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Purification and Sequence Analysis of 4-Methyl-5-Nitrocatechol Oxygenase from *Burkholderia* sp. Strain DNT

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4-Methyl-5-nitrocatechol (MNC) is an intermediate in the degradation of 2,4-dinitrotoluene by *Burkholderia* sp. strain DNT. In the presence of NADPH and oxygen, MNC monooxygenase catalyzes the removal of the nitro group from MNC to form 2-hydroxy-5-methylquinone. The gene (*dntB*) encoding MNC monooxygenase has been previously cloned and characterized. In order to examine the properties of MNC monooxygenase and to compare it with other enzymes, we sequenced the gene encoding the MNC monooxygenase and purified the enzyme from strain DNT. *dntB* was localized within a 2.2-kb *Apa*I DNA fragment. Sequence analysis of this fragment revealed an open reading frame of 1,644 bp with an N-terminal amino acid sequence identical to that of purified MNC monooxygenase from strain DNT. Comparison of the derived amino acid sequences with those of other genes showed that DntB contains the highly conserved ADP and flavin adenine dinucleotide (FAD) binding motifs characteristic of flavoprotein hydroxylases. MNC monooxygenase was purified to homogeneity from strain DNT by anion exchange and gel filtration chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single protein with a molecular weight of 60,200, which is consistent with the size determined from the gene sequence. The native molecular weight determined by gel filtration was 65,000, which indicates that the native enzyme is a monomer. It used either NADH or NADPH as electron donors, and NADPH was the preferred cofactor. The purified enzyme contained 1 mol of FAD per mol of protein, which is also consistent with the detection of an FAD binding motif in the amino acid sequence of DntB. MNC monooxygenase has a narrow substrate specificity. MNC and 4-nitrocatechol are good substrates whereas 3-methyl-4-nitrophenol, 3-methyl-4-nitrocatechol, 4-nitrophenol, 3-nitrophenol, and 4-chlorocatechol were not. These studies suggest that MNC monooxygenase is a flavoprotein that shares some properties with previously studied nitrophenol oxygenases.

Microorganisms can use either monooxygenase (33, 34, 36, 49) or dioxygenase (2, 15, 21, 25, 33, 37, 40) enzymes to catalyze the oxidative removal of nitro groups from nitroaromatic compounds. Although the recent literature contains numerous examples of oxygenase-catalyzed nitro group displacements (33), little is known about the enzymes involved in these reactions. Monooxygenases that oxidize 4- and 2-nitrophenol (34, 36, 49) have been described, but only the 2-nitrophenol monooxygenase from *Pseudomonas putida* B2 has been purified and characterized (49). Recent evidence has suggested that some of the dioxygenases involved in the removal of nitro groups from aromatic compounds are multicomponent enzyme systems similar to the naphthalene dioxygenase enzyme system (2, 40). *Burkholderia* sp. strain DNT (formerly *Pseudomonas* sp. strain DNT) uses both mono- and dioxygenase enzymes to remove nitro groups (13, 37, 40) in an oxidative pathway that leads to the mineralization of 2,4-dinitrotoluene (2,4-DNT) (37).

The initial attack on 2,4-DNT by *Burkholderia* sp. strain DNT occurs via a dioxygenation reaction to form 4-methyl-5-nitrocatechol (MNC) with concomitant release of nitrite (37, 40). A monooxygenase catalyzes the subsequent oxidation of MNC to produce 2-hydroxy-5-methylquinone and a second molecule of nitrite (13, 14). A quinone reductase then converts 2-hydroxy-5-methylquinone to 2,4,5-trihydroxytoluene, which is oxidized by a ring-cleavage enzyme to an unidentified product (Fig. 1) (13, 39). The genes encoding DNT dioxygenase (*dntA*), MNC monooxygenase (*dntB*), and trihydroxytoluene oxygenase (*dntD*) have been cloned and characterized elsewhere (40–43).

