Oxidative Transformation of Aminodinitrotoluene Isomers by Multicomponent Dioxygenases

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The electron-withdrawing nitro substituents of 2,4,6-trinitrotoluene (TNT) make the aromatic ring highly resistant to oxidative transformation. The typical biological transformation of TNT involves reduction of one or more of the nitro groups of the ring to produce the corresponding amine. Reduction of a single nitro substituent of TNT to an amino substituent increases the electron density of the aromatic nucleus considerably. The comparatively electron-dense nuclei of the aminodinitrotoluene (ADNT) isomers would be expected to be more susceptible to oxygenase attack than TNT. The hypothesis was tested by evaluating three nitroarene dioxygenases for the ability to hydroxylate the ADNT isomers. The predominant reaction was dioxygenation of the ring to yield nitrite and the corresponding aminomethylnitrocatechol. A secondary reaction was benzylic monooxygenation to form aminodinitrobenzyl alcohol. The substrate preferences and catalytic specificities of the three enzymes differed considerably. The discovery that the ADNT isomers are substrates for the nitroarene dioxygenases reveals the potential for extensive bacterial transformation of TNT under aerobic conditions.

The manufacture of 2,4,6-trinitrotoluene (TNT) for use in military and industrial explosives and its disposal have resulted in extensive environmental contamination. Most contamination is limited to manufacture and disposal sites, but it is still problematic, since TNT persists in the environment for long periods, is acutely toxic, and is readily transformed into carcinogenic compounds (13). Current strategies for remediation of TNT-contaminated soil involve a number of practices, including chemical treatment, physical processes, land farming, phytoremediation, and other combined biological processes (1, 16, 31). However, no current treatment short of incineration is widely used for complete detoxification and mineralization of TNT.

The most common biological transformation of TNT is non-specific reduction of the nitro substituents (Fig. 1A) via the sequential addition of three electron pairs to reduce a nitro group to a nitroso group, a hydroxylamino group, and finally an amino group (30). Under strictly anaerobic conditions, all three nitro substituents can be reduced to the amine (5, 18). In aerobic systems, partially reduced products accumulate. The predominant products under aerobic conditions are the aminodinitrotoluene (ADNT) isomers and, to a lesser extent, 2,4-diamino-6-nitrotoluene and azoxytetranitrotoluenes (4, 5, 12, 17, 24, 39). A productive reduction of TNT is catalyzed by the nitrate ester reductase from *Enterobacter cloacae* PB2. The reduction leads to liberation of nitrite and allows the strain to grow using TNT as the sole nitrogen source (9).

Previous work defined the oxidative pathways for biodegradation of nitrobenzene and 2,4-dinitrotoluene (2,4-DNT) (19, 32). The initial step in each case is dihydroxylation of the aromatic ring (Fig. 1B and C); the nitro substituent then serves as an anionic leaving group in the reaction to produce the

corresponding catechol. The genes encoding nitroarene dioxygenases have been cloned from *Burkholderia* sp. strain DNT (35), *Comamonas* sp. strain JS765 (D. J. Lessner, G. R. Johnson, R. E. Parales, J. C. Spain, and D. T. Gibson, submitted for publication), and *Burkholderia cepacia* R34 (B. E. Haigler, C. C. Somerville, J. C. Spain, and R. K. Jain, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. Q343, 1997). The three nitroarene dioxygenases have high amino acid sequence identity, but the substrate preference and catalytic specificity of the enzymes differ despite their sequence similarity (22; Lessner et al., submitted).

Various oxygenases catalyze oxidation of the methyl substituent of nitroarenes. It can be a productive reaction, as in the pathways for 4-nitrotoluene degradation in *Pseudomonas* sp. strain 4NT (11) and *Pseudomonas* sp. strain TW3 (25). Many oxygenases gratuitously transform the methyl group on nitroarenes to the corresponding benzyl alcohol (6, 20, 26). Other reports describe biological oxidation of TNT. *Pseudomonas* sp. strain JLR11 transforms TNT to 2,4,6-trinitrobenzaldehyde (7). Bruns-Nagel et al. (3) found 2,4,6-trinitrobenzoate in extracts obtained from TNT-contaminated soils, although they could not define the source of the oxidant in the complex system.

Structurally, TNT is similar to 2,4-DNT and 2,6-DNT, which suggests that the dioxygenases that catalyze the oxidation of dinitrotoluenes could also hydroxylate TNT. However, no compelling evidence of oxygenolytic removal of the nitro groups of TNT has been reported. Oxidative attack on the ring appears to be severely hampered by the combined electron-withdrawing effects of the three nitro substituents. The partial reduction of TNT to 2- or 4-ADNT creates an electron-donating amino group on the ring in place of the electron-withdrawing nitro group. The increase in electron density of the ring should increase the potential for electrophilic attack by dioxygenase enzymes (15).

