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Oxidative Release of Nitrite from 2-Nitrotoluene by a Three-Component Enzyme System from *Pseudomonas* sp. Strain JS42

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***Pseudomonas* sp. strain JS42 utilizes 2-nitrotoluene (2NT) as the sole source of carbon and energy for growth. Intact cells catalyze the oxidation of 2NT to 3-methylcatechol and nitrite in a reaction that requires molecular oxygen. Cell extracts oxidized 2NT to 3-methylcatechol and nitrite in the presence of NAD(P)H and ferrous iron. Ion-exchange chromatography yielded three protein fractions (A, B, and C) which were all required for the oxidation of 2NT to 3-methylcatechol and nitrite. Component B (reductase$_{2NT}$) catalyzed a NAD(P)H-dependent reduction of cytochrome *c*. Solutions of component A (ISP$_{2NT}$) were brown and showed absorption maxima at 458 and 324 nm. Two major bands with M_rs 52,500 and 28,000 were observed when ISP$_{2NT}$ was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Component C could be replaced by ferredoxin$_{NAP}$ from the *Pseudomonas putida* NCIB 9816-4 naphthalene dioxygenase system and was given the designation ferredoxin$_{2NT}$. Experiments with $^{18}O_2$ showed that both oxygen atoms were added to the aromatic ring of 2NT to yield 3-methylcatechol. The enzyme is a new multicomponent enzyme system which we have designated 2NT 2,3-dioxygenase.**

Nitroaromatic compounds are widely distributed in the environment because of their common use in the production of dyes, pesticides, and explosives. 2-Nitrotoluene (2NT) is one of the by-products from 2,4,6-trinitrotoluene production (32). However, little is known about its biodegradation.

Polar nitroaromatic compounds are more accessible to electrophilic attack by oxygenases than nonpolar molecules. Consequently, a considerable amount of information is available about the oxidation of nitrophenols (3, 27, 28, 34) and nitrobenzoates (5, 6, 14) by aerobic bacteria. Two nitrophenol monooxygenases oxidizing 4- or 2-nitrophenol have been studied (27, 28, 35), and the one for 2-nitrophenol oxidation from *Pseudomonas putida* B2 has been purified and characterized elsewhere (35). In contrast, enzymatic oxidation of nonpolar nitroaromatic compounds, such as nitrotoluenes and nitrobenzenes, has been reported only in the last 5 years (9, 10, 12, 18, 29). For example, the initial reaction in the oxidation of 2,4-dinitrotoluene (2,4DNT) by a *Pseudomonas* species is catalyzed by a dioxygenase. Oxygen is incorporated at the 4,5 position to form 4-methyl-5-nitrocatechol, and nitrite is released (29). The genes encoding DNT dioxygenase have been cloned in *Escherichia coli* (31). However, the enzyme has not been purified, and the enzymatic mechanism for the reaction is not known.

Toluene dioxygenases from *P. putida* F1 and *Pseudomonas* sp. strain JS150 oxidize the methyl group of 2NT and 3NT and the aromatic nucleus of 4NT (23). In contrast, toluene monooxygenase encoded by the TOL plasmid does not oxidize 2NT and oxidizes the methyl group of 3NT and 4NT (8).

Recently, a *Pseudomonas* sp. strain JS42 that is able to use 2NT as the sole source of carbon, energy, and nitrogen was isolated from nitrobenzene-contaminated soil. Studies with intact cells and cell extracts indicated that the initial reaction in 2NT degradation involves the oxidative release of nitrite, with the concomitant formation of 3-methylcatechol (19). This reaction could be catalyzed by two sequential monooxygenation reactions or by a dioxygenase. We now report that the reaction is catalyzed by a multicomponent enzyme system which requires three protein fractions for activity. We also present direct evidence to demonstrate that the initial reaction is catalyzed by a dioxygenase which we have designated 2NT 2,3-dioxygenase.