Although a number of previous reports suggested that benzoquinones were intermediates in the monooxygenase-catalyzed eliminations of nitro groups from nitrophenols (36, 49), the earlier study with MNC monooxygenase was the first to identify the postulated benzoquinone (13). The reaction catalyzed by MNC monooxygenase is also similar to that postulated for the removal of halogens by *p*-hydroxybenzoate hydroxylase (16) and pentachlorophenol hydroxylase (47). In order to further study MNC monooxygenase and to compare it with the other proteins that catalyze analogous reactions, we have purified and characterized MNC monooxygenase. We have also sequenced the gene that encodes MNC monooxygenase, *dntB*, and compared the deduced amino acid sequence to those of other proteins. To our knowledge, this report is the first published sequence of a monooxygenase that is specific for the removal of nitro groups from aromatic compounds. Our results indicate that the MNC monooxygenase is a flavoprotein that uses flavin adenine dinucleotide (FAD) as the cofactor. Preliminary reports of this work have been presented previously (14, 41).

MATERIALS AND METHODS

Growth of bacteria. *Burkholderia* sp. strain DNT was grown and maintained as described previously (37). Large quantities of cells were grown in 2.8-liter Fernbach flasks containing yeast extract (2 g) and DNT (50 mg) in 1 liter of minimal salts medium (pH 6.8). As the DNT was consumed, more was added as previously described (13). Cultures were incubated at 30°C with constant shaking at 200 rpm. Cells were harvested by centrifugation and washed with 0.05 M KH_2PO_4 (pH 7.5), and the cell pellets were stored at −70°C until used.

Protein purification. All procedures were carried out at 4°C, unless stated

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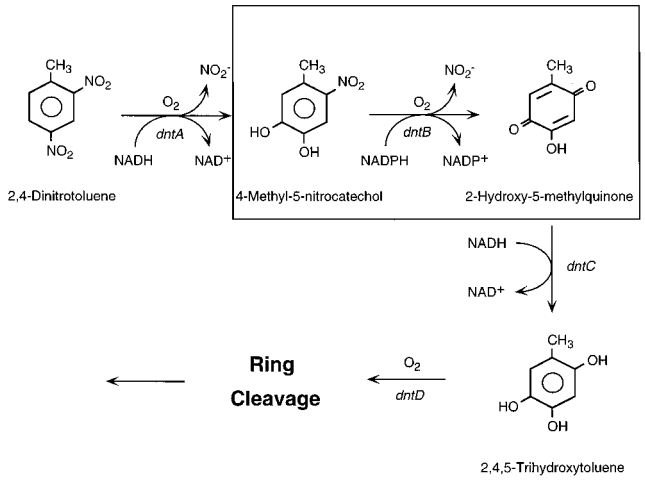

FIG. 1. Pathway for biodegradation of 2,4-DNT by *Burkholderia* sp. strain DNT (13, 37, 43).

otherwise. Cell extracts for protein purification were prepared from frozen cells (approximately 10 g [wet weight]) suspended in an equal volume (wt/vol) of 0.05 M KH_2PO_4 (pH 7.5) containing 0.5 mg of DNase I. After cell lysis and ultracentrifugation as previously described (35), the extract was loaded onto a DEAE-cellulose column (Whatman DE52; 2.6 by 16 cm) at a flow rate of 2 ml/min. The column was washed with 250 ml of phosphate buffer, and proteins were eluted with a linear NaCl gradient (0 to 0.5 M) in phosphate buffer (300 ml at 2 ml/min). The fractions (4 ml each) that exhibited maximum activity were pooled, dialyzed against two buffer changes to remove the salt, and concentrated by centrifugation in a Centricon 30 Centriprep tube (Amicon, Danvers, Mass.) to a final volume of 10 ml. The proteins in the concentrated solution were separated by two applications to a Mono Q HR 5/5 (1 ml; Pharmacia) column. After each application, the column was washed with 10 ml of buffer (1 ml/min) and proteins were eluted with a linear NaCl gradient (0 to 0.4 M) in phosphate buffer (30 ml at 1 ml/min). The fractions (2 ml each) that exhibited maximum activity were pooled and concentrated by centrifugation in a Centricon 30 Centriprep tube (Amicon) to a final volume of 1 ml. The concentrated protein solution was applied to a Sephacryl S-200 gel filtration column (Pharmacia; 1.6 by 95 cm) and eluted with phosphate buffer at a flow rate of 1.0 ml/min. Fractions (1 ml each) exhibiting maximum activity were pooled and loaded onto a Mono Q HR 5/5 (Pharmacia) column. The column was washed with 10 ml of phosphate buffer, and bound proteins were eluted as before with a 30-ml gradient (0 to 0.25 M NaCl). Fractions (1 ml each) with maximal enzyme activity were pooled and dialyzed as before prior to storage at −70°C.

