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A three-component dicamba *O*-demethylase from *Pseudomonas maltophilia*, strain DI-6: Purification and characterization ☆

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Abstract

Dicamba *O*-demethylase is a multicomponent enzyme that catalyzes the conversion of the herbicide 2-methoxy-3,6-dichlorobenzoic acid (dicamba) to 3,6-dichlorosalicylic acid (DCSA). The three components of the enzyme were purified and characterized. Oxygenase$_{DIC}$ is a homotrimer $(\alpha)_3$ with a subunit molecular mass of approximately 40 kDa. Ferredoxin$_{DIC}$ and reductase$_{DIC}$ are monomers with molecular weights of approximately 14 and 45 kDa, respectively. EPR spectroscopic analysis suggested the presence of a single $[2Fe–2S]^{(2+/1+)}$ cluster in ferredoxin$_{DIC}$ and a single Rieske $[2Fe–2S]^{(2+;\,1+)}$ cluster within oxygenase$_{DIC}$. Consistent with the presence of a Rieske iron–sulfur cluster, oxygenase$_{DIC}$ displayed a high reduction potential of $E_{m,7.0} = -21$ mV whereas ferredoxin$_{DIC}$ exhibited a reduction potential of approximately $E_{m,7.0} = -171$ mV. Optimal oxygenase$_{DIC}$ activity in vitro depended on the addition of Fe^{2+}. The identification of formaldehyde and DCSA as reaction products demonstrated that dicamba *O*-demethylase acts as a monooxygenase. Taken together, these data suggest that oxygenase$_{DIC}$ is an important new member of the Rieske non-heme iron family of oxygenases.

Keywords: Dicamba; *O*-Demethylase; Oxygenase; Ferredoxin; Reductase; Multicomponent enzyme; *Pseudomonas maltophilia*; Rieske non-heme oxygenase

The biodegradation of aromatic compounds in soil and groundwater is frequently a consequence of microbial activity [1]. Indeed, such activity is essential in preventing the build up of many pesticides in the environment and useful in the bioremediation of harmful pollutants [2]. Consequently, there has been an intensive effort to understand the structure and function of the enzymes that catalyze the degradation of several xenobiotic aromatic compounds [3]. Nonetheless, several chemicals used in large quantities over several years as pesticides in agricultural settings have received minimal attention. 2-methoxy-3,6-dichlorobenzoic acid (dicamba)[4] represents one such molecule. First introduced

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[4] *Abbreviations used:* Dicamba, 2-methoxy-3,6-dichlorobenzoic acid; DCSA, 3,6-dichlorosalicylic acid; TLC, thin layer chromatography; RCL, reduced-chloride media; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; IEF, isoelectric focusing.

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commercially in 1965, this herbicide has been used extensively to control broadleaf weeds in crops such as corn and wheat. Field studies have shown microbial degradation of dicamba under both aerobic and anaerobic conditions. However, the only evaluation of the metabolism and enzymatic degradation of dicamba has been limited to a single bacterium, *Pseudomonas maltophilia*, strain DI-6 [4–6]. This soil bacterium can completely mineralize dicamba to CO_2, H_2O, and Cl^- and utilize dicamba as a sole carbon and energy source [4]. Initial identification of 3,6-dichlorosalicylic acid (DCSA) as the first breakdown product of dicamba by strain DI-6 [4] has been confirmed by both in vivo and in vitro analyses [5]. Nonetheless, only preliminary studies of the enzyme responsible for this first-step reaction, dicamba *O*-demethylase, have been conducted [6].

In the present study we describe the purification of dicamba *O*-demethylase and its biochemical and physical characterization. In so doing, we provide corroborating evidence that dicamba *O*-demethylase activity requires three separate components (reductase$_{DIC}$, ferredoxin$_{DIC}$, oxygenase$_{DIC}$) and that these components provide for efficient conversion of dicamba to 3,6-dichlorosalicylic acid. Our demonstration that the enzyme is a monooxygenase that requires Fe(II) for full activity and that the homotrimeric $(\alpha)_3$ oxygenase component contains Rieske [2Fe–2S] clusters lead us to suggest that oxygenase$_{DIC}$ is a member of the large and diverse family of Rieske non-heme iron oxygenases [2].

Methods and materials

Chemicals

Dicamba, DCSA, and [^{14}C]dicamba were supplied by Sandoz Agro (Des Plaines, IL). Chemicals were purchased from Sigma Chemical (St. Louis, MO). All columns and packing materials were purchased from Pharmacia. HPLC column and accessories were purchased from Alltech. Thin layer chromatography (TLC) plates were made of silica gel containing a UV 254 indicator and were purchased from J.T. Baker Chemical.