MATERIALS AND METHODS

Cell growth and preparation on cell extract. *Pseudomonas* sp. strain JS42 was maintained on Luria broth agar plates. The organism was cultured in 2.8-liter flasks each containing 500 ml of tryptic soy broth medium at 30°C. Cells were collected at late log phase by centrifugation (13,800 × *g* for 10 min at 4°C) and were washed once with 20 mM NaK phosphate buffer, pH 7.0. Cell paste (8 g [wet weight]) was resuspended in a breakage buffer (MEG) containing 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 7.0), 5% (vol/vol) ethanol, and 5% (vol/vol) glycerol. Phenylmethylsulfonyl fluoride and dithiothreitol were added to final concentrations of 0.2 and 1.0 mM, respectively. DNase I was also added to a final concentration of 20 μg/ml before cell breakage. Cell extract was obtained by a single passage through a chilled French press cell at 18,000 lb/in^2. Whole cells and cell debris were removed by centrifugation at 13,800 × *g* and 4°C for 0.5 h. Ultracentrifugation was carried out at 146,000 × *g* for 1 h at 4°C. The clear supernatant solution was frozen in liquid nitrogen and stored at −70°C or was used immediately for purification.

Escherichia coli JM109(DE3)(pDTG135) contains the cloned *nahAb* gene encoding the ferredoxin$_{NAP}$ component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4

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(30). Expression of *nahAb* is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T_7 promoter vector PT7-7. Cells were cultured at 30°C in T_7 medium (30) containing 20 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter, 0.2% glycerol, and 50 mM phosphate buffer (pH 7.0). Expression of the *nahAb* gene was initiated by the addition of IPTG (200 μM) when the cell density at 600 nm reached 0.5. Cells were harvested at 3.5 h after the addition of IPTG. Cell extracts were obtained by the method described above for *Pseudomonas* sp. strain JS42.

Enzyme assays. 2NT dioxygenase activity was determined by the measurement of nitrite released from the substrate at 30°C. Nitrite was determined by a modification of the method described by Smibert and Krieg (26). The 1.0-ml reaction mixture contained 50 mM MES buffer (pH 6.5), 0.4 mM NADH, 0.2 mM ferrous ammonium sulfate, 1.0 mM 2NT, and 0.05 to 0.5 mg of protein (cell extract). The reaction was initiated by the addition of 4 μl of 250 mM 2NT in methanol, and incubation was carried out at 30°C and 220 rpm in a water bath shaker. At appropriate times, 50 μl of the reaction mixture was added to 1.0 ml of nitrite assay solution, which contained equal volumes of 1% (wt/vol) sulfanilamide and 0.02% (wt/vol) *N*-(1-naphthyl)ethylene diamine in 1.5 N HCl. The A_{540} of the pink complex was measured after 30 min. The amount of nitrite present was determined from a calibration curve of sodium nitrite over the concentration range of 10 to 400 μM. In control experiments, 2NT was omitted from the reaction mixtures. The concentrations of 2NT, NADH, and ferrous iron were optimized for maximum enzymatic activity. This assay procedure was also used to determine the activity of partially purified components (A, B, and C) resolved by anion-exchange chromatography (see below). One unit of 2NT dioxygenase activity was defined as the amount of protein required to convert 1 nmol of 2NT to nitrite in 1 min.

The enzymatic reduction of cytochrome *c* was also used to assay component B at room temperature as described by Haddock et al. (15). The reaction mixture (1.0 ml) contained 50 mM bis(2-hydroxyethyl)imino-Tris(hydroxymethyl)methane (bis-Tris) buffer, pH 7.0, 87 μM horse heart cytochrome *c*, 400 μM NADH, and 2 to 100 μg of protein. The reaction was started by the addition of NADH. The rate of cytochrome *c* reduction was determined by measuring the increase in A_{550}. Concentrations of reduced cytochrome *c* were calculated from a molar extinction coefficient of $\varepsilon = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$. One unit of NADH cytochrome *c* reductase activity was defined as the amount of protein required to reduce 1 nmol of cytochrome *c* per min.

Protein concentrations were measured by the Bradford method (2) with a Bio-Rad protein assay kit. Bovine serum albumin was used as the standard.

Q-Sepharose chromatography of cell extract. Crude cell extract prepared from *Pseudomonas* sp. strain 2NT (15 ml, 350 mg of protein) was applied to the top of a Q-Sepharose HP 26/10 column (2.6 by 10 cm; Pharmacia LKB Biotechnology). The column was equilibrated with 300 ml of 50 mM MEG buffer (pH 6.5) prior to the application of cell extract. Unbound protein was eluted from the column with 100 ml of the same buffer at a rate of 5 ml/min. Bound protein was eluted with a linear gradient of 0.0 to 0.45 M KCl in 50 mM MEG buffer. Fractions (5.0 ml) were collected and assayed for enzymatic activity. The protein concentration in each fraction was monitored by the A_{280} with a Pharmacia Monitor UV-M detector. The column was operated with a Pharmacia fast protein liquid chromatography system at 6°C. Three fractions (A, B, and C, based on elution order) were found to be necessary for 2NT oxidation. Active fractions were pooled and concentrated by an Amicon ultrafiltration system (Beverly, Mass.). The concentrated fractions were used immediately or stored at −70°C.

