Oxidation of Aminonitrotoluenes by 2,4-DNT Dioxygenase of *Burkholderia* sp. strain DNT

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Abstract: Aminonitrotoluenes form rapidly from the reduction of dinitrotoluenes (DNTs) which are priority pollutants and animal carcinogens. For example, 4-amino-2-nitrotoluene (4A2NT) and 2A4NT accumulate from the reduction of 2,4-DNT during its aerobic biodegradation. Here, we show that 2,4-DNT dioxygenase (DDO) from *Burkholderia* sp. strain DNT oxidizes the aminonitrotoluenes 2A3NT, 2A6NT, 4A3NT, and 5A2NT to 2-amino-3-nitrobenzylalcohol, 2-amino-4-nitro-*m*-cresol and 3-amino-5-nitro-*p*-cresol, 4-amino-3-nitrobenzylalcohol and aminonitrocresol, and 2-amino-5-nitro-*o*-cresol, respectively. 2A5NT and 3A4NT are oxidized to aminonitrocresols and/or aminonitrobenzylalcohols, and 4A2NT is oxidized to aminonitrocresol. Only 2A4NT, a reduced compound derived from 2,4-DNT, was not oxidized by DDO or its three variants. The alpha subunit mutation I204Y resulted in two to fourfold faster oxidization of the aminonitrotoluenes. Though these enzymes are dioxygenases, they acted like monooxygenases by adding a single hydroxyl group, which did not result in the release of nitrite. © 2005 Wiley Periodicals, Inc.

INTRODUCTION

The biological treatment of 2,4,6-trinitrotoluene (TNT)-contaminated soil and groundwater has been studied for decades (Hughes et al., 1999) since this was the most-common explosive (Nishino et al., 1999). 2,4-Dinitrotoluene (2,4-DNT) and 2,6-DNT are intermediates in the production of TNT and are found in the soil and ground water of TNT-production facilities (Johnson et al., 2002; Nishino et al., 2000). DNTs are also used for the production of polyurethane foams as a precursor of toluene diisocyanate (Nishino et al., 1999), and both 2,4-DNT and 2,6-DNT are listed as priority pollutants by the U.S. Environmental Protection Agency (EPA) (Nishino et al., 1999). 2,4-DNT and 2,6-DNT are carcinogenic to rats and mice (Anonymous, 1988), and the other DNT isomers (e.g., 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT) cause systemic intoxication as well as liver necrosis (Gosselin et al., 1976). According to the 1996 Toxics Release Inventory, 8,159 pounds of 2,4-DNT and 2,6-DNT were released into the environment from five processing facilities (http://www. atsdr.cdc.gov), and there are at least 122 current or former EPA National Priorities List hazardous waste sites that contain 2,4-DNT and 2,6-DNT (http://iier1.isciii.es/toxprofiles/phs109.html).

Biological treatment of DNT is potentially less expensive compared to chemical and physical methods, and the most widely recognized pathway for DNT biotransformation is reductive (Freedman et al., 1996); aminonitrotoluenes are formed as nitro reduction products of DNTs during their aerobic biodegradation (Hughes et al., 1999). For example, 4-amino-2-nitrotoluene (4A2NT) and 2A4NT were detected during the aerobic biodegradation of 2,4-DNT (Christopher et al., 2000; Freedman et al., 1996; Hughes et al., 1999), and 4A2NT was the predominant isomer formed (Christopher et al., 2000). In addition, 2A4NT and 4A2NT were also observed under anaerobic and anoxic conditions (Christopher et al., 2000). This is a concern because these reduced aromatic metabolites also pose a significant toxicological hazard; for example, 2A4NT is an experimental mutagen and carcinogen (Freedman et al., 1996).