Enzyme assays. MNC monooxygenase activity was determined spectrophotometrically by monitoring the disappearance of MNC at 420 nm (ε = 9,760 M^{-1} at pH 7.5). The assay system contained NADPH (2×10^{-4} M), MNC (1.67×10^{-6} M), and appropriate amounts of protein in 1 ml of KH_2PO_4 buffer (0.05 M, pH 7.5). The reaction was started by the addition of MNC. Specific activities are expressed as micromoles of substrate transformed per minute per milligram of protein. MNC monooxygenase activity towards 4-nitrocatechol was determined in a similar manner at 440 nm (ε = 10,390 M^{-1} at pH 7.5). Protein was measured by the method of Smith et al. (31). Assays for enzyme activity and the determination of K_m values for NADPH, NADH, and MNC were performed with a Cary 3E UV light-visible spectrophotometer. MNC monooxygenase activity used to determine K_m values for oxygen was assayed polarographically with a Clark-type electrode (35).

The activity of MNC monooxygenase towards other substrates was tested by measuring the disappearance of the test substrate (10 μM) by high-pressure liquid chromatography (HPLC). The assay system was as described above with 8 μg of MNC monooxygenase per assay. Reactions were performed at room temperature, and samples were analyzed at 5 and 20 min and compared with control reactions performed in the absence of enzyme and with enzyme and 10 μM MNC (positive control). HPLC was performed as previously described (37) with trifluoroacetic acid (13.5 mM)-acetonitrile (50:50) as the mobile phase.

SDS-PAGE and molecular weight determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8, 10, and 15% resolving gels was carried out with the discontinuous system described by Laemmli (19). Standard proteins used for molecular weight determinations of MNC monooxygenase were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Proteins were stained by the method of Fairbanks et al. (11).

The molecular weight of MNC monooxygenase was determined by gel filtration on a Sephacryl S-200 superfine column (1.6 by 95 cm). The column was calibrated with catalase, aldolase, bovine serum albumin, and ovalbumin (20).

TABLE 1. Purification of MNC monooxygenase

Purification step	Protein (mg)	Activity[a] (U)	Sp act (U/mg)	Recovery (%)	Purification (fold)
1. Cell extract[b]	592	90.0	0.15	100	1
2. DEAE cellulose chromatography	59.5	67.8	1.14	75	7.5
3. Mono Q HR 5/5 chromatography	22.7	31.5	1.39	35	9
4. Sephacryl S-200 chromatography	9.67	32.1	3.32	36	22
5. Mono Q HR 5/5 chromatography	5.60	23.0	4.11	26	27

[a] Activity was determined spectrophotometrically by monitoring the disappearance of the MNC at 420 nm. A unit of activity is defined as micromoles of substrate consumed per minute in the presence of MNC monooxygenase.
[b] Soluble protein obtained after ultracentrifugation.

DNA and amino acid sequencing and analysis. The nucleotide sequence of the 2.2-kb *Apa*I DNA fragment containing *dntB* on plasmid pJS53 (43) was determined by Bio-Synthesis, Inc. (Lewisville, Tex.). Nucleotide and deduced amino acid sequences were analyzed with MacDNASIS Pro sequence analysis software (Hitachi, San Bruno, Calif.) and the Lasergene software package (DNAStar, Inc., Madison, Wis.). The GenBank nucleotide sequence database (release 92, January 1996) and the NBRF-PIR protein database (release 45, January 1996) were used for the homology search (10).

Strain DNT was identified as a *Burkholderia* sp. by partial sequence analysis comparison of genes encoding 16S rRNA with currently available sequences of genes encoding 16S rRNA of gram-negative bacteria (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Chemicals. DNase I; FAD, flavin mononucleotide; 2-, 3-, and 4-nitrophenol; 3-methyl-2-nitrophenol; 3-methyl-4-nitrophenol; 3-nitrocatechol; 4-nitrocatechol; and 4-chlorocatechol were purchased from Sigma (St. Louis, Mo.). MNC was obtained from R. Spanggord (SRI International, Menlo Park, Calif.). 3-Methyl-4-nitrocatechol was produced biologically from 2,6-DNT by *Burkholderia cepacia* PR7 (24a, 26). All other chemicals were of the highest purity commercially available.

Nucleotide sequence accession number. The GenBank accession number of the sequence data shown in this paper is U68411.