The aim of the present study was to test the hypothesis that

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FIG. 1. (A) Pathway for bacterial reduction of TNT (30). The monoamino compounds are typical stable end products under aerobic conditions. Further reduction generally requires lower redox potentials (17). (B) Oxidative transformation of nitrobenzene to catechol in the nitrobenzene pathway from strain JS765 (19). (C) Oxidative pathway for 2,4-DNT degradation (32). The initial transformation is catalyzed by 2,4-DNT dioxygenase. a, 2A46DNT is formed in equivalent reactions.

partial reduction of TNT to ADNT isomers makes the aromatic ring susceptible to attack by multicomponent nitroarene dioxygenases. Oxygenolytic transformation of 2-amino-4,6-dinitrotoluene (2A46DNT) and 4-amino-2,6-dinitrotoluene (4A26DNT) was evident from transformation of ADNT to a dihydroxylated aromatic compound with concomitant evolution of nitrite. The oxidation of the ADNT isomers and identification of the hydroxylated products suggests that the electronic nature of TNT rather than steric hindrance is primarily responsible for its resistance to attack by nitroarene dioxygenases. The partial reduction of TNT to ADNT, followed by dioxygenation of the ADNT, could account for significant transformation of TNT under aerobic conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5 α was the host strain for recombinant plasmids containing the oxygenase genes. Bacteria were grown in Luria-Bertani medium (Difco Inc., Detroit, Mich.) containing glycerol (0.5%) and ampicillin (100 mg/liter) or kanamycin (50 mg/liter) to maintain plasmid selection. The genes encoding the dioxygenase operons were cloned into various vectors under control of the *lac* promoter to allow regulated gene expression. The 2,4-DNT dioxygenase from strain DNT was cloned within vector pGEM7f (Promega, Inc., Madison, Wis.) as a 6.8-kb *Nsi*I restriction fragment to yield plasmid pJS48 (34). The 2,4-DNT dioxygenase from strain R34 was cloned on a 5.6-kb *Sac*I/*Eco*RV fragment from pJS329a (Haigler et al., Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997) into vector pK18 (23) to yield plasmid pJS1329. The recombinant plasmid pDTG927 served as the source for nitrobenzene dioxygenase (Lessner et al., submitted). Plasmid pDTG927 con-

tains a 4.5-kb DNA fragment from *Comamonas* sp. strain JS765 (19) cloned into vector pUC18.

Transformation of aminodinitrotoluene. *E. coli* DH5 α strains were grown in Luria-Bertani medium–glycerol at 30°C with shaking to an A_{600} of 0.4 to 0.6, and then isopropyl- α -D-thiogalactopyranoside (IPTG; 1 mM) was added to induce synthesis of the oxygenase. After 4 h, the cells were harvested by centrifugation, washed in phosphate buffer (20 mM; pH 7.5), and then suspended in buffer.

Transformation assays were done in triplicate in 125-ml baffled flasks containing cells (final A_{600} , 0.3 to 0.5), 10 ml of phosphate buffer with pyruvate (1 mM), and the aminodinitrotoluene substrates (25 μ M). The suspensions were incubated with shaking (250 rpm; 30°C), and samples were collected at appropriate intervals for high-performance liquid chromatography (HPLC) and nitrite analysis to monitor the progress of the reaction. The reaction was stopped by mixing the samples with one-fifth volume of acetonitrile and centrifuging the mixture to remove the cells. Reaction rates were estimated from the initial slopes of the substrate disappearance curves.

The samples (25 ml) for gas chromatography-mass spectrometry analyses were obtained from cell suspensions that had been incubated with ADNT for 4 to 12 h. Following incubation, the cells were removed by centrifugation and the aqueous phase was acidified to pH 2.5. The reaction products were extracted three times with 8 ml of ethyl acetate (previously washed with 1 N NaOH). The organic phase was dried over anhydrous sodium sulfate, the solvent was evaporated under nitrogen, and the residue was dissolved in acetonitrile.

