A Three-Component Enzyme System Catalyzes the O Demethylation of the Herbicide Dicamba in *Pseudomonas maltophilia* DI-6†

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An enzyme activity which converts dicamba (2-methoxy-3,6-dichlorobenzoic acid) to 3,6-dichlorosalicylic acid in vitro has been detected in cell lysates of *Pseudomonas maltophilia* DI-6. Phenyl-Sepharose column chromatography of a partially purified lysate resulted in the separation of this enzyme into three separate protein components tentatively identified as an oxygenase, a ferredoxin, and a reductase. The activity of dicamba O-demethylase was dependent on oxygen and required NADH and Mg²⁺.

The chlorinated aromatic compound dicamba (2-methoxy-3,6-dichlorobenzoic acid) (trade name, Banvel) is a herbicide widely used for the control of annual and perennial broadleaf weeds. The fact that dicamba is chemically stable but nonpersistent in soils suggests that it is rapidly metabolized by the soil microflora. One particular soil bacterium, *Pseudomonas maltophilia* DI-6, has been shown to use dicamba as a sole carbon source and to completely degrade the herbicide to H₂O, CO₂, and chloride ion (10). However, the mechanism of dicamba degradation has not been completely elucidated. Studies with whole cells (5, 19) have shown that the first degradation product of dicamba is 3,6-dichlorosalicylic acid (DCSA).

In this report, we demonstrate that cell lysates of *P. maltophilia* DI-6 contain an O-demethylase which rapidly converts dicamba to DCSA. In addition, we show that this enzyme can be separated chromatographically into three interdependent protein components and discuss the initial classification of these components as a reductase, a ferredoxin, and an oxygenase. Finally, we compare the properties of this three-component enzyme system with those of other previously characterized oxygenase systems.

Bacterial strain and growth conditions. *P. maltophilia* DI-6 (11) (provided by Douglas Cork of the Illinois Institute of Technology, Chicago) was maintained on reduced chloride medium (10) supplemented with either dicamba (2 mg/ml) or a mixture of glucose (2 mg/ml) and Casamino Acids (2 mg/ml). The carbon sources were filter sterilized and added to the medium after it was autoclaved. Solid media were prepared by the addition of 1% (wt/vol) Gelrite (Scott Laboratories, West Warwick, R.I.).

Preparation of cell lysates. Cells were grown to an optical density at 550 nm of 1.3 to 1.5 in liquid reduced chloride medium containing a mixture of glucose and Casamino Acids on a rotary shaker (250 rpm at 30°C). The cells were harvested by centrifugation, washed twice with cold 100 mM MgCl₂, and centrifuged again. Cell pastes were either used immediately or quickly frozen in liquid nitrogen and stored at –80°C. At the time of enzyme purification, 25 g of frozen cells was thawed

and resuspended in 50 ml of isolation buffer containing 25 mM Tris buffer (pH 7.0), 10 mM MgCl₂, and 0.5 mM EDTA. Phenylmethylsulfonyl fluoride and dithiothreitol were added to final concentrations of 0.5 and 1 mM, respectively. After addition of 10 mg of lysozyme and 1 mg of DNase, cells were stirred for 10 min on ice and broken by sonication (model XL2020 sonicator; Heat Systems) on ice at a medium setting (setting 5) with 12 20-s bursts and 40-s resting intervals. The resulting cell lysates were diluted to 90 ml with isolation buffer and centrifuged at 56,000 × g for 1 h at 4°C. The supernatant was used as the source of cleared cell lysate.

Dicamba O-demethylase assay. Enzyme activity was assayed by measuring the formation of [¹⁴C]DCSA from [¹⁴C]dicamba (>98% radiochemical purity; provided by Sandoz Agro, Inc.). The standard assay was carried out at 30°C, and the mixture contained, in a total volume of 0.3 ml, 25 mM potassium phosphate buffer (pH 7.0), 10 mM MgCl₂, 0.5 mM NADH, 0.5 mM ferrous sulfate, 52.5 μM dicamba (including 2.5 μM [¹⁴C]dicamba to obtain a final specific activity of 1.9 mCi/mmol), and different amounts of cell lysate or partially purified enzyme. When a single component was assayed, the other two components were provided in excess. Reactions were initiated by the addition of the substrate, dicamba. At specific times, reactions were stopped by adding 50 μl of 5% (vol/vol) H₂SO₄. Dicamba and dicamba metabolites were then extracted twice with 1 volume of ether, and the extracts were evaporated to dryness. The efficiencies of recovery (means ± standard deviations) for the extraction procedure were 87% ± 2% for dicamba and 85% ± 3% for 3,6-DCSA (19).