RESULTS

Purification of MNC monooxygenase. A typical purification procedure (Table 1) yielded a 27-fold purification with a recovery of 26% of the MNC monooxygenase activity. Analysis of the purified protein by SDS-PAGE revealed a single band (Fig. 2). Comparison with crude cell extracts revealed that MNC monooxygenase constitutes a significant portion of the soluble cell protein. Calculations based on the specific activity of MNC monooxygenase and the activity in cell extracts indicate that it makes up 3.7% of the soluble protein of the cell.

Properties of MNC monooxygenase. The molecular weight of MNC monooxygenase was determined by gel filtration and SDS-PAGE to be 65,000 and 60,200, respectively. Sequence analysis (Fig. 3) revealed that *dntB* encodes a peptide of 548 amino acids with a molecular weight of 59,165. The isoelectric point of MNC monooxygenase was calculated from the derived amino acid sequence to be pH 4.96.

Solutions of purified MNC monooxygenase were yellow and gave absorption maxima at 273, 372, and 453. These spectral properties are consistent with those of a flavoprotein (38, 49). The flavin cofactor of the MNC monooxygenase was characterized by HPLC as previously described (32). The analysis indicated that 1.1 mol of FAD was bound per mol of purified protein.

Catalytic properties of MNC monooxygenase. Both NADH and NADPH served as electron donors to the purified enzyme. The apparent K_m values for NADPH and NADH were deter-

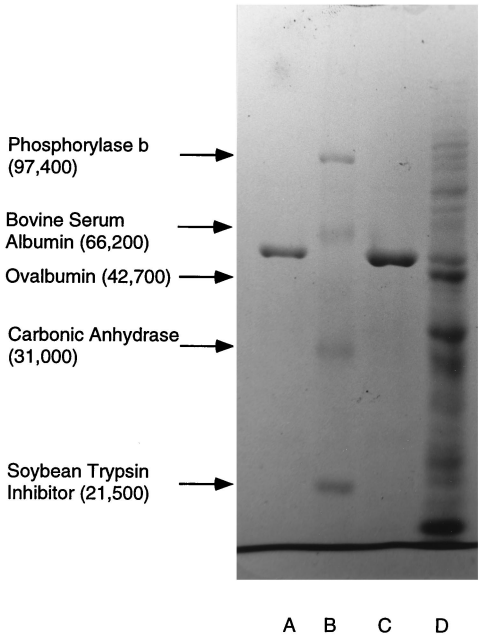

FIG. 2. SDS-PAGE of purified MNC monooxygenase. Purified MNC monooxygenase (lanes A and C) was compared with the crude cell extract (lane D) and a standard protein mix (lane B). Protein concentrations were 5 (lane A), 10 (lane C), and 148 (lane D) μg. The molecular weights of the standards are given in parentheses, and the arrows indicate their positions in the gel (lane B).

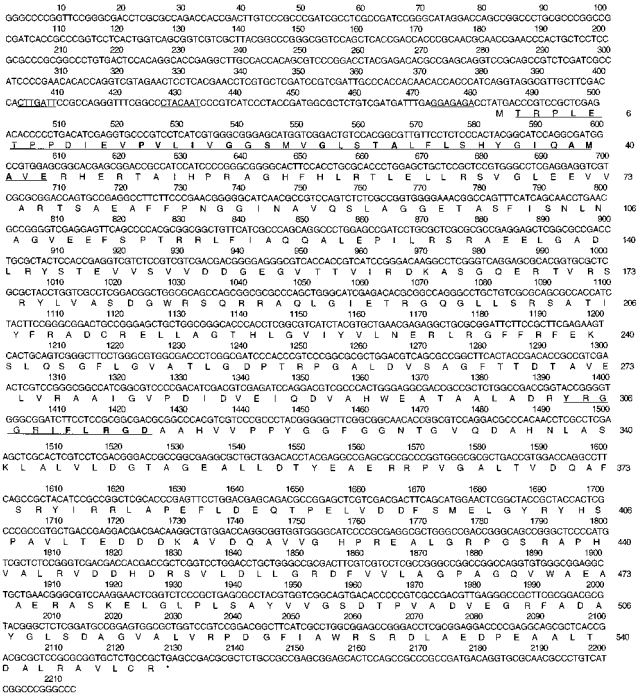

FIG. 3. Nucleotide sequence of the 2.2-kb *Apa*I fragment containing *dntB*. Single-letter symbols for amino acids are aligned with the second nucleotide of each codon. The underlined area indicates the sequence (403 to 433) which has similarity to the *Pseudomonas* consensus promoter region (7). Other boldface and underlined areas indicate the putative ADP binding region (residues 1 to 41) and the putative FAD-isoalloxazine ring binding region (residues 303 to 313) (8, 46).