$^{18}O_2$ incorporation experiments. The reaction mixture (5.0 ml) contained 50 mM MES buffer (pH 6.5), 0.4 mM ferrous ammonium sulfate, 0.4 mM NADH, 1.0 mM 2NT, 0.96 mg of component A, 0.72 mg of component B, and 10.6 mg of cell extract from JM109(DE3)(pDTG135). All components except for 2NT were added together in a 28-ml flask fitted with a Teflon-lined septum and a screw-on-hole cap (Supelco, Bellefonte, Pa.). The headspace (23 ml) contained air or an atmosphere containing approximately equal amounts of $^{16}O_2$ and $^{18}O_2$. After 5 min of equilibration, the atmosphere was sampled and analyzed by electron impact mass spectrometry. The reaction was initiated by injection of 10 μl of 0.5 M 2NT in methanol. The flask was incubated at 30°C with shaking at 220 rpm in a water bath. More NADH (0.4 mM) was added after 1 h of incubation. After 3 h of reaction, the headspace atmosphere was sampled and analyzed again. The reaction was stopped by heating the flask in a microwave oven for 10 s. Denatured proteins were removed by centrifugation at 14,000 × *g* for 5 min. The supernatant solution was extracted twice with 5 ml of ethyl acetate. The combined extracts were dried with anhydrous sodium sulfate, and solvent was removed under a stream of nitrogen to give a final volume of approximately 200 μl. Samples were analyzed by both thin-layer chromatography and gas chromatography-mass spectrometry.

Analytical methods. Gas chromatography-mass spectrometry was performed with a Hewlett-Packard model 5890 gas chromatograph linked to a Hewlett-Packard 5970 mass selective detector. Gas chromatography separations were carried out on a cross-linked methyl silicon (Ultra 1) capillary column (inner diameter, 0.2 mm by 25 m; film thickness, 0.33 μm; Hewlett-Packard, Avondale, Pa.). A 1-μl volume of the concentrated ethyl acetate extract was injected onto the column with a split ratio of 50:1. The carrier gas was helium at a flow rate of 0.49 ml/min. The column oven temperature was held at 50°C for 1 min and then raised to 250°C at a rate of 15°C per min. The injection port temperature and the detector temperature were 250 and 280°C, respectively. Electron impact ionization was performed at an ionizing voltage of 70 eV. A 5-μl sample of headspace atmosphere was analyzed in a similar way, except that the injection temperature was 220°C, and the column temperature was kept at 150°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels (70 by 80 by 0.75 mm) was used to monitor protein purification and to estimate molecular weights under denaturing conditions. The concentrations of polyacrylamide were 13.5% in separating gels and 4% in stacking gels. The gels were stained with 0.1% Coomassie brilliant blue R-250 in fixative (40% methanol and 10% acetic acid) for 30 min and destained with 40% methanol–10% acetic acid. SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were used to determine the molecular weights of unknown proteins.

Materials. The following were obtained from the sources indicated: NADH, NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), MES, DNase I, dithiothreitol, and horse heart cytochrome *c* type III, Sigma Chemical Co., St. Louis, Mo.; phenylmethylsulfonyl fluoride and IPTG, Boehringer Mannheim Biochemicals, Indianapolis, Ind.; bovine serum albumin, Pierce, Rockford, Ill.; 3-methylcatechol, Pfaltz and Bauer, Inc., Waterburg, Conn.; 2NT and 3NT, Aldrich Chemical Company, Inc., Milwaukee, Wis.; plastic-backed thin-layer silica gel plates, EM Reagents, Darm-

TABLE 1. Requirements for 2NT oxygenase activity in cell extract[a]