Bacterial strain and culture conditions

Pseudomonas maltophilia, strain DI-6, was grown in reduced-chloride media (RCL) [4] with glucose (2 mg/ml) and casamino acids (2 mg/ml) as the carbon sources. Cells were grown to an OD_{600} ranging from 1.5 to 2.0 by incubation at 30 °C on an orbital shaker at 225 rpm for 24–48 h. For enzyme purification, 6 liters of cells were harvested by centrifugation in a Beckman Avanti J-25I centrifuge at 4000*g* for 20 min. Pelleted cells were stored at −80 °C.

Preparation of cleared cell lysates

Frozen pelleted cells (~25.0 g wet weight) were suspended in 40 ml of 100 mM $MgCl_2$, centrifuged, and resuspended in 50 ml of sonication buffer (100 mM Mops (pH 7.2), 1 mM dithiothreitol, and 5% glycerol). Lysozyme (0.4% final conc.), Protease Inhibitor Cocktail for Bacterial extracts (Sigma; P 8465; 5 ml/20 g of cells), and phenylmethylsulfonyl fluoride (PMSF; 0.5 mM final conc.) were added in succession. Cells on ice were subjected to sonication with a Sonics & Materials sonicator, Model VCX 600, at an amplitude of 50% in 9.0 s bursts with 3.0 s resting periods for 30 min. Lysed cells were centrifuged at 56,000*g* for 75 min at 4 °C. The supernatant (cleared cell lysate) was decanted and glycerol was added to a final concentration of 15% prior to storage at −80 °C.

Enzyme purification

All purification steps were carried out at 4 °C. After each step, active fractions were determined by a rapid enzyme assay [6] described below. After each purification step, protein concentrations in the fractions were determined by the Bradford method using reagents from Bio-Rad and bovine serum albumin as the protein standard. Enzymes were stored at −80 °C for future characterizations. Modifications of a protein purification scheme described by Small and Ensign [7] were employed for purification of the three components of dicamba *O*-demethylase.

Fractionation of dicamba O-demethylase components

Crude cell extract (~2000 mg of protein) was loaded on a 26 × 200 mm DEAE–Sepharose Fast Flow column (Pharmacia XK; 25 ml bed volume) using a BioCAD Perfusion Chromatography Workstation. The column was equilibrated with 50 mM Mops (pH 7.2), 1 mM dithiothreitol, and 15% (v/v) glycerol (buffer A) and run at a flow rate of 5.0 ml/min. After the column was loaded, it was washed with buffer A until the 280 nm reading was <0.1 A. All three components were bound to the DEAE column under these conditions. The column was developed with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing ferredoxin$_{DIC}$ eluted at 400 mM NaCl and the reductase$_{DIC}$ and oxygenase$_{DIC}$ components co-eluted at 250 mM NaCl.

Purification of ferredoxin$_{DIC}$

DEAE–Sepharose column fractions containing ferredoxin$_{DIC}$ were pooled, buffer exchanged into 50 mM Mops (pH 7.2), 5% glycerol (v/v), and 200 mM NaCl (buffer B), and concentrated to approximately 2 ml using an Amicon Cell Concentrator with a YM10 membrane

and a Centricon 10 concentrator. This sample was then loaded onto a pre-packed 26 × 600 mm Sephacryl S-100 (Pharmacia Hi Prep) column at a flow rate of 0.5 ml/min using fast protein liquid chromatography (FPLC). Active fractions were pooled, buffer exchanged into 50 mM Mops (pH 7.2), 1 mM dithiothreitol, and 5% glycerol (v/v) (buffer C), and concentrated to ~2 ml. The partially purified protein was taken through a final Mono-Q (Pharmacia HR) 5 × 50 mm column equilibrated in buffer C. The column was eluted with a linear gradient ranging from 0 to 2.0 M NaCl in buffer C. Before storage at −80 °C, fractions containing peak ferredoxin activity were analyzed for protein concentration and relative purity by electrophoresis on 1-D SDS–PAGE gels and subsequent staining with Coomassie brilliant blue R250.

Purification of oxygenase$_{DIC}$

DEAE column fractions containing a combination of the oxygenase and reductase components were pooled and ammonium sulfate was added to a final concentration of 1.5 M. After incubating at 4 °C for 1.5 h the samples were centrifuged for 75 min at 56,000*g* at 4 °C. The supernatant was retained and loaded at a flow rate of 5.0 ml/min onto a 26 × 200 mm Phenyl-Sepharose 6 Fast Flow column (25 ml bed volume, Pharmacia XT) equilibrated in buffer A containing 1.5 M $(NH_4)_2SO_4$. Proteins were eluted with a decreasing gradient of $(NH_4)_2SO_4$ and fractions containing oxygenase activity were pooled, buffer exchanged into buffer B, and concentrated to approximately 2 ml (Amicon concentrator with a YM30 membrane and a Centricon 10 concentrator). The 2 ml sample was loaded onto a pre-packed 26 × 600 mm Sephacryl S-100 (Pharmacia Hi Prep) column equilibrated in buffer B and run at 0.5 ml/min. Fractions that showed oxygenase activity were analyzed for protein concentration and stored at −80 °C.