Burkholderia sp. strain DNT was isolated from water samples from Waconda Bay near the Volunteer Army Ammunition Plant in Chattanooga, Tenn. (Spanggord et al., 1991), and this strain uses 2,4-DNT as a sole carbon and

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energy source (Spanggord et al., 1991). Catabolism of 2,4-DNT is initiated by 2,4-DNT dioxygenase (DDO) which produces 4-methyl-5-nitrocatechol (4M5NC) (Suen and Spain, 1993). In DDO, electrons are transferred from NADH through a flavoprotein reductase (DntAa), an iron-sulfur [2Fe-2S] ferredoxin (DntAb), and an iron-sulfur oxygenase which consists of a large (α) subunit (DntAc) and a small (β) subunit (DntAd) (Kauppi et al., 1998; Suen et al., 1996). DDO alpha subunit variants I204L and I204Y were identified previously with enhanced activity for 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT (Leungsakul et al., 2005) (e.g., wild-type DDO has no activity on 2,3-DNT and 2,5-DNT).

The goal of this work was to investigate the activity of wild-type DDO and its DntAc variants I204L, I204Y, and I204F (Leungsakul et al., 2005) toward various aminonitrotoluenes including 2-amino-3-nitrotoluene (2A3NT) from the reduction of 2,3-DNT, 2A4NT and 4A2NT from the reduction of 2,4-DNT, 5A2NT and 2A5NT from the reduction of 2,5-DNT, 2A6NT from the reduction of 2,6-DNT, and 3A4NT and 4A3NT from the reduction of 3,4-DNT. This is the first report of enzymatic activity toward aminonitrotoluenes, and that DDO acts like a monooxygenase producing aminonitrocresols or aminonitrobenzylalcohols.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli JVQ2 (double nitroreductase mutant) containing a 177-bp deletion in *nfsA* and a 43-bp IS*5* element integrated into *nfsB* (Whiteway et al., 1998) was used to express DDO from pBS(Kan)DNT (Leungsakul et al., 2005) under the control of a *lac* promoter, and pBS(Kan) (Canada et al., 2002) without DDO was used as a negative control. Cells were grown from single colonies in Luria-Bertani (LB) medium (Sambrook et al., 1989) containing kanamycin (100 μg/mL) to maintain the plasmid and were incubated at 37°C overnight at 250 rpm (C25 incubator shaker, New Brunswick Scientific, Edison, NJ). One milliliter of the overnight culture was used to inoculate 75 mL of LB medium containing kanamycin (100 μg/mL) and 1 mM of isopropyl-β-D-thiogalactopyranoside, and cells were grown to an optical density at 600 nm (OD) of 1.5–2.0 (UV mini 1240 UV-VIS spectrophotometer, Shimadzu Scientific Instruments, Inc., Kyoto, Japan). Cultures harvested during the exponential phase were used in all experiments. Cells were centrifuged at 13,000*g* for 7 min at 25°C in a Beckman J2-HS centrifuge (Palo Alto, CA), washed with 100 mM sodium phosphate buffer (pH 6.5), and resuspended in this buffer to an OD of 5–15.

Burkholderia sp. strain DNT was used to confirm the activity of its native DDO on 2A3NT, 2A5NT, and 5A2NT. Cells were grown in LB for overnight at 30°C, then 2 mL of the overnight culture was used to inoculate 100 mL of MSB (Spain and Nishino, 1987) containing 75 μM of 2,4-DNT (to maintain DDO activity) and 20 mM of sodium succinate to an OD of 0.6. Cells were centrifuged, washed, and resuspended as described above.

Chemicals

2A3NT and 3A4NT were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI), 5A2NT was obtained from Lancaster Synthesis, Inc. (Pelham, NH), and 2A4NT, 2A5NT, 2A6NT, 4A2NT, and 4A3NT were purchased from Fisher Scientific Co. (Fairlawn, NJ). 2-Amino-6-nitrobenzyl alcohol (2A6NB) and 4A2NB were obtained from Professor Masa-aki Mori (Mori et al., 2000).