Reaction mixture	Sp act (nmol/min/mg protein)	Relative sp act (%)
CE + 2NT + Fe^{2+} + NADH	21.9	100
CE + 2NT + Fe^{2+} + NADPH	21.2	97
CE + 2NT	2.0	9
CE + 2NT + Fe^{2+}	3.3	15
CE + 2NT + NADH	11.8	54
CE + 2NT + NADPH	10.7	49
CE + 2NT + Fe^{2+} + NADH + FAD	21.2	97
CE + 2NT + Fe^{2+} + NADH + FMN	22.3	102
Boiled CE + 2NT + Fe^{2+} + NADH	0.02	0.1

[a] Reaction mixtures (1.0 ml) contained 0.7 mg of protein from cell extract (CE), 1 mM 2NT, 0.2 mM $Fe(NH_4)_2SO_4$, 0.4 mM NADH or NADPH, 1 μM FAD or FMN, and 50 mM MES buffer, pH 6.5. The reactions were terminated after 15 min, and the amounts of nitrite released were determined as described in Materials and Methods. Values reported are the averages of duplicate determinations.

stadt, Germany; and $^{18}O_2$ (98 atom%), ICON, Mt. Marion, N.Y.

RESULTS

2NT oxygenase activity in cell extracts. The oxidation of 2NT by cell extract was dependent on the presence of NADH or NADPH and of ferrous iron (Table 1). Activity was not observed with boiled cell extract and was not stimulated by the addition of FAD, FMN or a crude cell membrane preparation. Maximum enzyme activity was observed at pH 6.5 in 50 mM MES buffer. Activities at pH 5.5, 6.0, 7.0, and 7.5 were 39, 89, 78, and 40% of that at pH 6.5, respectively. The rate of the reaction was linear for at least 30 min. Crude cell extracts prepared in 50 mM MEG buffer were stable at 4°C for 2 days. A nonlinear relationship between enzymatic activity and protein concentration in cell extract (Fig. 1) suggested that 2NT oxygenase may consist of more than one component.

Multicomponent nature of 2NT oxygenase. Q-Sepharose chromatography of cell extract led to the separation of three protein fractions (Fig. 2) that were required for the release of nitrite from 2NT (Table 2). The individual components were designated A, B, and C according to the order of their elution from the column. Fractions containing components A, B, and C eluted at KCl concentrations of 0.15, 0.22, and 0.35 M and were red-brown, yellow, and pale yellow, respectively. Figure 2 shows that there was some overlap in the elution of components A and B. Solutions of all three components were concentrated by ultrafiltration, and their absorption spectra were recorded. Figure 3 shows the spectra of components A and B. Component C was quite dilute at this stage of the purification procedure and did not show any prominent absorption properties (see below). The inset in Fig. 3 shows the results of SDS-PAGE of fractions containing components A, B, and C. The major proteins in component A gave apparent molecular masses of 52.5 and 28 kDa, while those in components B and C showed prominent bands at 52 and 11 kDa. The molecular mass of the major protein in component C suggested that C may be similar to the Rieske-type [2Fe-2S] ferredoxins that function as electron transport proteins in other multicomponent oxygenase systems (see Discussion). This was supported by further purification and concentration procedures which showed that component C exhibits absorption maxima at 457 and 326 nm. In addition, $ferredoxin_{NAP}$, which was expressed by the cloned naphthalene dioxygenase *nahAb* gene in *E. coli* JM109(DE3)(pDTG135), could replace component C in the 2NT oxygenase assay (data not shown).

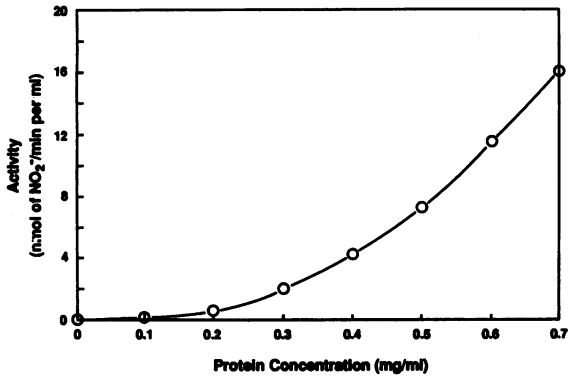

FIG. 1. The effect of protein concentration on 2NT dioxygenase activity.