Purification of reductase$_{DIC}$

Phenyl Sepharose column fractions (see purification of oxygenase$_{DIC}$) that contained the reductase component were pooled, buffer exchanged into buffer B, and concentrated to approximately 2 ml. The 2 ml sample was loaded onto a pre-packed 26 × 600 mm column of Sephacryl S-100 (Pharmacia Hi Prep) equilibrated in buffer B and run at 0.5 ml/min. Fractions containing reductase activity were pooled, buffer exchanged into buffer C, and concentrated to approximately 2 ml. The protein sample was loaded onto a pre-packed Mono-Q HR 5 × 50 mm column previously equilibrated in buffer C. Bound protein was eluted with a linear gradient of 0–2.0 M NaCl in buffer C at a flow rate of 0.5 ml/min. Fractions that showed reductase activity were analyzed and stored at −80 °C.

Assay of dicamba O-demethylase activity

Dicamba *O*-demethylase activity was assayed using three different methods. The rapid enzyme assay was used to monitor dicamba *O*-demethylase activity in various fractions during purification of the three components and was performed as described earlier [5,6]. In this assay, the activity of dicamba *O*-demethylase was detected by visual observation under UV light of fluorescence from the reaction product, DCSA. Experiments evaluating the requirement of dicamba *O*-demethylase activity for Fe^{2+} were performed as time course assays in which $FeSO_4$ was omitted or added to provide a final concentration of 0.5 mM. In these assays, [^{14}C]dicamba and its degradation products (primarily DCSA) at each time point were extracted and separated by thin layer chromatography as previously described [6]. Radioactivity in dicamba and in the degradation products was measured using a Molecular Dynamics PhosphorImager. The third type of assay utilized product identification by HPLC. Reactions were performed at 30 °C for 10 min in a total volume of 500 µl. Assays were initiated by addition of dicamba after a 5 min preincubation at 30 °C. The reaction was terminated by the addition of 80 µl of 5% sulfuric acid. Samples were centrifuged, filtered, and 250 µl of each sample was then injected into a C-18 reverse-phase micro-bondapack 4.6 × 150 mm column. The product mixture was separated using a linear gradient of 60–0% methanol in 40 mM Tris acetate (pH 7.2) using a Waters HPLC unit. DCSA retention time was determined to be 14.1 min (established using 250 µl of 500 mM DCSA as a standard). Set concentrations of DCSA were used as quantitation standards. For kinetic studies, DCSA in reaction samples was detected and quantified by fluorescence emission at 420 nm (excitation wavelength, 310 nm) after separation from other reaction products by reverse-phase high-performance liquid chromatography (HPLC). Enzymatic activity in fractions containing the particular component being purified was assayed in the presence of excess quantities of the other two components of dicamba *O*-demethylase, 25 mM potassium phosphate buffer (pH 7.2), 0.5 mM NADH, 10 mM magnesium chloride, 0.5 mM ferrous sulfate, and 0.5 mM dicamba.

Characterization of protein components

The molecular weights of purified proteins were estimated using electrophoresis on a 12.5% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) by the method of Laemmli [8]. The functional molecular mass of the native oxygenase$_{DIC}$ component was determined by gel filtration chromatography. Purified enzyme was loaded on a Superdex 200 HR 10/30 column (Amersham Biosciences) and separated by FPLC in 50 mM $NaPO_4$, pH 7.0, containing 150 mM NaCl, at a flow

rate of 0.25 ml/min. Size determination was made by comparison to molecular weight standards (Amersham Biosciences) separated by chromatography under the same conditions. The molecular weight standards used were as follows: thyroglobulin, 699 kDa; ferritin, 416 kDa; catalase, 219 kDa; aldolase, 176 kDa; albumin, 67 kDa; ovalbumin, 47 kDa; chymotrypsinogen A, 20 kDa; and RNase A, 15 kDa. Resolution was sufficient to calculate a molecular mass of 128 kDa for oxygenase$_{DIC}$.

Isoelectric points were determined using isoelectric focusing (IEF) gels (pH 3–10; Bio-Rad). The anode and cathode buffers were 0.1 M β-alanine and 0.1 M acetic acid, respectively. Gels were run for 2 h at a constant voltage of 200 V and then for an additional 2 h at 400 V. The N-terminal amino acid sequence of purified components separated from contaminant proteins by SDS–PAGE was determined by automated microscale Edman degradation of protein blotted onto a polyvinylidene fluoride membrane.

UV–Vis spectroscopy of ferredoxin$_{DIC}$ and oxygenase$_{DIC}$

All UV–visible spectra were obtained under anaerobic conditions at room temperature using a Cary Varian Model 100 Bio UV spectrophotometer. Ferredoxin$_{DIC}$ and oxygenase$_{DIC}$ samples were prepared in 50 mM Mops buffer, pH 7.2, under a N_2 atmosphere. The spectrum of each oxidized component was recorded for the protein as isolated and the spectrum of the reduced component was obtained after incubation with 1 mM sodium dithionite.