Nitrite Formation

Nitrite production from the oxidation of the eight aminonitrotoluenes was assayed spectrophotometrically as described previously (Keenan et al., 2004) by resuspending exponentially grown cells in sodium phosphate buffer and contacting them with 300 μM of each aminonitrotoluene at a cell density of OD 5–10 in sodium phosphate buffer (100 mM, pH 6.5) for 60 min at 37°C in sealed 15 mL serum vials with Teflon septa. The nitrite concentration was determined using a linear calibration curve (0.72 to 145 μM), the sensitivity of the nitrite assay is 0.72 μM

Reaction Rates by HPLC

Reverse-phase high performance liquid chromatography (HPLC) was used to determine the substrate depletion rates of 2A3NT, 2A5NT, 2A6NT, 4A3NT, and 5A2NT and to identify products from the oxidation of 2A3NT, 2A4NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT. Ten milliliter samples of exponentially grown, washed cells (OD 5 to 15) were placed in sealed 60 mL serum vials and were contacted with 300 μM of each of the aminonitrotoluenes (acetonitrile was the diluent and had no effect on cell growth) at 37°C at 300 rpm (IKA-KS 250 Basic shaker, Cincinnati, OH) for 60 min. Samples (750 μL) were taken at 15, 30, 60, and 90 min using a 3 mL syringe, were centrifuged in a Spectrafuge 16M microcentrifuge (Labnet, Inc., Woodbridge, NJ) for 5 min at 16,000*g*, and supernatants (20 μL) collected and analyzed via HPLC with a Chlomolith™ Performance RP-18e column (Merck KGaA, 4.6 × 100 mm) and a Waters Corporation (Milford, MA) 515 solvent delivery system. Compounds were detected by a photodiode array detector (Waters 996). A gradient elution was used with H_2O (0.1% formic acid) and acetonitrile (100:0 at 0 min at 2 mL/min, 0:100 at 15 min at 2 mL/min, 100:0 at 25 min at 2 mL/min, 100:0 at 30 min at 2 mL/min) as the mobile phase. All experiments were performed at least twice.

Product Identification by GC-MS and NMR

The putative aminonitrocresols and aminonitrobenzylalcohols from aminonitrotoluene oxidation were identified by gas chromatography-mass spectrometry (GC-MS) using a

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Hewlett-Packard 5970B GC-MS instrument equipped with a HP-1 column (12 m × 0.2 mm, 0.33 μM thickness) operated at an ionization voltage of 70 eV. The initial column temperature was 120°C for 2 min which was increased at 10°C/min to 270°C followed by an isothermal operation for 6 min. The injector and detector temperatures were 270°C and 275°C. Samples were prepared in a similar manner to the samples for HPLC and were contacted for 1 h with 300 μM of the aminonitrotoluene. Supernatants were extracted with ethyl acetate, the solvent was evaporated under nitrogen, and the residue was dissolved in 40 μL of ethyl acetate. Trimethylsilyl derivatives were prepared by adding 40 μL *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Alltech Associates, Inc., Deerfield, Ill.) into the samples, and the samples were incubated for 30 min at room temperature. All experiments were performed at least twice, and GC-MS was conducted with derivatization for all the aminonitrotoluenes except for the oxidation product of 2A3NT, which was identified both with and without derivatization.

Samples for semi-preparative LC were prepared in the same way as the samples for GC-MS except 500 μL of DMSO (deuterated) was used as a solvent instead of ethyl acetate. Semi-preparative LC (IBM Instruments, model 9533) with a Reliasil BDX-C18 column (Column Engineering, Inc., 10 × 150 mm) packed with 5-micron C18 derivatized porous particles was performed at a flow rate of 2 mL/min to purify samples of 2A3NT, 2A6NT, 4A3NT, and 5A2NT oxidation products for ^{1}H nuclear magnetic resonance (NMR). A gradient elution was used in the same manner as for analytical HPLC, and compounds were detected by a UV detector (MiltonRoy, Model Spectro Monitor 3100) at 300 nm. NMR analysis was performed on a DRX-400 (400.144-MHz) instrument (Bruker BioSpin Corp., Billerica, Mass.) for 2-amino-4-nitro-*m*-cresol, 3-amino-5-nitro-*p*-cresol, and 2-amino-5-nitro-*o*-cresol and on an Avance 500 (500.133 MHz) instrument (Bruker BioSpin Corp., Billerica, Mass.) for 2-amino-3-nitrobenzylalcohol and 4-amino-3-nitrobenzylalcohol with a standard 30° pulse and 1.5-s relaxation delay. DMSO was used as the solvent.

