

Alcaligenes eutrophus JMP134 “2,4-Dichlorophenoxyacetate Monooxygenase” Is an α -Ketoglutarate-Dependent Dioxygenase

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The *Alcaligenes eutrophus* JMP134 *tfdA* gene, encoding the enzyme responsible for the first step in 2,4-dichlorophenoxyacetic acid (2,4-D) biodegradation, was overexpressed in *Escherichia coli*, and several enzymatic properties of the partially purified gene product were examined. Although the *tfdA*-encoded enzyme is typically referred to as 2,4-D monooxygenase, we were unable to observe any reductant-dependent activity. Rather, we demonstrate that this enzyme is a ferrous ion-dependent dioxygenase that uses α -ketoglutarate as a cosubstrate. The α -ketoglutarate is converted to succinate concomitant with 2,4-D conversion to 2,4-dichlorophenol. By using [1-¹⁴C] α -ketoglutarate, we established that carbon dioxide is the second product derived from α -ketoglutarate. Finally, we verified the proposal that glyoxylate is the second product derived from 2,4-D.

The 2,4-dichlorophenoxyacetic acid (2,4-D) biodegradation pathway is a paradigm for microbial metabolism of chloroaromatic compounds (10, 27). The scheme for mineralization of 2,4-D was first established in an *Arthrobacter* species (2, 3, 18, 32, 33) and was shown to involve side chain removal, hydroxylation of the resulting 2,4-dichlorophenol (2,4-DCP), opening of the 3,5-dichlorocatechol ring, and subsequent conversion of 2,4-dichloro-*cis,cis*-muconate to succinate, which is metabolized by the cells. This pathway also is operative in *Alcaligenes eutrophus* JMP134, in which several of the 2,4-D degradation genes (*tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*) have been localized to the transmissible plasmid pJP4 (7–9). Three of the downstream genes, *tfdCDE*, exhibit extensive nucleotide sequence similarity to the well-studied chlorocatechol degradation genes *clcABD* (9a) and *tcBCDE* (34), and the corresponding chlorocatechol dioxygenase, cycloisomerase, and hydrolase steps are biochemically well understood (9, 26). Similarly, the *A. eutrophus* *tfdB* gene (24) exhibits significant sequence similarity to the phenol monooxygenase gene *pheA* (22), and the nicotinamide-dependent, flavin-containing 2,4-DCP hydroxylase that it encodes has been purified and well characterized (17). In contrast to the other *tfd* genes, the *A. eutrophus* *tfdA* gene sequence (31) exhibits no obvious evolutionary relationship to other known sequences and no biochemical characterization of the *tfdA* gene product (TFDA) has been reported.

Here, we identify the complete enzymatic reaction catalyzed by the product of the *tfdA* gene. Although TFDA is generally referred to as 2,4-D monooxygenase (9, 11, 15, 23, 25, 31), we demonstrate that the enzyme is a ferrous ion-dependent and α -ketoglutarate-dependent dioxygenase that converts α -ketoglutarate to succinate and carbon dioxide concomitant with the conversion of 2,4-D to 2,4-DCP and glyoxylate.

MATERIALS AND METHODS

Plasmid construction and cell growth. The 3.1-kbp *SacI* fragment of plasmid pKJS31 (31), containing the *tfdA* gene, was subcloned into the *SacI* site of plasmid pUC19, followed by elimination of a 1.5-kbp *XbaI* fragment and religation to yield plasmid pUS311. *Escherichia coli* JM109 (35) cells were transformed with pUS311, grown overnight at 30°C on modified LB medium (containing [per liter] 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl, pH 7.2) supplemented with 100 μ g of ampicillin per ml, and harvested by centrifugation. All restriction enzyme digestions, ligations, transformations, and other common DNA manipulations were performed by standard procedures (29).