Electron paramagnetic resonance spectroscopy

For EPR spectroscopy the purified components of the dicamba *O*-demethylase system (reductase$_{DIC}$, ferredoxin$_{DIC}$, and oxygenase$_{DIC}$) were concentrated over a microcon YM10 ultra membrane (Amicon, Beverly, MA) to a protein concentration suitable for EPR studies. X-band electron paramagnetic resonance spectroscopy was performed on a Varian E-9 spectrometer.

Redox titration

To investigate the redox properties of the iron–sulfur clusters of *P. maltophilia* oxygenase$_{DIC}$ and ferredoxin$_{DIC}$ the two components were titrated in concert in the presence of redox mediators. Mediated redox titrations versus NHE were performed as described previously [9], using sodium dithionite and potassium ferricyanide as reductant and oxidant, respectively. The proteins were reduced or oxidized in a stepwise manner and the respective EPR spectra were recorded at redox equilibrium. Titrations were performed at room temperature in 0.1 M Hepes buffer, pH 7.0.

Identification of reaction products

Products of an in vitro assay using lysates of strain DI-6 and [^{14}C]dicamba were analyzed by TLC [6] and fluorometry [10]. At each time point, equal aliquots were removed from the reaction mixture. One aliquot was subjected to acidified ether extraction and subsequent TLC for detection of radioactively labeled dicamba and DCSA. The amount of dicamba converted to DCSA was determined using a PhosphorImager as described above. The other aliquot was mixed with two volumes of Nash reagent and immediately incubated at 60 °C for 10 min [11]. The reaction mixture was centrifuged at full speed in an Eppendorf microfuge for 30 s and the supernatant was transferred to a quartz cuvette. Fluorescence was measured (excitation = 412 nm, emission = 505 nm) using a Perkin-Elmer LS-50 fluorometer. A standard curve derived from reactions with various concentrations of reagent-grade formaldehyde was used to calculate the amount of formaldehyde formed in reactions containing dicamba as the substrate. All measurements were performed in triplicate with appropriate controls and blanks included.

Results

A purification scheme for dicamba *O*-demethylase significantly different from the one previously employed [6] resulted in the isolation of three separate protein fractions necessary for enzymatic activity. As such, these observations provided additional evidence of the requirement for at least three separate and freely dissociable components for dicamba *O*-demethylase activity (Table 1). Tentative designation of these components [6] as an oxygenase (oxygenase$_{DIC}$), a ferredoxin (ferredoxin$_{DIC}$), and a reductase (reductase$_{DIC}$) was retained in naming components isolated in the present study. The procedure employed resulted in preparations of nearly homogeneous ferredoxin$_{DIC}$ and purified oxygenase$_{DIC}$ and reductase$_{DIC}$ (Fig. 1). The physical and biochemical properties determined for each purified component are described separately below.

Ferredoxin$_{DIC}$

Ferredoxin$_{DIC}$ purified to homogeneity was brownish in color. The final purification step yielded 1.5 mg of pure protein (Table 1), which represents the lowest recovery among the three protein components. The molecular weight and p*I* of the single polypeptide were determined to be approximately 14 kDa (Fig. 1) and 4.0 (data not shown), respectively. The N-terminal amino acid sequence of the purified protein was: MPQITVVN QSGEESSVEASEGRTLME.

Table 1
Purification of the three components of dicamba *O*-demethylase

Step	Volume (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield	Fold purification
Reductase						
Cell lysate	70	1855	7606	4.1	100	1
DEAE Sepharose	95	1358	6695	4.93	88	1.2
Phenyl Sepharose	2	106	2618	24.7	34	6
S-100	2	5.2	432	83	6	20
Mono-Q	5	1.8	243	135	3	33
Ferredoxin						
Cell lysate	70	1855	2968	1.6	100	1
DEAE Sepharose	2	17	651	38.3	22	24
s-100	2	3.9	406	104	14	65
Mono-Q	7	1.5	315	210	7	131
Oxygenase						
Cell lysate	70	1855	5380	2.9	100	1
DEAE Sepharose	95	1358	4753	3.5	88	1.2
Phenyl Sepharose	2	80.3	2610	32.5	49	11
S-100	19	37	2257	61	42	21

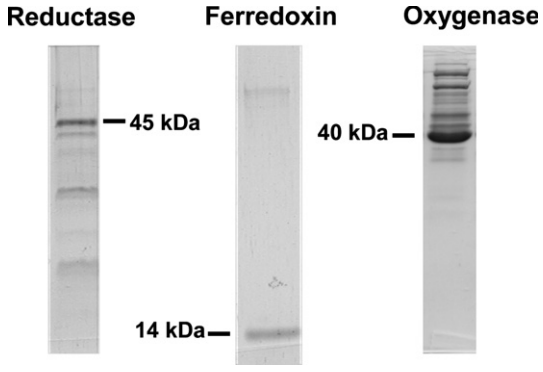

Fig. 1. Purified components of dicamba *O*-demethylase analyzed by SDS–polyacrylamide gel electrophoresis and visualized by staining with Coomassie brilliant blue R-250.