mined to be 15.5 and 1,220 μM. This indicates that NADPH is the preferred cofactor. Concentrations of MNC above 2 μM were inhibitory (Fig. 4). At these concentrations, the rate of MNC consumption increased with time as the substrate level decreased. Repeated additions of noninhibitory concentrations of the substrate did not result in significant reduction of initial activity. These results suggest that inhibition of the enzyme activity is due to MNC and not the products of the reaction. Substrate inhibition of *meta* fission by 2,3-dihydroxybiphenyl in *Pseudomonas* sp. strain CB15 has been described previously by the polynomial expression $[S]/V = K_m/V_{max} + (1/V_{max})[S] + (1/K_{SS} \times V_{max})[S]^2$, where S is the substrate concentration, V is the observed rate, V_{max} is the maximum rate, K_m is the Michaelis constant, and K_{SS} is the inhibition constant (1). The inhibition of MNC monooxygenase by MNC describes a curve that fits the expression well ($r^2 = 0.993$) and gave a theoretical K_m of 1.2 μM, a K_{SS} of 1.0, and a V_{max} of 3.6 μmol/min/mg of protein. The measured K_m value for oxygen was determined to be 50 ± 47 μM (95% confidence limit, $n = 8$) when an inhibitory concentration (37 μM) of MNC was used. The apparent low K_m for MNC and the inhibition by the substrate made it difficult to accurately determine K_m values for oxygen.

The activity of *o*-nitrophenol oxygenase was stimulated by the addition of magnesium or manganese ions but not FAD (49). The addition of 4 mM magnesium sulfate had no effect on MNC monooxygenase activity, and the addition of flavin (1 μM) had only a slight stimulatory effect (data not shown).

MNC monooxygenase is highly specific for MNC. 4-Nitrocatechol was the only other compound tested that was oxidized at detectable rates (58% of the activity for MNC). 4-Nitrocatechol was inhibitory at concentrations of >1 μM, which is similar to the inhibition by MNC. Other substituted phenols, such as 2-, 3-, and 4-nitrophenol, 3-methyl-2-nitrophenol, 3-methyl-4-nitrophenol, 3-nitrocatechol, 3-methyl-4-nitrocatechol, 4-methyl-2-nitrophenol, 2-methyl-5-nitrophenol, 5-methyl-2-nitrophenol, or 4-chlorocatechol, did not serve as substrates for MNC monooxygenase.

Sequence analysis of *dntB*. *dntB* was previously localized within a 2.2-kb *Apa*I fragment (41, 43). Sequence analysis (Fig. 3) of this fragment indicated that it contained a single open

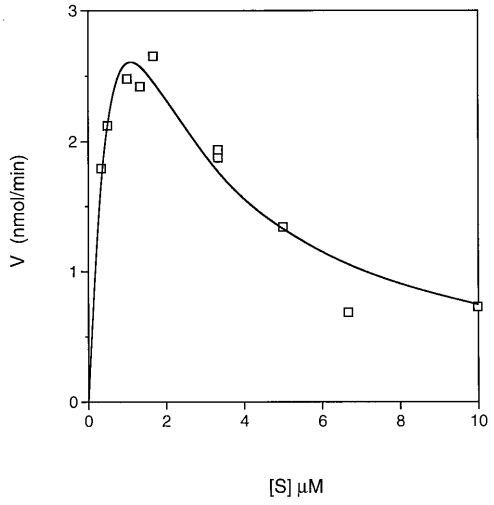

FIG. 4. Dependence of MNC monooxygenase activity on MNC. Enzyme assays were performed as described in Materials and Methods with 2.3 μg of a preparation of MNC monooxygenase with a specific activity of 1.2 U/mg and varying amounts of MNC.

