Characterization of the First Enzyme in 2,4-Dichlorophenoxyacetic Acid Metabolism

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This paper reviews the properties of the Alcaligenes eutrophus JMP134 tfdA gene product, the enzyme responsible for the first step in 2,4-dichlorophenoxyacetic acid (2,4-D) biodegradation. The gene was overexpressed in Escherichia coli and several of its enzymatic properties were characterized. Although this enzyme catalyzes a hydroxylation reaction, it is not a monoxygenase. Rather, TfdA is an Fe(III) and α-ketoglutarate-dependent dioxygenase that metabolizes the latter cosubstrate to succinate and carbon dioxide. A variety of other phenoxyacetates and α-ketoacids can be used by the enzyme, but the greatest catalytic efficiencies were found using 2,4-D and α-ketoglutarate. The enzyme possesses multiple essential histidine residues, whereas catalytically essential cysteine and lysine groups do not appear to be present. — Environ Health Perspect 103(Suppl 9):37–39 (1995)

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The biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D), a broadleaf herbicide, has been shown to involve side chain removal, hydroxylation of the resulting 2,4-dichlorophenol (2,4-DCP), ortho cleavage of 3,5-dichlorocatechol, conversion of 2,4-dichloro-cis,cis-muconate to succinate, and subsequent metabolism of this intermediate by the cells (Figure 1) as reviewed by Håggblom (1). The genes encoding the enzymes involved in these processes in Alcaligenes eutrophus JMP134 have been localized to the pJP plasmid, cloned, and sequenced (2). Whereas the sequences of the tfdB gene (encoding 2,4-DCP hydroxylase) and the tfcC, tfcD, and tfdE genes (responsible for dichlorocatechol degradation) exhibit similarities to sequences of genes involved in mineralization of nonchlorinated or monochlorinated analogues (3), the sequence of the tfdA gene (4) exhibits no obvious evolutionary relationship to other known sequences. Furthermore, until recently no biochemical characterization of the tfdA gene product (TfdA) had been reported. Here, we describe our efforts to characterize the first enzyme in the 2,4-D pathway. We have shown that the enzyme is not a 2,4-D monoxygenase, as commonly stated in the literature but is rather a ferrous and α-ketoglutarate (α-KG)-dependent dioxygenase.

A 3.1-kilobase pair (kb) SacI fragment of pJP containing the tfdA gene was subcloned into the SacI site of pUC19, followed by elimination of a 1.5-kbp Xbal fragment to yield plasmid pUS311. This plasmid was transformed into Escherichia coli JM109 and the recombinant cells were shown to synthesize high levels of a peptide with relative molecular mass (Mr) 32,000. Despite the abundance of TfdA, the 2,4-D-degrading activity in cell extracts was very low (0.003 μmol of 2,4-D converted to 2,4-DCP/min/mg protein) compared to the published rate of degradation in whole cells of A. eutrophus JMP134(pJP4) (0.105 μmole/min/mg) (5). The trace level of activity was abolished upon addition of chelators and restored upon addition of ferrous ion, consistent with a Fe(II) requirement for the enzyme. The presence or absence of reducing agents had no effect on activity, which was inconsistent with the behavior of a monoxygenase. Rather, we found (6) that the enzyme is a dioxygenase that requires α-KG as a cosubstrate and converts this compound to carbon dioxide and succinate, as illustrated in Figure 2.