Larger quantities of the putative 3-amino-4-methyl-5-nitrocatechol (3A4M5NC) were produced by incubating 2A46DNT with *E. coli* DH5 α (pJS48) until the 2A46DNT was depleted. The 3A4M5NC was collected from reaction supernatants on a C_{18} 35 cm^3 solid-phase extraction cartridge (Millipore, Milford, Mass.) and eluted from the matrix with methanol-water (50:50). The eluate was diluted to 20% methanol, acidified to pH 2.5, and extracted with ethyl acetate. The ethyl acetate was removed under vacuum, and the product purity was confirmed by HPLC. The 3A4M5NC gave UV-visible light absorbance maxima at 459 nm (ϵ =

FIG. 2. Transformation of ADNTs by nitroarene dioxygenases. The data are averages of triplicate reactions. ■, 4A26DNT (A) and 2A46DNT (B and C); ▲, nitrite. (A) Nitrobenzene dioxygenase [*E. coli* DH5 α (pDTG927)]; 0.15 mg of protein ml⁻¹. (B) 2,4-DNT dioxygenase from strain DNT [*E. coli* DH5 α (pJS48)]; 0.08 mg of protein ml⁻¹. (C) 2,4-DNT dioxygenase from strain R34 [*E. coli* DH5 α (pJS1329)]; 0.13 mg of protein ml⁻¹.

4,130 M⁻¹) and 207 nm (ϵ = 17,140 M⁻¹) with a slight shoulder at 230 nm in water at pH 7.0.

Analytical methods. HPLC analysis was done with a Hewlett-Packard (Santa Clarita, Calif.) model 1050 chromatography system equipped with a model 1040 M diode array detector. Compounds were separated on a Supelcosil LC-ABZ-Plus column (25 by 4.6 mm) (Supelco, Bellefonte, Pa.) with a mobile phase of acetonitrile–0.1% trifluoroacetic acid (1 ml min⁻¹). The mobile phase was held at 25% acetonitrile for 6 min, increased to 60% acetonitrile over 1 min, held at 60% acetonitrile for 8 min, and then returned to initial conditions. The UV absorbance was monitored at 230 and 330 nm.

Trimethylsilyl derivatives were prepared with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Alltech Associates Inc., Deerfield, Ill.) according to methods provided by the distributor. Gas chromatography-mass spectrometry analyses were done on a Hewlett-Packard series 5971 mass spectrometer and Hewlett-Packard model 5890 gas chromatograph with an HP-5 M.S. capillary column (30 m by 0.25 mm; 0.25- μ m film thickness; Hewlett-Packard). Helium was the carrier gas at a constant flow rate of 0.8 ml min⁻¹; the injector and transfer line temperatures were 280 and 300°C, respectively. The chromatography program was as follows: initial column temperature of 100°C for 1 min, increased at 10°C min⁻¹ to 300°C, and isothermal for 10 min. The ionization voltage and electron multiplier settings were 70 eV and 2,000 V, respectively.

Nitrite concentrations were measured using standard methods (28). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) (29).

Chemicals. 2A46DNT and 4A26DNT were from Accustandards Inc. (New Haven, Conn.); other chemicals were from Aldrich Chemicals (Milwaukee, Wis.).

RESULTS AND DISCUSSION

Transformation of ADNT. In preliminary experiments, ADNT isomers were transformed by recombinant *E. coli* strains carrying genes for nitroarene dioxygenases (data not shown). The nitrobenzene dioxygenase appeared to hydroxylate both the 2-amino- (2A46DNT) and 4-amino-dinitrotoluene (4A26DNT) isomers. However, nitrite only accumulated in reactions with 4A26DNT as the substrate. A single product was found during analysis of the ethyl acetate extract from 2A46DNT reaction mixtures. The mass spectrum of the derivatized reaction product (retention time [R_t], 11.3 min; predominant ions [*m/z*], 285, 270, 222, 195, 176, 149, 75, 73 [trimethylsilane]) was consistent with oxidation of the methyl substituent of the ring to yield 2-amino-4,6-dinitrobenzyl alcohol. The BSTFA-derivatized compound gave a molecular ion of 285 atomic mass units (amu), consistent with the monohydroxylated 2A46DNT derivatized at a single functional group. Monooxygenation of the methyl substituent of nitroaromatic compounds is commonly catalyzed by multicomponent dioxygenases (21, 26). The mass spectrum of the reaction product, previously described reactions of multicomponent dioxygenases, and absence of any nitrite accumulation were consistent with benzylic hydroxylation of 2A46DNT rather than with attack on the aromatic ring. 2,4-DNT dioxygenases did not hydroxylate 4A26DNT but did transform 2A46DNT with concomitant accumulation of nitrite, which provided presumptive evidence for dioxygenation of the substrate. Subsequent experiments, therefore, focused on substrate-enzyme combinations that showed evidence for dioxygenation of the substrate.

