has been shown previously to hydroxylate only (*S*)-dichloroprop, a phenoxypropionate herbicide [14]. Additionally, a fellow member of the superfamily of α-kG-dependent dioxygenases, EpoA, catalyzes the enantiomer specific production of fosfomycin ((−)-*cis*-1,2-epoxypropylphosphonic acid) from *cis*-propenylphosphonic acid in *Penicillium decumbens* [25]. Likewise, styrene monooxygenase (from a different superfamily of oxygenases) is capable of carrying out the enantioselective conversion of cinnamic acid methyl ester to phenylglycidic acid methyl ester [16]. The studies described here lay the groundwork for future efforts to develop this potential biocatalyst.

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