The thermolabile enzyme (stable only up to 30°C) was purified to apparent homogeneity (specific activity of 16.9 μmoles substrate converted per minute per milligram of protein) by a simple two-step procedure (Table 1) and extensively characterized (7). The presence of protease inhibitors during early stages of TfdA purification enhances the stability of the enzyme by preventing conversion of the subunit to an inactive TfdA fragment of apparent Mr 27,000. Whereas N-terminal sequence analysis of the nondegraded subunit revealed the residues (S–V–V–A–N–) expected from DNA sequence analysis, the amino-terminal sequence of the proteolytic fragment of TfdA (F–K–Y–A–E–L–) was consistent with hydrolytic cleavage after arginine (residue #77). By using anti-TfdA IgG in Western blot analysis of various samples, the conversion was found to occur after cell disruption rather than during the cell cultivation period. The same methods were used to demonstrate that the Mr of 32,000 form of the protein was present in cell extracts of A. eutrophus JMP134. The native protein has an apparent Mr of 50,000 ± 2500, which is consistent with a homodimeric structure. The enzyme exhibits maximum activity at pH 6.5 to 7; however, it is stable over a pH range of 6.5 to 11.

Ferrous ion is absolutely required for activity and cannot be replaced by Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II), or Zn(II). As shown by a time-dependent decrease in enzyme activity, however, ferrous ion alone is unable to sustain enzyme catalysis over long time periods. The rate of activity loss was greatly reduced (although not completely eliminated) by inclusion of ascorbic acid in the assay.
Figure 1. Pathway for degradation of 2,4-D in Alcaligenes eutrophus JMP134. Modified from Fukumori and Hausinger (7).

Catalytic turnover of the enzyme is not required for inactivation, as shown by the loss of activity during enzyme incubation with ferrous ion before addition of substrate. Although not completely characterized, the inactivation clearly results from a metal ion-mediated event as demonstrated by the retention of activity when the enzyme is stored in the absence of metal ions and the presence of EDTA. To minimize enzyme inactivation in kinetic studies, reactions were initiated by addition of enzyme to assay mixtures, and short assay periods were used to calculate initial rates.

Although capable of hydroxylating a wide range of phenoxyacetates and related compounds (Table 2), the enzyme exhibits the greatest affinity and highest catalytic efficiency for 2,4-D. Nonhalogenated phenoxyacetate possesses a larger (Km) value than the halogenated substrates. Similarly, the (Km) value for 2-phenoxypropionate is substantially larger than that of 2-(2,4-dichlorophenoxy)propionate. The additional methyl group in the side chain of these two compounds, however, greatly decreases their kcat values for hydroxylation, perhaps due to the change from a secondary to a tertiary carbon atom. Although 3-phenoxypropionate is a very poor substrate, it is hydroxylated by the enzyme, demonstrating that the substrate binding site can accommodate one extra methylene carbon in the side chain. In contrast, TfdA exhibits no activity toward 4-(2,4-dichlorophenoxy)butyrate, 2-phenoxybenzoate, 2-phenoxyethanol, hydrocinnamic acid, indolylacetic acid, and methyl esters of phenoxyacetate and 4-chlorophenoxyacetate.

Although α-KG is the preferred cosubstrate for the enzyme, TfdA can use a range of other α-ketoacids with lower efficiency (Table 3). The non-α-ketoacid carboxyl group is not required for recognition by the enzyme; the (Km) values for the two substrates possessing a secondary acidic group are significantly less, and the catalytic rates are generally higher compared to those for the other substrates. Addition of an extra methylene group between the α-ketoacid group and the free carboxyl group, in α-ketoacidipate, leads to small changes in the kinetic constants. In contrast, removal of one of the methylene groups, as in oxalacetate, led to an ineffective substrate. Furthermore, β-ketoglutarate, malonate, succinate, and glutarate were unable to support hydroxylation. Finally, in the absence of 2,4-D, no decomposition of α-KG was observed.

Chemical modification studies were used to provide evidence consistent with the absence of essential thiol or arginine residues and the presence of multiple essential histidine residues in the enzyme. Whereas iodoacetamide, N,N-dimethylmethamine, and butanedione failed to affect TfdA activity, the addition of diethylpyrocarbonate (DEP), a histidine-selective reagent, led to rapid pseudo-first-order loss of activity. The ability of 2,4-D, α-KG, Fe(II) plus ascorbate and combinations of these substances to protect the enzyme against DEP inactivation was examined. Whereas none of the individual compounds are able to significantly protect the enzyme from inactivation by DEP, the combinations of 2,4-D plus Fe(II) or α-KG with Fe(II) decrease the inactivation rate. Furthermore, the combined presence of 2,4-D and α-KG is very effective in protecting the enzyme from inactivation by DEP.