Preparation of cell extracts and partial purification of TFDA. Cultures (2 liters) of *E. coli* JM109(pUS311) were grown overnight at 30 or 37°C with moderate aeration. Cells were harvested by centrifugation (6,000 $\times$ g) for 20 min and then suspended in 25 ml of 20 mM Tris–1 mM EDTA–0.4 μ g of leupeptin per ml (pH 7.2). Crude cell extracts were obtained by passing the cells twice through a precooled French pressure cell at 120 MPa (after adjusting the buffer to 1 mM phenylmethylsulfonyl fluoride) and clarifying the debris by centrifugation at 100,000 $\times$ g for 30 min at 4°C. TFDA was purified 19-fold from cell extracts (obtained from cultures that were grown at 30°C) by chromatography on DEAE-Sepharose (2.6-by-30-cm column, 2 ml/min) at 4°C, followed by Mono-Q 10/10 chromatography (2 ml/min) at room temperature in the presence of 1 mM EDTA and 20 mM Tris-HCl buffer (pH 7.7 and 7.2, respectively) and by using NaCl gradients. The enzyme elutes from the resins at approximately 0.1 and 0.05 M NaCl, respectively.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (16) and included a 12% running gel and a 4.5% stacking gel. Gels were stained with Coomassie brilliant blue (5). Molecular weight markers included phosphorylase b (M_r , 92,500), bovine serum albumin (M_r , 66,200), ovalbumin (M_r , 45,000), carbonic anhydrase (M_r , 31,000), soybean trypsin inhibitor (M_r , 21,500), and lysozyme (M_r , 14,400).

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Assays. The 2,4-D degrading activities of cell extracts and partially purified TFDA were determined by measuring 2,4-DCP formation after 10 min of incubation at 30°C in 1 mM 2,4-D–10 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.5) containing the additions indicated in Results and Discussion. 2,4-DCP was assayed by a previously described 4-aminoantipyrine method (15). The A_{510} was determined after 20 min with a Gilford Response spectrophotometer (Gilford Instrument Laboratory, Inc., Oberlin, Ohio). Alternatively, 2,4-D disappearance and 2,4-DCP formation were monitored with an HP-1050 high-performance liquid chromatograph (HPLC) (Hewlett-Packard Co.) equipped with a C_{18} (octadecyl silane) reverse-phase column (4.0 by 250 mm) at a flow rate of 1.5 ml/min in various compositions of methanol–0.1% H_3PO_4 in H_2O with concomitant monitoring of the A_{230} . One unit of activity is defined as the amount of enzyme that forms 1 μ mol of phenol derivative per min at 30°C. The protein concentration was measured by colorimetric methods (19) using bovine serum albumin as a standard.

Organic acid determinations. The succinate concentration was determined by using an Aminex HPX-78H column (Bio-Rad) with an eluent of 8 mM sulfuric acid at a flow rate of 0.5 ml/min with concomitant monitoring with a differential refractive index detector. By using the same organic acid separation column, glyoxylate and α -ketoglutarate were partially resolved from each other (elution times of ~11.7 and ~11.0 min, respectively) and well separated from succinate (elution time of ~15.2 min). The identities of succinate and glyoxylate in reaction mixtures were confirmed by well-established gas chromatography-mass spectrometry (GC-MS) methods (4).

Identification of CO_2 as a product of the reaction. Partially purified TFDA was incubated with 2.1 nmol of $[1-^{14}C]\alpha$ -ketoglutarate (1.8 GBq/mmol; New England Nuclear) in the presence of unlabeled α -ketoglutarate (99 μ M), 2,4-D (100 μ M), ascorbate (50 μ M), and $(NH_4)_2Fe(SO_4)_2$ (100 μ M) in 2 ml of 10 mM MOPS buffer (pH 6.5) at 30°C. The reaction was stopped by the addition of 1 ml of HCl, and the released $^{14}CO_2$ was trapped in phenethylamine-wetted filter paper by published procedures (5). The amount of radioactivity in the filter paper trap was assessed by scintillation counting.