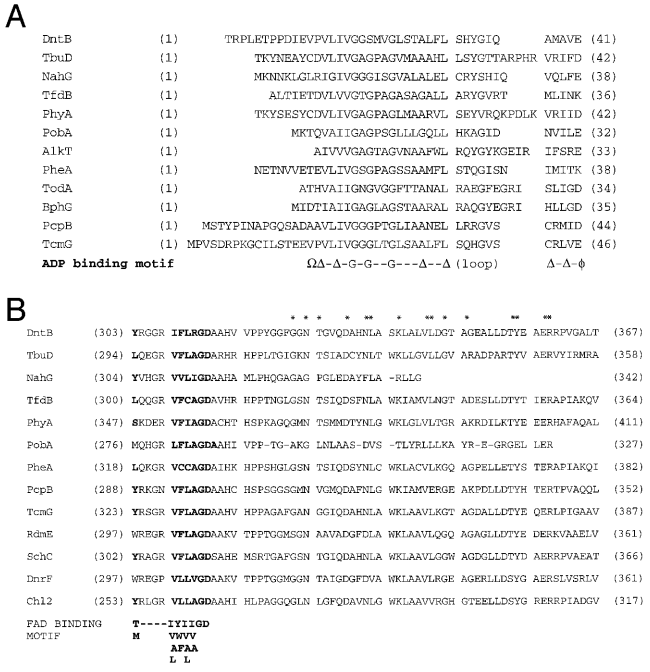

FIG. 5. Putative ADP and FAD binding sequences of DntB aligned with those of other proteins (DntB, MNC monooxygenase from *Burkholderia* sp. strain DNT; TbuD [18], phenol-cresol hydroxylase from *Pseudomonas pickettii* PKO1; NahG [48], salicylate hydroxylase from *P. putida* PpG7; TfdB [29], 2,4-dichlorophenol hydroxylase from *Alcaligenes eutrophus* JMP134; PhyA [17], phenol hydroxylase from *T. cutaneum*; PobA [9], *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; AlkT [8], rubredoxin reductase from *Pseudomonas oleovorans*; PheA [27], phenol 2-monooxygenase from *Pseudomonas* sp. strain EST1001; TodA [50], ferredoxin oxidoreductase of toluene dioxygenase from *P. putida* F1; BphG [10], ferredoxin oxidoreductase of biphenyl dioxygenase from *Pseudomonas* strain LB400; PcpB [28], pentachlorophenol-4-monooxygenase from *Flavobacterium* sp. strain ATCC 39723; TcmG [6], tetracenomycin A2 hydroxylase from *Streptomyces glaucescens*; RdmE [24], putative aklavinone-11-hydroxylase from *Streptomyces purpurascens*; SchC [4], putative hydroxylase involved in spore pigment synthesis from *S. halstedii*; DnrF [12], putative aklavinone-11-hydroxylase from *Streptomyces paucetius*; Chl2 [5], tetracycline 6-hydroxylase from *Streptomyces aureofaciens*). In the fingerprint of the ADP binding motif (A) proposed by Wierenga et al. (46), Ω represents the amino acids H, K, N, Q, R, S, and T; Δ represents A, C, I, L, M, and V; and ϕ is D and E. The short sequence important in FAD binding for many FAD-containing enzymes (8, 30) is shown in panel B. The asterisks in panel B indicate the 15 residues that DntB has in common with residues noted previously by Kukor and Olsen (18) as being conserved among the phenol hydroxylases TfdB, PheA, PhyA, and TbuD. The number in parentheses indicates the location of the amino acid residue in the protein sequence.

reading frame of 1,644 bp with an N-terminal amino acid sequence identical to that of purified MNC monooxygenase (8 amino acids sequenced). The deduced amino acid sequence of MNC monooxygenase contains a single highly conserved FAD binding motif (Fig. 5B) that is found in many flavoprotein hydroxylases that act on aromatic compounds (22). In addition, the amino acid sequence between residues 13 and 41 of DntB is similar to a consensus fingerprint ADP binding motif proposed by Wierenga et al. (46). This conserved motif, found in a number of flavin-containing hydroxylases and oxidoreductases, has been proposed to be involved in binding of the ADP moiety of FAD to flavin-containing proteins (Fig. 5A) (8). The amino acid sequence encoded by *dntB* is similar to the sequences of several phenol and chlorophenol hydroxylases (Table 2) and the sequences of hydroxylases involved in anthracycline antibiotic production by *Streptomyces* species.

Analysis of the nucleotide sequence upstream from *dntB* revealed a TTGATT-N_{18}-TACAAT sequence (55 bases upstream of the ATG start codon of MNC monooxygenase) at positions 403 to 433 that is similar to *Pseudomonas* TTGACA -$N_{17 \pm 1}$-TATAAT promoters recognized by *Escherichia coli* RNA polymerase (7). This suggests that the 403-to-433 sequence forms part of the promoter region of *dntB*.