Initial transformation rates differed substantially among the strains expressing the different oxygenases. 4A26DNT was transformed at 0.53 nmol min⁻¹ mg of protein⁻¹ by the strain carrying the nitrobenzene dioxygenase genes, and 2A46DNT was transformed at 9.26 nmol min⁻¹ mg of protein⁻¹ by the strain carrying the 2,4-DNT dioxygenase genes from strain DNT and at 6.33 nmol min⁻¹ mg of protein⁻¹ by the strain

TABLE 1. Dioxygenase specific activity for recombinant *E. coli* strains carrying the nitroarene dioxygenases

Dioxygenase (strain)	Sp act ^a				Relative activity
	Nitrobenzene ^b	2,4-DNT ^b	2-ADNT ^c	4-ADNT ^c	
Nitrobenzene dioxygenase	4.6			0.87	0.19
2,4-DNT dioxygenase (DNT)		15.2	4.4		0.23
2,4-DNT dioxygenase (R34)		22.2	5.4		0.24

^a Nanomoles of nitrite per minute per milligram of protein.

^b 50 μ M.

^c 25 μ M.

carrying the 2,4-DNT dioxygenase genes from strain R34 (Fig. 2). *E. coli* DH5 α did not catalyze transformation of either ADNT isomer. No further experiments were done to optimize oxygenase activities for the individual strains. Additional assays compared dioxygenase activities with ADNT and the physiological substrate of each oxygenase (Table 1). The range of relative activities was narrow, which suggested that the oxygenases had comparable preferences for their respective ADNT isomers.

ADNT disappearance coincided with accumulation of nitrite in the reaction supernatant. Substrate disappearance and nitrite accumulation were stoichiometric in reactions catalyzed by the nitrobenzene dioxygenase (Fig. 2A) and 2,4-DNT dioxygenase (Fig. 2B) from strain DNT. The ratios of nitrite accumulation to substrate loss measured over the time course reactions were 1.00 and 0.98, respectively. In contrast, the ratio of nitrite accumulation to substrate loss was 0.42 for the reaction catalyzed by the 2,4-DNT dioxygenase from strain R34 (Fig. 2C). The discrepancy suggested that oxidative transformation of 2A46DNT by the dioxygenase from strain R34 was not limited to hydroxylation of the aromatic ring. The ratios of 3-amino-4-methyl-5-nitrocatechol to substrate loss were 1.07 and 0.50 for the reactions with the 2,4-DNT dioxygenases from strains DNT and R34, respectively, consistent with that predicted from nitrite concentration.

Identification of products. The recombinant strain carrying the nitrobenzene dioxygenase [*E. coli* DH5 α (pDTG927)] transformed 4A26DNT into a single compound. The BSTFA-derivatized product had a retention time of 10.85 min. The mass spectrum was consistent with an aminomethylnitrocatechol (molecular weight, 184) derivatized on the two hydroxyl substituents of the ring (Fig. 3A). Although an amino substituent can be derivatized by trimethylsilane (14), the reaction conditions used here allowed substitution of the protons only on the more reactive hydroxyl substituents. The only catechol isomer that could result from dioxygenation of 4A26DNT and generate the mass spectrum in Fig. 3A is 3-amino-6-methyl-5-nitrocatechol.

The recombinant strain carrying the 2,4-DNT dioxygenase from strain DNT [*E. coli* DH5 α (pJS48)] transformed 2A46DNT into a single product. BSTFA derivatization yielded a single compound with a gas chromatography retention time of 10.65 min (Fig. 3B). The mass spectrum of the compound was very similar to that of the product from the nitrobenzene dioxygenase-catalyzed transformation of 4A26DNT (Fig. 3A). Either the *ortho*- or the *para*-nitro substituent of 2A46DNT could be removed in the reaction to yield three possible dihy-

FIG. 3. Mass spectra of metabolites from oxidation of 4A26DNT by nitrobenzene dioxygenase [*E. coli* DH5 α (pDTG927)] (A) and oxidation of 2A46DNT by 2,4-DNT dioxygenase [*E. coli* DH5 α (pJS48)] (B) or [*E. coli* DH5 α (pJS1329)] (C and D).

droxy compounds. Because the enzyme was unable to transform and denitrate the 4A26DNT isomer, hydroxylation at the 5,6 position seems improbable. In addition, the 311-amu fragment is $[M-O-H]^+$, a fragment characteristic of a nitro group *ortho* to a methyl group. By analogy to the transformation of 2,4-DNT (Fig. 1C), the most likely product is 3-amino-4-methyl-5-nitrocatechol. Rigorous identification will require nuclear magnetic resonance analysis.