The UV–visible absorbance spectrum of oxidized ferredoxin$_{DIC}$ is presented in Fig. 2B. The absorbance spectrum of the oxidized enzyme (i.e., the enzyme as isolated) displayed absorbance maxima at 315, 416, and 457 nm. On reduction with sodium dithionite, the enzyme appeared to be slightly bleached and showed a small, new absorbance peak at 546 nm. The broad absorbance peak at 326 nm was the result of sodium dithionite added as a reductant. As-isolated ferredoxin$_{DIC}$ (2.2 mg/ml) was EPR silent. Adding 1 mM sodium dithionite resulted in the appearance of an axial $S = 1/2$ EPR spectrum with effective g-values of 2.017 and 1.933 (Fig. 3A, lower spectrum). Its g_{ave} (1.961) and relaxation behavior (Fig. 3B) are indicative of adrenodoxin and putidaredoxin [2Fe–2S] clusters [12,13].

Oxygenase$_{DIC}$

Active fractions eluting from the final sizing column were reddish-brown in color and suggested that this component might contain one or more iron–sulfur clusters or heme groups. The mobility of the purified protein on SDS–PAGE suggested an apparent molecular mass of 40 kDa (Fig. 1) while elution from a gel filtration column indicated a molecular mass for the native protein of approximately 128 kDa (data not shown). The isoelectric point was estimated by IEF to be 5.0 (data not shown). Results of amino acid sequencing indicated that the first 27 amino acids of the N-terminal region were: TFVRNAWYVAALPEELSEKPLGRTILD.

Results of kinetic studies with oxygenase$_{DIC}$ were used to compute the K_m for dicamba as 8 μM and the V_{max} as 150 nmol/min/mg. The UV–visible spectrum of oxidized oxygenase$_{DIC}$ is presented in Fig. 2A. The oxidized protein (as isolated) had absorbance maxima at 327, 462, and 572 nm, whereas the reduced protein showed peaks at 328 and 520 nm. As with the spectrum of reduced ferredoxin$_{DIC}$, the absorption at 328 nm can be ascribed to sodium dithionite. The EPR spectrum of the as-isolated oxygenase$_{DIC}$ (24 mg/ml) exhibited a weak, near-isotropic low-field signal at $g \approx 4.3$, characteristic for high-spin, rhombic ferric iron ($S = 5/2$) (not shown). Upon adding 1 mM ferricyanide this signal did not increase in intensity. Upon reduction with 1 mM dithionite, the signal disappeared, and another rhombic signal was observed (Fig. 3A, upper spectrum). The g values of this $S = 1/2$ spectrum ($g_1 = 2.008$, $g_2 = 1.911$, $g_3 = 1.780$, $g_{ave} = 1.899$) and its power saturation behavior (Fig. 3B) identify it to originate from a Rieske [2Fe–2S] cluster [14]. Upon adding 10 mM dicamba (added as a solution in 50% methanol) no change in the EPR spectrum was observed.

Reductase$_{DIC}$

The purified reductase$_{DIC}$ component was yellowish in color and exhibited fluorescence under UV light, suggesting that the enzyme might use a flavin as a cofactor.

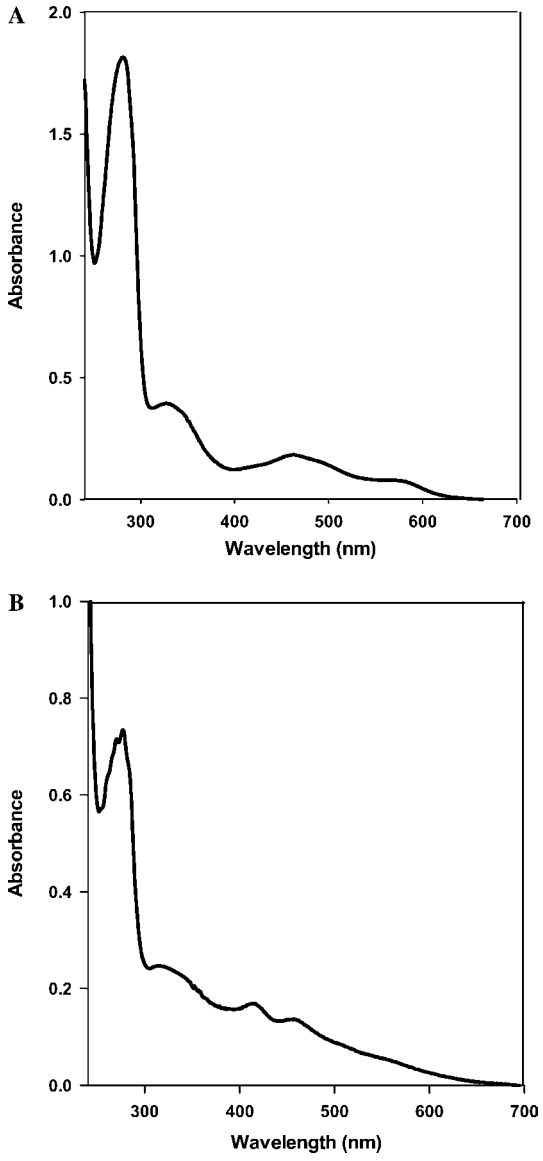

Fig. 2. UV–visible absorption spectra of oxygenase$_{DIC}$ (A) and ferredoxin$_{DIC}$ (B) in the oxidized state as isolated.