TABLE 2. Amino acid similarity of MNC monooxygenase (DntB) to other proteins[a]

Protein	No. of amino acids	% Identity	% Similarity	Reference
DntB	548	100	100	
TcmG	572	41.9	59.8	6
RdmE	535	38.1	58.5	24
PheA	610	34.9	56.4	27
TfdB	598	34.7	56.4	29
TbuD	671	27.8	48.6	18
PcpB	538	26.1	48.0	28
PhyA	665	25.0	51.6	17

[a] Amino acid sequence comparisons were obtained by Gerben Zylstra (Rutgers, The State University of New Jersey, New Brunswick) by using the Genetics Computer Group program sequence analysis package. Proteins being compared are DntB, MNC monooxygenase from *Burkholderia* sp. strain DNT; TcmG, tetracenomycin A2 hydroxylase from *Streptomyces glaucescens*; RdmE, putative aklavinone-11-hydroxylase from *Streptomyces purpurascens*; PheA, phenol 2-monooxygenase from *Pseudomonas* sp. strain EST1001; TfdB, 2,4-dichlorophenol hydroxylase from *Alcaligenes eutrophus* JMP134; TbuD, phenol-cresol hydroxylase from *Pseudomonas pickettii* PKO1; PcpB, pentachlorophenol-4-monooxygenase from *Flavobacterium* sp. strain ATCC 39723; PhyA, phenol hydroxylase from *T. cutaneum*.

DISCUSSION

The reaction catalyzed by MNC monooxygenase (13) is similar to those catalyzed by 2-nitrophenol and 4-nitrophenol monooxygenases (34, 36, 49). In reactions catalyzed by nitrophenol monooxygenases, 2 mol of NADPH is required and benzoquinone intermediates were postulated but have not been demonstrated in the formation of hydroquinone (34, 36) or catechol (49).

The nitrophenol and MNC monooxygenases all are inhibited by their substrates at low concentrations (≥2 μM for MNC monooxygenase; ≥20 and 50 μM for *o*-nitrophenol and *p*-nitrophenol monooxygenases, respectively), the apparent K_m values for their physiological substrates are similar (MNC, 1.2 μM; *o*-nitrophenol, 8 μM; and *p*-nitrophenol, 6 μM), and all three use NADPH as the preferred electron donor (34, 36, 49). The MNC and *o*-nitrophenol monooxygenases are soluble enzymes with similar molecular weights (both are approximately 60,000), whereas the *p*-nitrophenol monooxygenase obtained from a *Moraxella* sp. is membrane bound. The fact that purified MNC monooxygenase contains bound FAD suggests that it is more similar to the *p*-nitrophenol monooxygenase (36), which is stimulated by additional flavin, than to the *o*-nitrophenol monooxygenase, which is stimulated by magnesium ions (49). It is clear from our results that MNC monooxygenase contains a single tightly bound FAD molecule, whereas the cofactors of the nitrophenol monooxygenases have not been rigorously determined (36, 49).

When *Burkholderia* sp. strain DNT utilizes 2,4-DNT as a sole source of carbon and energy, growth is slow and is often preceded by an extended, unpredictable acclimation period (37, 43). This may be a consequence of sequential induction of initial enzymes that are encoded by separate operons (43). The presence of a potential promoter region upstream of *dntB* is consistent with previous observations that *dntB* gene expression is controlled separately from that of *dntA* (43). The inhibition of the MNC monooxygenase by very low concentrations

of MNC may contribute to the unpredictable acclimation period. The transient accumulation of MNC to concentrations of >2 μM is frequently observed in uninduced cultures after the addition of 2,4-DNT (37). The high levels of enzyme that are produced in induced cells of strain DNT might compensate for this inhibition. Similarly, an inverse relationship between high concentrations of an enzyme and low enzyme activity has been reported for the phenol hydroxylase in *Trichosporon cutaneum* (23) and the 2,4-dichlorophenol hydroxylase in an *Acinetobacter* species (3).