RESULTS

Because of the prevalence of aminonitrotoluenes and the need to remediate them due to their toxicity, we have investigated the activity of dinitrotoluene enzymes on these substrates with the aims of determining if nitrite is released (as with dinitrotoluenes) and whether the methyl group or benzene rings are oxidized. To achieve these goals, we expressed the DDO enzymes in whole cells of *E. coli* so that competing dioxygenase reactions from the original soil bacteria would be eliminated and so that NADH would be provided for these reactions (it is likely bioremediation schemes would require whole cells, too, due to the NADH requirement). We also used an *E. coli* host with significantly reduced, non-specific nitroaromatic reduction activity so that all the products may be attributed to the expression of the dioxygenases rather than due to non-specific nitro group reduction.

Reaction Rates

It was discovered that wild-type DDO and its alpha subunit (DntAc) variants I204L, I204Y, and I204F have significant activity on aminonitrotoluenes. Figure 1 shows the reactions of the DDO enzymes with the aminonitrotoluenes 2A3NT, 2A4NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT.

HPLC was used to obtain the substrate depletion rates for the oxidation of the aminonitrotoluenes by wild-type DDO and the variant DntAc enzymes and to help identify some of the oxidation products based on the retention time and UV-visible spectra. It was found that wild-type DDO oxidizes 2A3NT, 2A5NT, 2A6NT, 4A3NT, and 5A2NT rapidly from 0.3 to 0.6 nmol/min/mg protein (Table I). These rates compare well to the rate for 2,4-DNT, the natural substrate (2.2 nmol/min/mg protein, (Leungsakul et al., 2005)). Additionally, it was found that the alpha subunit (DntAc) mutation I204Y enhances significantly (2- to 4-fold) the oxidization of 2A3NT, 2A5NT, 2A6NT, 4A3NT, and 5A2NT (Table I); therefore, mutation at this position is beneficial for creating enzymes with elevated activity for these substrates. For substrates 2A3NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT, the same oxidation products with the same retention times and UV-visible spectra were found with both wild-type DDO and the DDO DntAc I204L, I204Y, I204F variants whereas the negative control *E. coli* JVQ2 with pBS(Kan) did not have activity; hence, the results were corroborated using the different variant enzymes.

Product Identifications

Along with HPLC, GC-MS and NMR were used to identify the products from the oxidation of aminonitrotoluenes using the DDO DntAc I204Y variant (since it had the greatest activity, Table I). For 2A6NT, two distinct product peaks were detected via GC-MS (Table II) indicating the presence of either aminonitrocresols (actual MW 168 but seen as MW 240 since BSTFA was added for trimethylsilyl derivatization) or aminonitrobenzylalcohol (2A6NB, MW 168) were detected via GC-MS which had the same molecular weight (MW 168). HPLC and the authentic 2A6NB standard revealed 2A6NT was transformed to aminonitrocresols since the reaction products had different retention times and UV-visible spectra than the 2A6NB standard. Via NMR (Table II), the two products were identified as 2-amino-4-nitro-*m*-cresol and 3-amino-5-nitro-*p*-cresol (Fig. 1).

For the oxidation of 2A3NT by the DDO DntAc I204Y variant, GC-MS showed either the aminonitrocresol or the aminonitrobenzylalcohol was formed (MW 168); but, NMR (Table II) was used to distinguish that 2A3NB was formed (Fig. 1). Also, a molecular ion at m/z 168 was found using GC-MS without derivatization confirming the result with derivatization.