The 2,4-DNT dioxygenase from strain R34 was less specific; it produced two hydroxylated products. The first (Fig. 3C) was identical to the putative 3-amino-4-methyl-5-nitrocatechol produced by the 2,4-DNT dioxygenase from strain DNT (Fig. 3B). The mass spectrum of the second product (Rt 11.2) (Fig. 3D) was consistent with a monohydroxylated aminodinitrotoluene derivatized on the hydroxyl group. Benzylic hydroxylation is often catalyzed by dioxygenases and was shown specifically for nitroarene dioxygenases with various nitroaromatic compounds as substrates (20; Lessner et al., submitted). Accordingly, the second product appears to be 2-amino-4,6-dinitrobenzyl alcohol.

The reaction products were identified by analogy to known compounds and characteristic fragments in their mass spectra. To our knowledge, this is the first report of the aminomethylnitrocatechol isomers. 2-Amino-4,6-dinitrobenzyl alcohol and 4-amino-2,4-dinitrobenzyl alcohol have been chemically synthesized (27). Mass spectra of the underivatized 2-amino-4,6-dinitrobenzyl alcohol ($[M]^+$ 213, 196, 179) from our reactions (not shown) correspond with the major ion fragments reported by Schmidt et al. (27).

The substrate preferences of the dioxygenases can be related to the regiospecific hydroxylation of their physiological substrates. The 2,4-DNT dioxygenases appear to require a nitro group *para* to the methyl substituent of the ring for the dioxygenase reaction (Fig. 4). The proposed oxidation of 2A46DNT is analogous to the formation of 4-methyl-5-nitrocatechol in the 2,4-DNT pathway. The nitrobenzene dioxygenase showed the opposite specificity. When the amino substituent was in the *ortho* position, the nitrobenzene dioxygenase only hydroxylated the methyl group by a monooxygenase reaction (Fig. 4). If the amino substituent was in the *para* position, nitrobenzene dioxygenase catalyzed the typical dioxygenase reaction to form the catecholic product and remove one of the nitro substituents (Fig. 4). So, not unexpectedly, substitutions on the aromatic ring influence the regiospecificity of the oxygenase reaction. It is probable that the substitutions alter the positioning of the substrate in the enzyme active site. Additional studies of the three-dimensional structures of the nitroarene dioxygenases will determine the true basis for the catalytic differences.

To date, no oxygenase enzymes have been shown to attack the aromatic ring of trinitroaromatic compounds. The combination of steric effects from the four ring substituents, along with the electron deficiency of the ring due to the nitro groups, make electrophilic oxidative attack on TNT an extreme challenge. The reduction of one nitro substituent to an amine changes the electronic properties of the compound and allows oxidative transformation. The results of recent work indicate that ligninolytic fungi mineralize ADNTs via an oxidative route, but no degradation intermediates have been identified (10, 36, 37). Other studies indicated that certain bacteria transform ADNTs to the corresponding benzoic acid derivatives

FIG. 4. Dioxygenase-catalyzed oxidation of 2A46DNT and 4A26DNT. NBz, nitrobenzene; X, reaction was not detected.

(38) and other unidentified polar compounds in oxygen-dependent reactions (2). *Pseudomonas pseudoalcaligenes* JS52 reduces 4A26DNT to 2-hydroxylamino-4-amino-6-nitrotoluene, which is transformed to 4-amino-2-nitroso-6-nitrotoluene and other unidentified compounds in oxygen-dependent reactions (8). Another pathway is used by *Clostridium acetobutylicum* to hydroxylate the aromatic ring. Two of the nitro groups are reduced to form 2,4-dihydroxylamino-6-nitrotoluene; the *para* substituent is then reduced to form 4-amino-2-hydroxylamino-6-nitrotoluene. The 4-amino-2-hydroxylamino-6-nitrotoluene is subsequently transformed to a compound tentatively identified as 2-amino-5-hydroxy-4-hydroxylamino-6-nitrotoluene (J. Hughes, personal communication). Ozone-mediated oxidation of ADNTs yields nitrate, nitrite, and small organic molecules, including pyruvic and glyoxylic acids (33), which could be assimilated in other biodegradative pathways.

The results presented here reveal that bacterial oxygenases can specifically oxidize ADNT isomers. The findings expand the known substrate ranges of the nitroarene dioxygenases beyond the mono- and dinitrotoluenes. Subsequent degradation of the ADNT oxidation products has not been examined. We plan to discover whether the dihydroxy compounds that result from the dioxygenase reaction are substrates for ring fission enzymes.

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