Comparison of the deduced amino acid sequence of DntB with those of other proteins in the NBRF-PIR database revealed that DntB contains a short motif which was identified by Russel and Model (30) and refined by Eggink et al. (8) as being important in FAD binding for many FAD-containing proteins. Beyond the FAD binding region, DntB contains 15 of the 18 residues that are conserved among the phenol hydroxylases PheA, TfdB, TbuD, and PhyA (18). TcmG, RdmE, and PcpB also exhibit a high degree of conservation of residues within this region of their respective sequences (Fig. 5B). In addition, the βαβ-fold ADP binding motif in the N-terminal region of DntB has also been identified in many aromatic ring hydroxylases containing FAD as the cofactor (Fig. 5). Unfortunately, there are no reported sequences of other nitrophenol monooxygenases with which to make comparisons.

The predicted amino acid sequence for DntB exhibits the highest degree of identity and similarity to the deduced sequences of TcmG, RdmE (Table 2), and SchC (39% identity) (4). TcmG and RdmE are hydroxylases involved in the biosynthesis of anthracycline antibiotics in strains of *Streptomyces* (6, 24). SchC is involved in spore pigment biosynthesis in *Streptomyces halstedii* (4). These proteins have not been purified, so direct comparisons of function and mechanism with those of DntB cannot be made. The hypothesized reaction mechanism for TcmG involves the insertion of two atoms of molecular oxygen and one atom of oxygen from water into tetracenomycin A2 to form tetracenomycin C (6). It is not known what cofactors or coenzymes are required during this reaction.

Although benzoquinones have been postulated as intermediates in the elimination of nitro- (33, 34, 49) and fluoro- (16) substituents from phenols, it was studies with the MNC monooxygenase that first identified the benzoquinone intermediate (13) and demonstrated a stoichiometry of 1 mol of NADPH per mol of substrate converted. The removal of the nitro group by MNC monooxygenase is analogous to the removal of chloride from pentachlorophenol by pentachlorophenol 4-monooxygenase (47), an enzyme with considerable amino acid sequence similarity (28). Pentachlorophenol 4-monooxygenase uses 2 mol of NADPH per mol of pentachlorophenol transformed, which suggests that the mechanism is similar to that of MNC monooxygenase; however, a quinone intermediate has not been detected in the latter reaction (47).

Previous studies with pentachlorophenol hydroxylase demonstrated that the stoichiometry of NADPH utilization depended on the substituent in the *para* position of the substrate (47). When the substituent was an electron-donating group, such as an amino group or a hydrogen, 1 mol of NADPH was required to substitute a hydroxyl group for the leaving group. In contrast, when the leaving group was an electron-withdrawing group, such as a nitro group, a cyano group, or a halogen, 2 mol of NADPH was required for replacement of the substituent with a hydroxyl group. Similar NADPH requirements were observed for fluoride elimination by *p*-hydroxybenzoate hydroxylase (16) and nitro group eliminations from *p*-nitrophenol (36) and *o*-nitrophenol (49) by their respective nitrophenol oxygenases. In each of the reactions mentioned above, benzoquinones were postulated to be intermediates in the formation of the hydroxylated products. The conversion of MNC to 2,4,5-trihydroxytoluene is similar to the reactions mentioned above in the overall requirement for 2 mol of NADPH. However, the fact that two enzymes that each use 1 mol of NADPH are required in the reaction sequence allowed the postulated benzoquinone, 2-hydroxy-5-methylquinone, to be identified as the product of the MNC monooxygenase (13).

The monooxygenase-catalyzed removal of nitro groups also is reminiscent of the displacement of the carboxyl substituent of salicylate with a hydroxyl group by salicylate hydroxylase (45). The fact that salicylate hydroxylase can catalyze the replacement of the nitro group of *o*-nitrophenol (44) supports the idea that the reaction mechanisms are related.

The substrate range of MNC monooxygenase suggests that the hydroxyl groups *meta* and *para* to the nitro group are critical to the reaction mechanism. The methyl group does not seem to be required as indicated by the substantial activity with 4-nitrocatechol. However, the absence of either hydroxyl group results in a complete loss of activity. Only one hydroxyl group is essential for formation of the quinone after nitro group removal. The function of the second hydroxyl group may be to position the substrate in the active site of the enzyme. The absence of activity with 3-methyl-4-nitrocatechol suggests that the methyl group might also play a role in orienting the substrate in the enzyme active site. Alternatively, any large substituent *ortho* to the hydroxyl group, such as the methyl group in 3-methyl-4-nitrocatechol, may hinder MNC monooxygenase activity. More studies are required to determine the role of the substituents of MNC in substrate binding and enzyme activity.

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