Dicamba and its degradation products were analyzed by one-dimensional silica gel thin-layer chromatography (TLC). The ether-extracted dicamba and its metabolites were redissolved in 50 μl of ether prior to being spotted onto a TLC plate. The solvent system for running the TLC was chloroform-ethanol-acetic acid (85:10:5 [vol/vol/vol]). Dicamba, 3,6-DCSA, and other metabolites were visualized under UV light. Radioactivity was quantitated by exposing the TLC plate to a phosphor screen for 24 h and then scanning the screen in a PhosphorImager SF (Molecular Dynamics, Sunnyvale, Calif.). Estimates of the amount of radioactivity in a particular spot on the TLC plate were determined by comparing the total pixel count in that spot relative to a spot on the same plate containing a known amount of [¹⁴C]dicamba. One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 nmol of DCSA from dicamba per min at 30°C. Specific

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activities were based on the total protein concentration of the assay mixture.

Partial purification of dicamba *O*-demethylase. All procedures were performed at 4°C unless otherwise stated. Solid ammonium sulfate was slowly added to a 90-ml volume of cleared cell lysate to 40% (wt/vol) saturation, with constant stirring. After 15 min of stirring, the mixtures were centrifuged at $15,400 \times g$ for 15 min, and the precipitate was discarded. Additional solid ammonium sulfate was added, to 70% (wt/vol) saturation, with constant stirring of the supernatant. After 15 min of stirring, the mixtures were centrifuged under the conditions described above. The supernatant was discarded, and the precipitate was resuspended in a minimal volume of buffer A (20 mM Tris [pH 8.0], 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5% [vol/vol] glycerol, and 1 mM dithiothreitol).

The resuspended 40 to 70% ammonium sulfate fraction was loaded onto a phenyl-Sepharose (Pharmacia) column (2.5 by 10 cm) connected to a Pharmacia fast protein liquid chromatography apparatus. The column was preequilibrated with buffer A containing 10% (wt/vol) ammonium sulfate. The flow rate was 1 ml/min. Protein concentrations were continuously monitored at A_{280} during column elution. The column was washed with 120 ml of buffer A containing 10% (wt/vol) ammonium sulfate until baseline A_{280} readings were obtained. Bound proteins were eluted with a decreasing gradient of $(NH_4)_2SO_4$ in buffer A [10 to 0% (wt/vol) $(NH_4)_2SO_4$ in a total volume of 210 ml]. Fractions of 2 ml were collected. Aliquots of 10 μ l were taken from each fraction and added to the standard dicamba *O*-demethylase assay mixture (see above) except that nonradioactive dicamba was used as the substrate. Initial indications of enzyme activity in single fractions or in mixtures of fractions could be rapidly monitored with a hand-held UV lamp (312 nm; Fotodyne) because of the strong fluorescence of DCSA at this wavelength. This procedure, coupled with subsequent standard radioactive assays (see above), allowed resolution of dicamba *O*-demethylase into three pools (designated components I, II, and III) (Fig. 1). Fractions containing a single type of activity were combined (component I, fractions 128 to 145; component II, fractions 12 to 33; and component III, fractions 62 to 92).

Component I, which bound strongest to the phenyl-Sepharose column and eluted at very low salt concentrations, had a reddish brown color. The UV-visible absorption spectrum of this component in its oxidized state showed absorption maxima at 330, 460, and 560 nm (data not shown), which implied that it could be an iron-sulfur protein.

Component II did not bind to the phenyl-Sepharose column and had a yellowish color. *Clostridium pasteurianum* ferredoxin could substitute for component II in reactions with components I and III, but with markedly lower reaction rates. In a set of reactions in which 55 μ g of partially purified component II was mixed with excess amounts of components I and III to catalyze the conversion of dicamba to DCSA at a rate of approximately $5 \text{ nmol min}^{-1} \text{ mg}^{-1}$, substitution of 100 μ g of highly purified ferredoxin from *Clostridium* resulted in an activity of only $0.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$. The *Clostridium* ferredoxin clearly functioned in place of component II, but given the highly impure nature of the component II used in these experiments, the efficiency of the *Clostridium* enzyme was significantly lower than that of the putative ferredoxin from strain DI-6.