We interpret results from the above studies in terms of the model illustrated in Figure 3. Consistent with the expected requirements for positive charges at the binding sites of 2,4-D and α-KG, we propose that essential histidine residues are present at the binding sites for each of

Table 1. Purification of 2,4-D/α-ketoglutarate dioxygenase.

| Purification step                        | Total protein, mg | Total activity, U (%) | Activity, U/mg | Purification, -fold |
|-----------------------------------------|-------------------|-----------------------|----------------|-------------------|
| Cell extracts                           | 750               | 1690 (100)            | 2.2            | 1                 |
| (NH₄)₂Fe(SO₄)₂ precipitation            | 225               | 1400 (83)             | 6.2            | 2.8               |
| Monô Q                                  | 58                | 980 (58)              | 16.9           | 7.7               |

Table 2. Phenoxyacetate substrate specificity and kinetic parameters for 2,4-D/α-ketoglutarate dioxygenase.

| Substrate                        | K_m (µM) | k_cat (min⁻¹) | k_cat/K_m (µM⁻¹ min⁻¹) |
|----------------------------------|----------|---------------|------------------------|
| Phenoxyacetate                   | 460      | 443           | 960                    |
| 2-Chlorophenoxyacetate           | 110      | 380           | 3,450                  |
| 4-Chlorophenoxyacetate           | 117      | 595           | 5,090                  |
| 3,5-Dichlorophenoxyacetate       | 102      | 298           | 2,820                  |
| 2,4-Dichlorophenoxyacetic acid   | 17.5     | 529           | 30,200                 |
| 3,4-Dichlorophenoxyacetate       | 219      | 307           | 1,610                  |
| 2,4,5-Trichlorophenoxyacetic acid| 59.6     | 96            | 1,400                  |
| 4-Chloro-2-methylphenoxyacetate   | 89.0     | 233           | 2,620                  |
| α-2-Phenoxypropionate            | 1,170    | 5.1           | 4.4                    |
| 2-(2,4-Dichlorophenoxy)propionate| 191      | 61            | 320                    |
| 3-Phenoxypropionate              | 12,900   | 3.2           | 0.25                   |

*All experiments were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM α-KG, 50 µM ascorbate, and 50 µM (NH₄)₂Fe(SO₄)₂. Modified from Fukumori and Hausinger (7).
Table 3. α-Ketosac substrate specificity and kinetic parameters for 2,4-D/α-ketoglutarate dioxygenase.²

| Substrate              | \(k_w\) (µM) | \(k_{cat}\) (min⁻¹) | \(k_{cat}/k_w\) (min⁻¹ x mM⁻¹) |
|------------------------|--------------|----------------------|---------------------------------|
| α-KG                   | 3.20         | 643                  | 2.0 \times 10⁵                  |
| α-Ketoadipate          | 20.6         | 290                  | 14,100                          |
| Pyruvate               | 1020         | 58                   | 60                              |
| α-Ketobutyrate         | 464          | 89                   | 190                             |
| α-Ketovalerate         | 607          | 404                  | 660                             |
| α-Ketocaprate          | 583          | 158                  | 270                             |
| α-Ketoisovalerate      | 745          | 16                   | 20                              |

² Enzyme reactions were performed at 30°C in 10 mM imidazole buffer (pH 6.75) containing 1 mM 2,4-D, 50 µM ascorbate, and 50 µM \((NH_4)_2Fe(SO_4)_2\). Modified from Fukumori and Hausinger (7).

Figure 3. Model of the TfdA active site. Modified from Fukumori and Hausinger (7).

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