For the oxidation of 4A3NT, two distinct product peaks were detected via GC-MS (Table II) indicating either

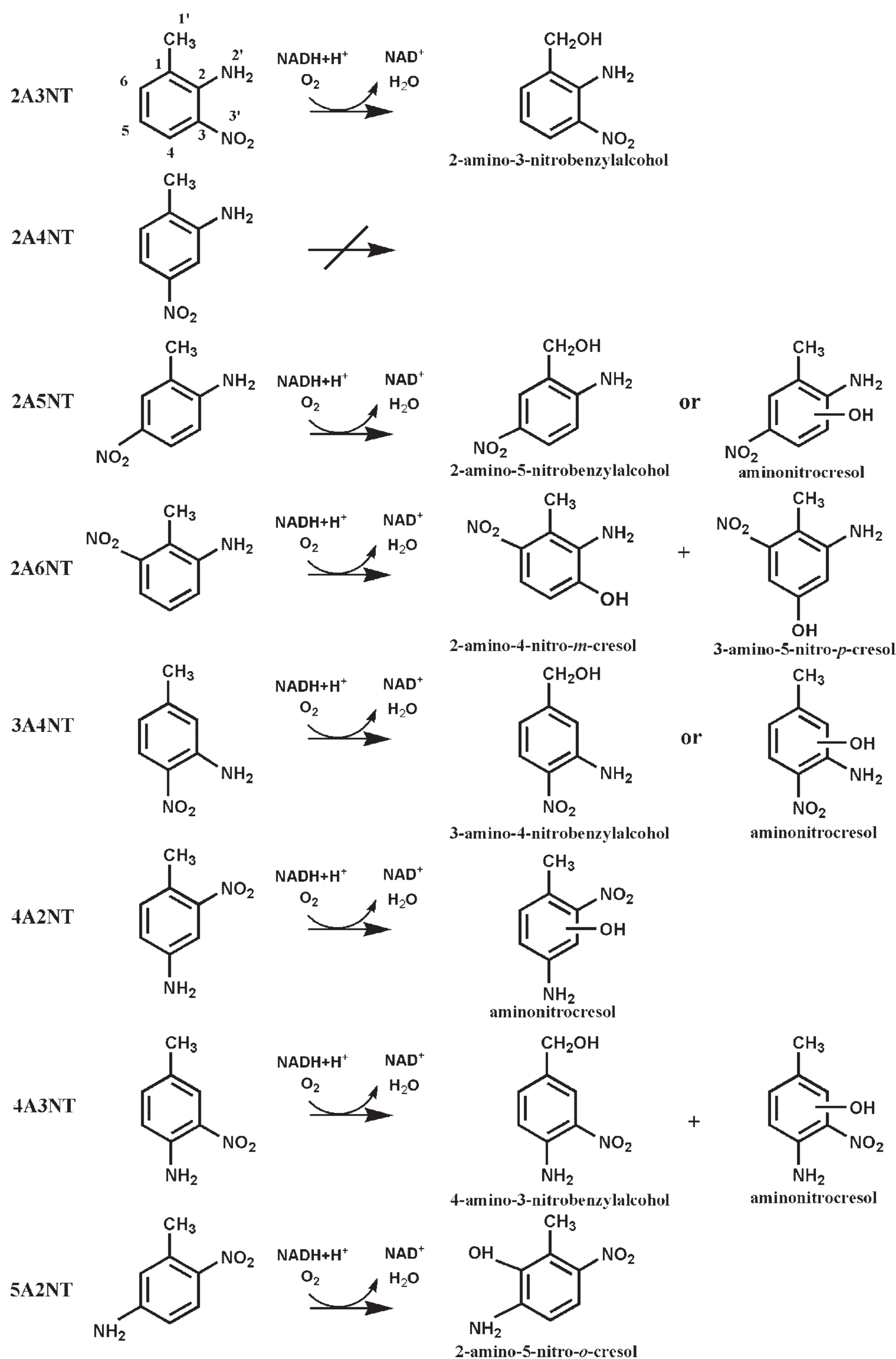

Figure 1. Oxidation of 2A3NT, 2A4NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT by *E. coli* JVQ2/pBS(Kan)DNT expressing DDO. The position of the single hydroxyl group that has not been confirmed for the aminonitrocresols are shown as an extended bond in the middle of the benzene ring.

aminonitrocresols or the aminonitrobenzylalcohol were formed by the DDO DntAc I204Y variant (MW 168). NMR analysis (Table II) was used to determine that 4A3NB was formed and the second product must be one of aminonitrocresol isomers.