Component III, which was eluted from the phenyl-Sepharose column with 4% (wt/vol) ammonium sulfate and which had a light yellow color, could be replaced with 100 μ g of cytochrome *c* reductase from porcine heart (type I; Sigma). In this set of reactions, the use of 50 μ g of partially purified

FIG. 1. Phenyl-Sepharose chromatography of a 40 to 70% (wt/vol) ammonium sulfate fraction of cleared cell lysate from *P. maltophilia* DI-6. The column was eluted with a gradient of decreasing concentrations of ammonium sulfate (---) while the A_{280} (○) was monitored. The activities of each of the three components were measured by combining 10 μ l from each fraction with excess levels of the two other components (partially purified by Mono Q chromatography and stored at -80°C) in a 300- μ l standard [^{14}C]dicamba reaction mixture. The mixtures were incubated at 30°C for 1 h, separated on a TLC plate, and then quantitated on a PhosphorImager. Symbols: Δ , component I; $\circ$, component II; $\blacksquare$, component III.

component III also produced a reaction rate of approximately $5 \text{ nmol min}^{-1} \text{ mg}^{-1}$ when mixed with an excess of components I and II. The highly purified cytochrome *c* reductase showed a specific activity of approximately $2.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$ in the reaction, an activity well below that provided by component III when one considers the impurity of the crude component III used in these assays. In addition, component III exhibited reductase activity when incubated with cytochrome *c* or 2,6-dichlorophenol-indophenol (DCPIP) in the presence of NADH (data not shown). Neither component I nor component II showed activity in either of these two reductase assays.

Oxygen requirement for dicamba *O*-demethylase. Preliminary experiments using a Clark oxygen electrode indicated oxygen consumption during a standard dicamba *O*-demethylase assay with dicamba as a substrate (data not shown). To verify a requirement for oxygen in the *O* demethylation of dicamba by dicamba *O*-demethylase, reactions were conducted in an anaerobic chamber which contained less than 1 ppm of oxygen. The procedures for displacement of oxygen from the reaction mixture were performed at 4°C . Reaction mixtures lacking enzyme were placed in a vial and sealed with a rubber stopper. For displacement of oxygen, the vial was evacuated twice by vacuum and flushed each time with nitrogen. After a third evacuation, the vial was flushed with 90% nitrogen plus 10% hydrogen. The enzyme solution was likewise purged of oxygen (with care taken not to bubble the enzyme solution). Both the reaction mixtures and enzyme solutions were transferred into an anaerobic chamber (95% N_2 -5% H_2 atmosphere). Two hundred forty microliters of cleared cell lysate was injected through the rubber stopper with a microsyringe and gently mixed with 960 μ l of oxygen-free reaction mixture. Reactions were carried out at 30°C .

An examination of the reaction products on TLC plates showed that the rate of [^{14}C]DCSA production from [^{14}C]dicamba under anaerobic conditions was significantly lower than the rate of reactions with the same amount of enzyme under

FIG. 2. Conversion of dicamba to DCSA by dicamba *O*-demethylase in vitro under aerobic and anaerobic conditions at 30°C. Lanes: 1 to 3, enzyme activity under aerobic conditions; 4 to 6, enzyme activity under anaerobic conditions; 7 to 9, enzyme activity after incubation of reaction mixture under anaerobic conditions for 30 min and subsequent removal to aerobic conditions. Activity was assayed by incubating 240 μ l of cleared cell lysate (8 mg of total protein/ml) in a standard [^{14} C]dicamba reaction mixture for the indicated times. The [^{14} C]-labeled components of each reaction mixture were separated on a TLC plate and visualized on a PhosphorImager. D, dicamba; S, DCSA; O, origin.

aerobic conditions (Fig. 2, lanes 4 to 6 versus lanes 1 to 3). Under anaerobic conditions, there was virtually no conversion of dicamba to DCSA within 1 h. However, when a parallel reaction mixture was taken from the anaerobic chamber after 30 min and incubated with air, a significant quantity of dicamba was converted to DCSA within 30 min (Fig. 2, lanes 7 to 9). These experiments demonstrated the strict dependence of the dicamba demethylation reaction on O_2 and confirmed that one of the components of the dicamba *O*-demethylase enzyme complex was an oxygenase.

It may be noted that the in vitro conversion of [^{14} C]dicamba to [^{14} C]DCSA mimics the in vivo conversion pathway documented earlier (5, 19). In these studies, DCSA was identified as a reaction product by both TLC and capillary electrophoresis. Stringent identification of the first major product of dicamba degradation as DCSA both in vivo and in vitro has been obtained by gas chromatography-mass spectrometry analyses (20).