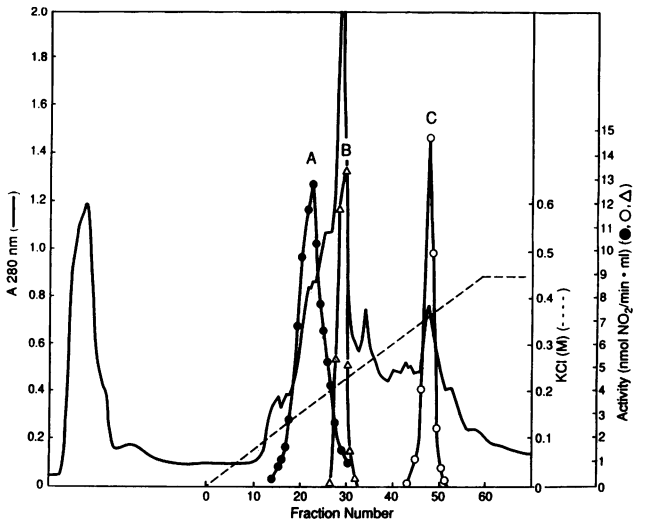

FIG. 2. Resolution of cell extract by anion-exchange chromatography into three protein components, A (●), B (△), and C (○), which are all required for 2NT dioxygenase activity.

Cytochrome *c* reductase activity of component B. The absorption spectrum of component B showed maxima at 449 and 320 nm, a property shared with the reductase component of naphthalene dioxygenase (16). The cytochrome *c* reductase activity of component B is shown in Table 3. NADH was a more effective electron donor than NADPH, and the reduction

TABLE 2. Requirement of three components for 2NT oxygenase activity

Component	Activity (nmol/min)[a]
A + B	0.15
A + C	0.10
B + C	1.15
A + B + C	13.5

[a] Reaction mixtures (final volume, 1.0 ml) contained 1 mM 2NT, 0.2 mM $Fe(NH_4)_2SO_4$, 0.4 mM NADH, and, when indicated, components A, B, and C containing 66.5, 83, and 62.5 μg of protein, respectively, in 50 mM MES buffer, pH 6.5.

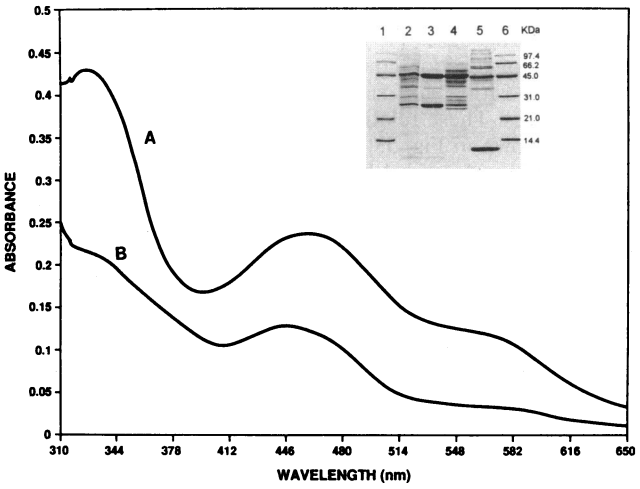

FIG. 3. Absorption spectra (310 to 650 nm) of the protein components A and B isolated by anion-exchange chromatography. Inset shows SDS-PAGE of cell extract and the three components isolated by anion-exchange chromatography. Lanes: 1 and 6, standard protein markers (0.5 μg of protein); 2, cell extract (8 μg of protein); 3, component A (5 μg of protein); 4, component B (5 μg of protein); and 5, component C (5 μg of protein).

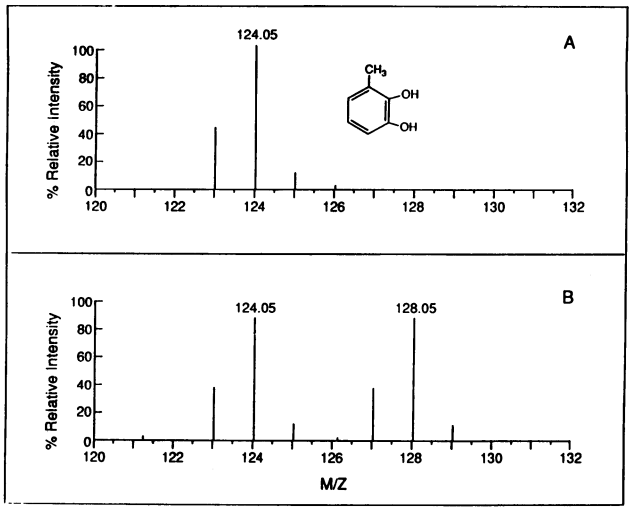

FIG. 4. Mass spectra of 3-methylcatechol produced from 2NT by partially purified components A and B and cell extract from *E. coli* JM109(DE3)(pDTG135) containing ferredoxin$_{NAP}$ in the presence of air (A) and an atmosphere containing a mixture of $^{16}O_2$-$^{18}O_2$ (47:53)(B).

rate was significantly stimulated by FAD or FMN. The presence of components A and C did not affect the rate of the reaction.