For 5A2NT, GC-MS showed either aminonitrocresol or aminonitrobenzylalcohol (MW 168) was formed by the DDO DntAc I204Y variant, and NMR revealed (Table II) that the absolute configuration of the product was 2-amino-5-nitro-*o*-cresol (Fig. 1). Hence, the DDO DntAc I204Y variant oxidized either the methyl group or benzene ring depending on the isomers of the aminonitrotoluene.

GC-MS showed 4A2NT was transformed to either an aminonitrocresol or aminonitrobenzylalcohol (4A2NB) by

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Table I. Reaction rates with 2A3NT, 2A4NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT (300 μm substrate concentrations by *E. coli* JVQ2 expressing wild-type DDO and the alpha-subunit variant I204Y.

Reduced compound	Nitroaromatic source	Yield[1]	DDO Rate[2]	I204Y Rate[2]
2A3NT	2,3-DNT	27 ± 2	0.43 ± 0.07	1.69 ± 0.03
2A4NT	2,4-DNT	n/a	n/a	n/a
2A5NT	2,5-DNT	34.4 ± 0.6	0.6 ± 0.6	1.161 ± 0.003
2A6NT	2,6-DNT	20 ± 4	0.3 ± 0.1	1.0 ± 0.2
3A4NT	3,4-DNT	9 ± 4[3]	~0	0.22 ± 0.09
4A3NT	3,4-DNT	19	0.31	0.71 ± 0.09
4A2NT	2,4-DNT	13 ± 2[3]	~0	0.07 ± 0.01
5A2NT	2,5-DNT	24 ± 3	0.33 ± 0.09	1 ± 1

[1]Percent substrate converted by wild-type DDO after 60 min.
[2]Substrate depletion rate (nmol/min/mg protein).
[3]Percentage substrate converted by the variant DntAc DDO I204Y after 60 min.
n/a, not applicable.

the DDO DntAc I204Y variant. However, HPLC indicated the product must be an aminonitrocresol since the product had different retention times and UV-visible spectra compared to the authentic 4A2NB standard. GC-MS also showed that 2A5NT and 3A4NT were converted to either aminonitrocresols or the respective aminonitrobenzylalcohol (MW 168).

2A4NT was not oxidized by both wild-type DDO and the I204L, I204Y, I204F DDO DntAc variants. Further, there was no significant nitrite formed from the oxidation of any of the aminonitrotoluenes by the whole cells expressing DDO or its variants. These results corroborate the HPLC, GC-MS, and NMR results in that they show a single hydroxyl group was added to the substrate but a dioxygenase reaction with two hydroxyl groups added and nitrite removed (to form catechol) did not occur (e.g., 2,4-DNT is converted to 4M5NC but a similar reaction did not occur here). We also did not see activity on 2A4NT with the wild-type naphthalene dioxygenase from *Ralstonia* sp. strain U2 (NDO) when this dioxygenase was expressed from a previously constructed vector, pBS(Kan)U2 (Keenan et al., 2005).

Wild-type DDO and the alpha subunit I204L and I204F variants also oxidized 2A3NT to 2A3NB, 2A5NT to 2A5NB or aminonitrocresol, 2A6NT to 2-amino-4-nitro-*m*-cresol and 3-amino-5-nitro-*p*-cresol, 3A4NT to 3A4NB or aminonitrocresols, 4A2NT to aminonitrocresol, 4A3NT to 4A3NB and aminonitrocresol, and 5A2NT to 2-amino-5-nitro-*o*-cresol. Hence, these results served to corroborate those of the DntAc I204Y variant. Furthermore, in addition to the recombinant *E. coli* strains, it was found that *Burkholderia* sp. strain DNT has activity on 2A3NT, 2A5NT, and 5A2NT and produces the same oxidation products with the same retention times and UV-visible spectra as *E. coli* JVQ2/pBS(Kan)DNT.