Component and cofactor requirements for dicamba *O*-demethylase. After the initial separation of the three components of dicamba *O*-demethylase by phenyl-Sepharose column chromatography, the partially purified preparations were taken individually through one additional purification on a Q-Sepharose column (2.5 by 6 cm). Samples were applied to a Q-Sepharose (Fast Flow) fast protein liquid chromatography column (Pharmacia) in buffer A and eluted with a 100-ml linear gradient of 0 to 0.6 M KCl (for the oxygenase component) or 0 to 1.0 M KCl (for the ferredoxin and reductase components) in 1.5-ml fractions. Appropriate pooled fractions from separate columns for oxygenase purification (fractions 29 to 37), for ferredoxin purification (fractions 30 to 37), or for reductase purification (fractions 26 to 38) were used for the determination of component and cofactor requirements.

The three components were assayed for dicamba *O*-demethylase activity in various combinations. None of the components showed enzyme activity when assayed alone. Indeed, a significant amount of *O*-demethylase activity could be detected only when all three components were combined. A mixture of components I and II exhibited small amounts of enzyme activity, probably due to traces of component III contaminating the component I fractions.

Table 1 shows the effects of various cofactors on dicamba

O-demethylase activity. Both NADH and NADPH supported enzyme activity, with NADH being markedly more effective than NADPH. Mg^{2+} was necessary for enzyme activity. Fe^{2+} , flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) produced little or no stimulation of enzymatic activity with the partially purified protein preparations in these experiments.

Dicamba *O*-demethylase is similar to other three-component oxygenase systems. The initial characterization presented here suggests that dicamba *O*-demethylase is similar to other non-heme, iron-containing, three-component dioxygenase and monooxygenase systems which are involved in the degradation of natural and xenobiotic compounds (1–3, 6, 9, 14, 16, 17, 21). First, dicamba *O*-demethylase has a stringent oxygen requirement; second, the enzyme complex can be separated into three distinct components with the properties of an oxygenase, a ferredoxin, and a reductase; and third, NADH and Mg^{2+} are two cofactors required for maximal enzymatic activity.

In general, multicomponent enzyme systems contain a flavoprotein which transfers electrons from reduced pyridine nucleotides to a small ferredoxin protein which, in turn, reduces a terminal non-heme, iron-containing oxygenase. A typical short electron transfer chain is as follows:

where Fe-S_{Fd} and Fe-S_{R} represent ferredoxin-type and Rieske-type (2Fe-2S) iron-sulfur clusters, respectively (14).

In most cases, three-component oxygenases are known to use hydrophobic, uncharged substrates, such as naphthalene (6), benzene (1), biphenyl (8), and toluene (21), whereas two-component oxygenases use hydrophilic substrates containing a charged side chain, such as benzoate (18), 4-chlorophenylacetate (13), 2-halobenzoate (7), 4-sulfobenzoate (12), and vanillate (4). The first exception to this rule was (chloro)benzoate 1,2-dioxygenase from *Pseudomonas aeruginosa* 142 (15), which was shown to be a three-component enzyme capable of degrading a hydrophilic substrate. The dicamba *O*-demethylase identified in our studies thus appears to be a second example of a three-component oxygenase capable of degrading a hydrophilic substrate.

TABLE 1. Cofactor requirements for dicamba *O*-demethylase activity

Constituents of assay mixture	Mean enzymatic activity (%) $\pm$ SE ^a
NADH, Fe^{2+} , FAD, Mg^{2+}	99.5 $\pm$ 0.3
Fe^{2+} , FAD, Mg^{2+}	7.4 $\pm$ 1.2
NADH, FAD, Mg^{2+}	86.6 $\pm$ 5.3
NADH, Fe^{2+} , Mg^{2+}	92.2 $\pm$ 6.1
NADPH, Fe^{2+} , FAD, Mg^{2+}	43.5 $\pm$ 3.9
NADH, Fe^{2+} , FMN, Mg^{2+}	94.7 $\pm$ 5.1
NADH, Fe^{2+} , FAD, FMN, Mg^{2+}	89.6 $\pm$ 3.6
NADH, Fe^{2+} , FAD	0.12 $\pm$ 0.03

^a Activity was assayed by standard incubations of a mixture of the three demethylase components after Q-Sepharose chromatography with [^{14} C]dicamba for 30 min at 30°C. The amounts of partially purified protein (provided in an optimized ratio) in the reaction mixtures were 85 μ g of oxygenase, 55 μ g of ferredoxin, and 50 μ g of reductase. The concentrations of cofactors used in the reaction mixtures were 0.5 mM NADH, 0.2 mM FAD, 0.5 mM $FeSO_4$, 10 mM $MgCl_2$, 0.5 mM NADPH, and 0.2 mM FMN. The highest enzyme activity in these cofactor experiments was 5.5 nmol min⁻¹ mg⁻¹ (measured with a combination of NADH, Fe^{2+} , FAD, and Mg^{2+}). Each value is the average from two independent experiments.

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