The single polypeptide of approximately 45 kDa had an isoelectric point of 4.5. The N-terminal amino acid sequence of the first 23 amino acids was deduced to be: SKADVVIVGAGHGGAQ(C)AIALQN. As-isolated reductase$_{DIC}$ was EPR silent. Attempts to stabilize the flavin in the semiquinone radical state by adding 1 mM ferricyanide, 1 mM sodium dithionite, or 0.1 mM NADH failed. No Fe/S EPR signals were detected in either as-isolated or dithionite-reduced reductase$_{DIC}$ suggesting the absence of a Fe/S ligation motif (or less likely, its loss during purification).

Redox titration

For the oxygenase$_{DIC}$ the derivative signal at $g = 1.911$ and for the ferredoxin$_{DIC}$ the derivative signal at $g = 1.933$ were monitored during redox titration. Fig. 3C shows EPR intensities of the oxygenase$_{DIC}$ [2Fe–2S] and ferredoxin$_{DIC}$ [2Fe–2S] plotted versus the chemically applied redox potential. Fitting the two redox titration curves to a Nernst equation assuming one-electron transitions resulted in a reduction potential $E_{m,7.0} = -21$ mV (versus NHE) for the oxygenase$_{DIC}$ Rieske [2Fe–2S] cluster, and a reduction potential $E_{m,7.0} = -171$ mV for the ferredoxin$_{DIC}$ [2Fe–2S] cluster.

Reaction requirements and characteristics

In experiments in which $FeSO_4$ was omitted from the reaction mixture, the rate of disappearance of dicamba was significantly slower than the rate observed when Fe^{2+} was added (Fig. 4). The initial rate of dicamba demethylation was approximately six times faster in the presence of exogenously added iron (II) than in the reaction deficient in iron (II). In addition to the marked stimulation of enzyme activity by exogenous Fe^{2+} demonstrated here, earlier investigations established requirements for O_2, Mg^{2+}, and NADH (in preference to NADPH) [6]. The dicamba *O*-demethylase reaction proceeded optimally at 30 °C and at pH values between 6.5 and 7.5 (data not shown). Characterization of reaction products of dicamba *O*-demethylase using ^{14}C-labeled dicamba as substrate demonstrated nearly equal quantities of 3,6-dichlorosalicylic acid and formaldehyde produced during the course of the reaction (Table 2). This result strongly suggests that dicamba *O*-demethylase functions as a monooxygenase in converting dicamba to DCSA and formaldehyde.

Discussion

The first step in the mineralization of dicamba to CO_2, H_2O, and Cl^- by *P. maltophilia*, strain DI-6 [4] has been proposed to be catalyzed by a three-component enzyme, dicamba *O*-demethylase [6], that converts dicamba to 3,6-dichlorosalicylic acid (DCSA). Development of a new purification scheme for each of the components has allowed isolation of purified preparations of each component and their initial biochemical characterization.

The Rieske non-heme iron oxygenases are multicomponent enzyme systems that promote the bacterial degradation of aromatic compounds by catalyzing the incorporation of oxygen into organic substrates [3,15]. The minimal configuration of such a ring-hydroxylating system consists of a flavin-containing reductase that accepts reducing equivalents from NADH, and a terminal oxygenase containing a Rieske [2Fe–2S] cluster plus a non-heme mononuclear iron center [16,17]. At least one [2Fe–2S] cluster is also involved; this cluster is either located on a separate ferredoxin-type protein, or it is a