DISCUSSION

These results show clearly that the DDO wild-type enzyme and its variants oxidize aminonitrotoluenes, the reduced

Table II. Oxidation products from 2A3NT, 2A4NT, 2A5NT, 2A6NT, 3A4NT, 4A2NT, 4A3NT, and 5A2NT by *E. coli* JVQ2 expressing wild-type DDO.

Reduced compound	Products	MW	Retention time (min)	GC-MS major fragment ions [M+ (% relative intensity)]	^{1}H NMR chemical shifts (δ)[1]
2A3NT	2-amino-3-nitrobenzylalcohol (1)	168 (240)	9.0	240 (84), 225 (74), 207 (87), and 75 (100)	7.94 (d, $J = 8$, H-4), 6.65 (dd, $J = 7$ and 8.5, H-5), 7.47 (d, $J = 7$, H-6), 4.50 (d, $J = 4.5$, $\underline{CH_2}$OH), 5.42 (t, $J = 5$, $CH_2\underline{OH}$)
2A4NT	None	—	—	—	
2A5NT	2-amino-5-nitrobenzylalcohol or aminonitrocresol (1)	168 (240)	10.7	240 (88), 225 (100), 207 (27), 151 (43), and 104 (37)	
2A6NT	2-amino-4-nitro-*m*-cresol and	168 (240)	8.2	240 (100), 179 (44), 164 (23), and 104 (21)	8.12 (s, OH), 7.14 (d, $J = 8$, H-4), 6.66 (d, $J = 8$, H- 5), 2.06 (s, CH_3)
	3-amino-5-nitro-*p*-cresol (2)		9.6	240 (67), 223 (100), 179 (35), and 164 (20)	8.45 and 8.57 (s, H-3 and H-5), 12.7 (bs, OH), 2.1 (s, CH_3)
3A4NT	3-amino-4-nitrobenzylalcohol or aminonitrocresols (2)	168 (240)	9.3	240 (100), 225 (100), 178 (30), and 122 (16)	
			10.4	240 (63), 225 (100), 195 (48), and 104 (20)	
4A2NT	aminonitrocresol (1)	168 (240)	9.8	240 (88), 223 (100), 178 (59), and 73 (100)	
4A3NT	4-amino-3-nitrobenzylalcohol and	168 (240)	8.1	240 (8), 147 (30), 70 (69), and 61 (100)	7.88 (s, H-2), 7.37 (s, NH_2), 7.33 (d, $J = 9$, H-5), 6.97 (d, $J = 9$, H-6), 4.36 (s, $\underline{CH_2}$OH), 8.16 (s, $CH_2\underline{OH}$)
	aminonitrocresol (2)		10.1	240 (57), 225 (36), 207 (100), and 151 (67)	
5A2NT	2-amino-5-nitro-*o*-cresol (1)	168 (240)	10.2	240 (31), 147 (52), 70 (82), and 61 (100)	8.02 (d, $J = 9.4$, H-3), 6.65 (d, $J = 9$, H-4), 10.40 (s, OH), 2.09 (s, CH_3)

The products were determined from HPLC, GC-MS and NMR (300 μM substrate concentrations). The number of product peaks from GC-MS are shown in parenthesis (products column). The molecular weights of the products are shown (derivatization values in parenthesis).
[1]Chemical shift multiplicities are abbreviated as follows: S, singlet; d, doublet; t, triplet; bs, broad singlet; dd; doublet of doublets. Coupling constants (J values) are given in hertz.

compounds of DNTs, to aminonitrocresols and aminonitrobenzylalcohols indicating that DDO and its variants act like monooxygenases by adding one hydroxyl group to the benzene ring or methyl group. Interestingly, the variant I204Y DDO DntAc produces the same products as the wild-type, but has two to four fold higher activity than the wild-type enzyme. Using HPLC, GC-MS, and NMR, the products from five of the seven reactions were determined. It is very interesting that each product from oxidation of the aminonitrotoluenes is different. For example, the oxidation products of 2A3NT and 4A3NT are 2A3NB and 4A3NB, respectively, but the oxidation product of 5A2NT is 2-amino-5-nitro-*o*-cresol. Hence, wild-type DDO and its variants hydroxylate both the methyl group and the aromatic ring of these aminonitrotoluene substrates.