RESULTS AND DISCUSSION

Preliminary cell extract analysis. The pJP4-derived *tfdA* gene was subcloned into pUC19 and transformed into *E. coli* JM109. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis verified that the recombinant cells synthesized high levels of a peptide with an M_r of 32,000, consistent with the reported properties of TFDA (31). Similar gel electrophoresis analysis of cell fractions revealed that TFDA formed insoluble aggregates in bacterial cells at 37°C, whereas the overexpressed protein was soluble when cells were grown at 30°C. Additional experiments focused on the cells that were grown at the lower temperature. Despite the apparent abundance of TFDA, the 2,4-D degradation activity in cell extracts of these samples remained very low, with typical specific activities of approximately 0.003 μ mol of 2,4-D converted to 2,4-DCP per min per mg of protein. For comparison, whole cells of *A. eutrophus* JM134(pJP4) grown on 2,4-D degrade this substrate at a rate of 0.105 μ mol/min/mg of protein, as shown by substrate disappearance (25). The trace level of activity in recombinant *E. coli* cell extracts was abolished by the addition of EDTA and other chelating agents or by gel filtration chromatography or dialysis. Addition of ferrous ions restored the low levels of

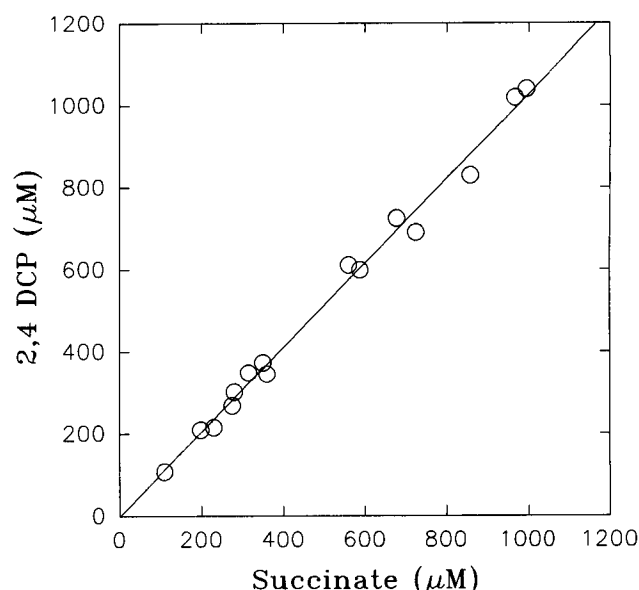


FIG. 1. Correlation between formation of 2,4-DCP and the concentration of succinate during 2,4-D degradation. The concentrations of 2,4-DCP and succinate were assessed by HPLC methods at various times after the addition of TFDA to an assay mixture containing 1 mM 2,4-D, 1 mM α -ketoglutarate, 50 μ M ascorbate, 100 μ M ferrous ammonium sulfate, and 10 mM MOPS buffer (pH 6.5).

activity to chelator-treated samples but not to protein fractions that were freed of low-molecular-weight components. Addition of NADPH or NADH had no effect on any of the samples. The latter results prompted us to question whether this gene product is indeed a monooxygenase, as is generally stated in the literature (9, 11, 15, 23, 25, 31).

Identification of TFDA as an α -ketoglutarate-dependent dioxygenase. Conversion of 2,4-D to 2,4-DCP by partially purified TFDA requires both ferrous ion and α -ketoglutarate. The activity of the partially purified enzyme was assayed in 10 mM MOPS buffer (pH 6.5) containing 1 mM 2,4-D with no additions and found to be 0.0 μ mol/min/mg of protein. The activity was identical when ferrous ion alone (as ferrous ammonium sulfate) or α -ketoglutarate alone was added to the buffer mixture. When α -ketoglutarate (1 mM) and ferrous ion (100 μ M) were both added, however, the activity of TFDA was 2.2 μ mol/min/mg of protein. In addition, oxygen was confirmed to be required for this reaction (data not shown) and ascorbate (100 μ M) was found to

TABLE 1. TFDA-catalyzed production of $^{14}CO_2$ from $[1-^{14}C]\alpha$ -ketoglutarate

Amt of TFDA in assay (μ g) ^a	Reaction time (min)	Radioactivity trapped as CO_2	
		kcpm	%
10	10	89.5	58.5
	30	97.1	63.5
25	10	110.4	72.2
None	10	2.4	1.6
	30	3.6	2.4

^a TFDA was incubated with 100 μ M α -ketoglutarate (containing 1.53×10^5 cpm of radiolabeled material)–100 μ M 2,4-D–50 μ M ascorbate–100 μ M ferrous ion in 2 ml of 10 mM MOPS buffer (pH 6.5) at 30°C. The released $^{14}CO_2$ was trapped as described in the text.