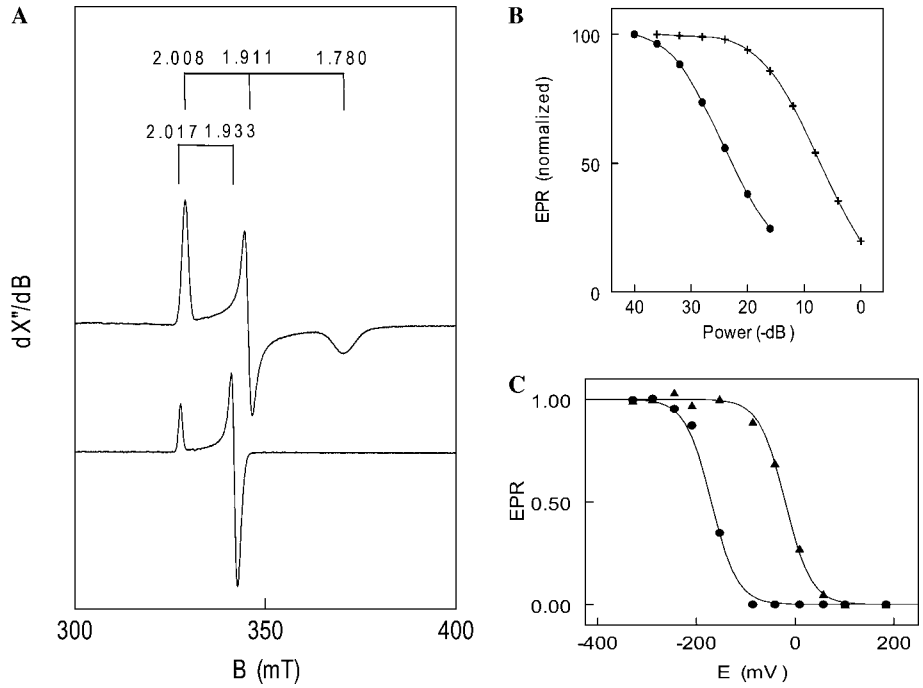

Fig. 3. EPR-based analysis of oxygenase$_{DIC}$ and ferredoxin$_{DIC}$. (A) EPR spectrum of reduced oxygenase$_{DIC}$ (upper spectrum) and reduced ferredoxin$_{DIC}$ (lower spectrum). Microwave power, 0.8 mW (oxygenase$_{DIC}$) or 0.05 mW (ferredoxin$_{DIC}$); modulation amplitude, 0.25 mT; modulation frequency, 100 kHz; temperature, 21 K; and frequency, 9224 MHz. (B) Power EPR saturation curve. Ferredoxin [2Fe–2S] (solid circles) and oxygenase Rieske [2Fe–2S] (crosses). (C) Mediated EPR redox titration (pH 7) of ferredoxin$_{DIC}$ (circles) and oxygenase$_{DIC}$ (triangles). *E* (mv), redox potential in millivolts; EPR, fraction of EPR signal strength at $g = 1.911$ (oxygenase$_{DIC}$) and $g = 1.933$ (ferredoxin$_{DIC}$). EPR conditions: microwave power, 2 mW; modulation amplitude, 0.32 mT; modulation frequency, 100 kHz; temperature, 22 K; and frequency, 9224 MHz.

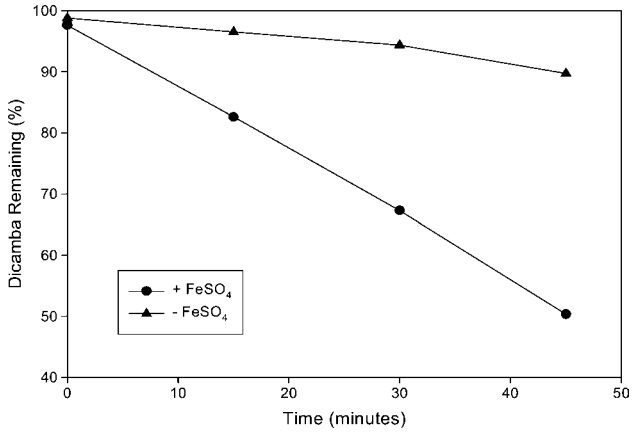

Fig. 4. Kinetics of degradation of [^{14}C]dicamba by dicamba *O*-demethylase in the presence (circles) and absence (triangles) of $FeSO_4$.

prosthetic group of the reductase. Rieske [2Fe–2S] clusters differ from classical ferredoxin (or plant type) [2Fe–2S] clusters in that they exhibit a higher reduction potential −100 to +250 mV [17,18]. The presence of the high-potential Rieske [2Fe–2S] cluster ($E_{m,7.0} = -21$ mV) in the oxygenase$_{DIC}$ component and the low-potential [2Fe–2S] cluster ($E_{m,7.0} = -171$ mV) in the ferredoxin$_{DIC}$ component (Fig. 3C) is congruent with the difference in electric potential along the reaction axis, where electrons are shuttled from the low-potential donor NADH to O_2 (Fig. 5).

Table 2
Analyses of the reaction products of dicamba *O*-demethylase using dicamba as substrate

Time of incubation (min)	μmoles formaldehyde	μmoles DCSA
60	6.7 ± 0.3	5.7 ± 0.2
120	10.0 ± 0.2	9.3 ± 0.8