Only 2A4NT was not oxidized by DDO and its variants. The substrate preference of DDO has been related previously to the position of the aromatic substituents. For example, 2,4-DNT dioxygenases require a nitro group *para* to the methyl substituent of the ring for the dioxygenase reaction with aminodinitrotoluenes (e.g., 2A46DNT) (Johnson et al., 2001). This is in contrast to nitrobenzene dioxygenase of *Comamonas* sp. strain JS765 that requires an amino group *para* to the methyl substituent of the ring for the dioxygenase reaction with aminodinitrotoluenes (e.g., 4A26DNT) (Johnson et al., 2001). In our case for 2A4NT, even though there is a nitro group *para* to the methyl substituent of the ring, there is no DDO activity if there is an amino group at the *ortho* position; whereas, the other 3 aminonitrotoluene isomers with an *ortho* amino group are good substrates (e.g., 2A3NT, 2A5NT, 2A6NT). In addition, the methyl groups of the aminonitrotoluene isomers are not hydroxylated if they are *ortho* to the nitro group (e.g., 2A6NT, 4A2NT, 5A2NT). Furthermore, there was no confirmed addition of a hydroxyl group *ortho* to the nitro group with this enzyme.

The aminonitrotoluene oxidation rates by DDO are slower compared to the original substrate, 2,4-DNT (2.2 nmol/min/mg protein), but are significant since this is the first report of the enzymatic activity toward aminonitrotoluenes. Furthermore, these rates are comparable to those reported for the transformation of 4A26DNT by nitrobenzene dioxygenase of *Comamonas* sp. strain JS765 (0.53 nmol/min/mg protein) (Johnson et al., 2001). Since the oxidation rates of aminonitrotoluene are relatively slow compared to toluene oxidation by monooxygenases (Tao et al., 2004), directed evolution might be a good way to improve the enzyme activity. For example, protein engineering has been used by us to create NDO variants that transform 2,6-DNT to 3-methyl-4-nitrocatechol as well as 2A46DNT to 2-amino-4,6-dinitrobenzylalcohol and 3-amino-4-methyl-5-nitrocatechol (Keenan et al., 2005) and has been used to engineer toluene dioxygenase from *Pseudomonas putida* F1 to create a variant that transforms 4-picoline 5.6-fold higher than the wild-type (Sakamoto et al., 2001).

Nitroaromatic dioxygenases may behave as monooxygenases by performing a single hydroxylation as has been shown previously (Keenan et al., 2004). Here, we show a whole family of aminonitrotoluenes are oxidized through a single hydroxylation. Other examples of monooxygenase reactions with dioxygenases include nitrobenzene dioxygenase (Lessner et al., 2002) and DDO (Leungsakul et al., 2005; Parales et al., 1998) on 4-nitrotoluene (forming 4-nitrobenzylalcohol) and on 2-nitrotoluene (forming 2-nitrobenzylalcohol), nitrobenzene dioxygenase and R34 DDO on 2-amino-4,6-dinitrotoluene (forming 2-amino-4,6-dinitrobenzylalcohol) (Johnson et al., 2001), as well as the R34 DDO alpha subunit variant V350F on *o*-cresol (forming 2-hydroxy benzylalcohol and methylhydroquinone) (Keenan et al., 2004). Naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816 also behaves like a monooxygenase toward a number of benzocyclic and alkyl-substituted aromatic compounds such as indan (forming indanol) and toluene (forming benzylalcohol) (Resnick et al., 1996).

In addition, isomers of 2-amino-4-nitro-*m*-cresol and 3-amino-5-nitro-*p*-cresol (generated here from the oxidation of 2A6NT), 6-amino-4-nitro-*o*-cresol and 2-amino-5-nitro-*p*-cresol, have been evaluated as a fungistatic compound (Zsolnai, 1961) and as an intermediate for an anti-parasitic agent (Haugwitz et al., 1982), respectively. Hence, we present not only new enzymatic activity of DDO toward aminonitrotoluenes but also allow suggest that some of these compounds may be useful as intermediates in biocatalysis.

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