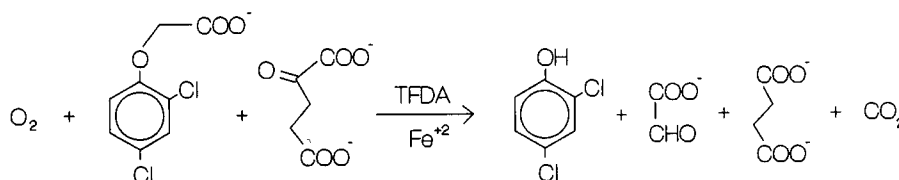


FIG. 2. Overall reaction for the activity of the enzyme encoded by the *tfdA* gene.

stimulate the activity when added along with ferrous ion and α -ketoglutarate (6.2 μ mol/min/mg of protein). The α -ketoglutarate does not simply regulate the activity of the enzyme but rather is a cosubstrate in this reaction. The concentration of α -ketoglutarate decreases at the same rate as that observed for the disappearance of 2,4-D (data not shown), and a compound that coelutes with succinate is produced concomitantly with the release of 2,4-DCP (Fig. 1). Identification of succinate as a product of the reaction was confirmed by GC-MS analysis. Analysis of the enzyme kinetics demonstrated that the reaction was nearly linear, with an apparent inhibition or slow inactivation at long time points. By using the 10 min time points as reasonable estimates for initial rates, the K_m s for 2,4-D and α -ketoglutarate were determined. Over substrate concentration ranges of 10 to 50 μ M for 2,4-D and 1.5 to 20 μ M for α -ketoglutarate, the K_m s were estimated to be 21 ± 1.9 and 3.3 ± 0.4 μ M, respectively.

Two products are generated by TFDA in addition to 2,4-DCP and succinate: glyoxylate and carbon dioxide. Early biochemical studies conducted with cell extracts of an *Arthrobacter* species provided compelling evidence that, at least in this microbe, 2,4-D is converted to 2,4-DCP and glyoxylate (32). Comparable studies have not been reported for *A. eutrophus*. Using partially purified *A. eutrophus* enzyme synthesized in *E. coli* and an organic acids-HPLC protocol, we found that a substance coeluting with glyoxylate is produced stoichiometrically with succinate. Identification of glyoxylate was verified by GC-MS analysis. By using a reaction mixture containing $[1-^{14}C]\alpha$ -ketoglutarate, the second product arising from α -ketoglutarate was demonstrated to be carbon dioxide (Table 1). We conclude that the overall reaction catalyzed by TFDA is that shown in Fig. 2 and propose the name 2,4-D/ α -ketoglutarate dioxygenase for the product of the *tfdA* gene.

Comparison of TFDA to other α -ketoglutarate-dependent dioxygenases. The reaction carried out by 2,4-D/ α -ketoglutarate dioxygenase is very similar to the reactions catalyzed by a class of hydroxylases that require ferrous ion and α -ketoglutarate (1). However, using the BESTFIT algorithm (6), we were unable to detect any significant sequence similarity between TFDA and several of these enzymes (prolyl hydroxylase [13], lysyl hydroxylase [21], hyoscyamine 6 β -hydroxylase [20], aspartyl β -hydroxylase [14], and deacetoxycephalosporin synthase/hydroxylase [30]) or with the mechanistically related 4-hydroxyphenylpyruvate dioxygenase (28). Indeed, the TFDA sequence exhibited no significant similarity to any sequences available in Release 71.0 of GenBank by using the WORDSEARCH algorithm (6). Although the TFDA sequence is distinct from all other reported sequences, we propose that an enzyme analogous to TFDA functions in the catabolism of other phenoxyacetates, such as 2,4,5-trichlorophenoxyacetic acid (12), and perhaps in other biodegradative pathways. Experiments are in progress to purify and further characterize the 2,4-D/ α -ketoglutarate dioxygenase.

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