As-isolated, *P. maltophilia* oxygenase$_{DIC}$ exhibited a $g = 4.3$ high-spin ferric iron EPR signal. Assuming a rhombicity *E*/*D* of 0.33 the spin concentration of this signal was estimated to be 8% relative to the Rieske [2Fe–2S] signal concentration. Attempts to increase the spin intensity of the $g = 4.3$ signal by adding ferricyanide failed. We surmise that the ferric iron atom that afforded this signal could be the oxidized form of the active-site ferrous iron. Alternatively, the spectrum may be generated by adventitiously bound iron to the protein. This would be analogous to 4-methoxybenzoate monooxygenase from *Pseudomonas putida*, which is an *O*-demethylase. In as-isolated 4-methoxybenzoate monooxygenase high-spin, rhombic iron (III) EPR signals were observed with *g* values between 4.3 and 9.6 [19]. These signals were assigned to two species of ferric iron with different rhombicities. Adding substrate reduced only one of the species while the other species remained unaffected. However, although adding sodium dithionite to *P. maltophilia* oxygenase$_{DIC}$ resulted in disappearance of the ferric iron EPR signals, adding the substrate

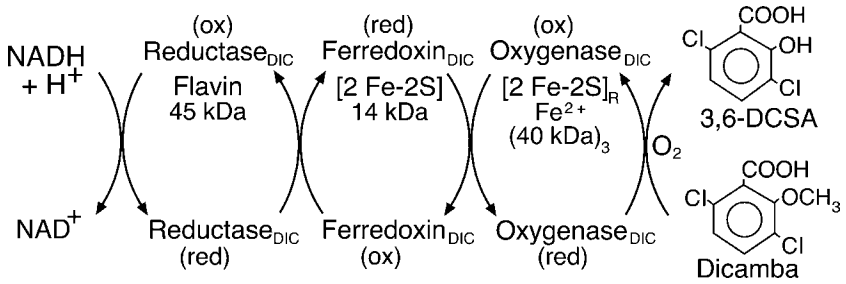

Fig. 5. Proposed scheme for the enzymatic conversion of dicamba to 3,6-dichlorosalicylic acid by *P. maltophilia*, strain DI-6.

dicamba did not affect the EPR spectrum of as-isolated oxygenase$_{DIC}$. These observations suggest that the $g = 4.3$ signal observed in oxidized oxygenase$_{DIC}$ is probably from non-specifically bound iron and that active-site iron may be always in the ferrous state, and cannot be oxidized by air or by ferricyanide. Alternatively, the as-isolated oxygenase$_{DIC}$ may be largely devoid of active-site mononuclear iron. This possibility is strengthened by the observation that adding iron (II) to the assay mixture stimulates the rate of enzymatic activity by approximately sixfold (Fig. 4). Such stimulation of oxygenase activity by added Fe^{2+} has been observed with several Rieske non-heme iron oxygenases including toluene dioxygenase [20], 4-chlorophenylacetate dioxygenase [21], phthalate dioxygenase [22], and naphthalene dioxygenase [23].

Because monoiron(II) centers are EPR silent, data presented here cannot identify the ligation motif of the active-site iron atom of oxygenase$_{DIC}$. In many other non-heme iron oxygen activating enzymes the active-site monoiron(II) center is ligated by a so-called 2-His-1-carboxylate facial triad [24–28]. This motif, which has been identified in extradiol cleaving catechol dioxygenases and in *cis*-hydroxylating Rieske dioxygenases, ligates a single iron atom, which in the case of naphthalene dioxygenase has been shown to be the site for the activation of dioxygen or substrate [17,24,26,27]. Our present data demonstrate dicamba *O*-demethylase is a monooxygenase (Table 2) that shares many features with other dioxygenase and monooxygenase systems that contain a terminal Rieske non-heme iron oxygenase [2].

In the classification scheme of Gibson and Parales [2], dicamba *O*-demethylase most closely aligns with those members of the phthalate family of Rieske non-heme iron oxygenases that function as monooxygenases. This designation receives additional support from the amino acid sequence data for the three components of dicamba *O*-demethylase that are presented elsewhere [29]. The sequence of oxygenase$_{DIC}$ contains a conserved domain with two His and two Cys ligands that are indicative of a Rieske iron–sulfur cluster [29]. The amino acid sequence of reductase$_{DIC}$ contains a conserved region that specifies a binding domain for FAD [29]. The sequence of ferredoxin$_{DIC}$ has a conserved domain typical of the [2Fe–2S] cluster found in the adrenodoxin family [29]. Finally, enzymatic assays with recombinant oxygenase in the presence of recombinant ferredoxin and recombinant reductase were fully active [29]. This last observation, coupled with the biochemical analyses described in the current study, strongly suggests that oxygenase$_{DIC}$ is a homotrimer $(\alpha)_3$ with no β subunit.

The herbicidal activity of dicamba is eliminated when it is converted to 3,6-dichlorosalicylic acid by dicamba *O*-demethylase. In a report to be published elsewhere, this property has recently been utilized to develop transgenic plants that are tolerant to dicamba levels that are 10 to 20 times higher than the typical field application rate. The dicotyledonous plants contain a genetically engineered version of the oxygenase$_{DIC}$ gene that allows them to withstand treatment with normally toxic levels of dicamba. These results suggest that the herbicide dicamba may potentially be used for more economic and efficient weed control in agronomic settings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2005.02.024.

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