$^{18}O_2$ incorporation experiments. The formation of 3-methylcatechol from 2NT could be due to two successive monohydroxylation reactions or the simultaneous incorporation of both atoms of dioxygen into the aromatic nucleus. To distinguish between these two reaction sequences, 2NT was incubated with components A and B and *E. coli* cell extract containing ferredoxin$_{NAP}$ in the presence of an approximately 50:50 mixture of $^{16}O_2$ and $^{18}O_2$. Ferredoxin$_{NAP}$ from *E. coli* replaced component C because preparations of the latter also contained 3-methylcatechol 2,3-dioxygenase. Reaction mixtures were extracted with ethyl acetate and analyzed by gas chromatography-mass spectrometry. The only compounds detected were 2NT (retention time, 8.50 min) and 3-methylcatechol (retention time, 9.65 min). The mass spectra of the *m/z* 120 to 130 region of the 3-methylcatechol formed in air or in air containing $^{18}O_2$ are shown in Fig. 4. The molecular ion of 3-methylcatechol formed in air is seen at *m/z* 124, whereas a value of *m/z* 128 was obtained from the 3-methylcatechol formed in the presence of $^{18}O_2$. In addition, Fig. 4B shows ions at *m/z* 124, 126, and 128 values in a ratio of 49.4:1.3:49.3, which corresponds well with ratio of 45.2:1.0:53.8 that was obtained by headspace analysis for $^{16}O_2$:^{16}O-^{18}O:$^{18}O_2$. The experiment was repeated with an atmosphere containing $^{16}O_2$:^{16}O-^{18}O:$^{18}O_2$ ratio of 61.0:0.8:38.2. The ratios of the 3-methylcatechol ions seen at *m/z* 124, 126, and 128 were 63.9:1.8:34.3. The results clearly show that both atoms of molecular oxygen are incorporated into the aromatic nucleus.

TABLE 3. Cytochrome *c* reductase activity of component B

Component and cofactors[a]	Activity (nmol/min)	Relative activity (%)
B	0	0
B + NADH	10.0	100
B + NADH + FAD	14.4	144
B + NADH + FMN	15.1	151
B + NADH + A	9.4	94
B + NADH + C	10.2	102
B + NADPH	5.3	53
B + NADPH + FAD	10.4	104

[a] Reaction mixtures (1.0 ml) contained 87 μM cytochrome *c*, 0.4 mM NADH or NADPH, 1 μM FAD or FMN, 4.1 μg of protein component B, and when indicated, 14.5 μg of protein component A or 3.6 μg of protein component C in 50 mM bis-Tris buffer, pH 7.0.

DISCUSSION

Oxidative release of nitro groups from nitroaromatic compounds by monooxygenases (27, 28, 34) and dioxygenases (9, 29) has been described. However, *o*-nitrophenol oxygenase, which catalyzes the conversion of 2-nitrophenol to catechol and nitrite, is the only enzyme that has been purified and characterized (35). This enzyme consists of a single polypeptide chain and requires NAD(P)H for enzymatic activity. To our knowledge, the present study is the first in vitro demonstration of the oxidative removal of an aromatic nitro group by a multicomponent enzyme system.

The JS42 2NT dioxygenase appears to be expressed constitutively. The activity of the dioxygenase in cell extracts prepared from cells grown with tryptic soy broth was almost the same as that in extracts from cells grown in mineral salts medium with 2NT as the sole source of carbon and energy. Cell-free activity was dependent on the presence of NADH or NADPH and ferrous iron. The nonlinear relationship between protein concentration in 2NT cell extracts and enzyme activity provided the first indication that 2NT oxygenase may be a multicomponent enzyme system, and this was subsequently confirmed by anion-exchange chromatography. These preliminary observations have been noted previously for other three-component dioxygenases, including benzene (1), toluene (33), naphthalene (11), biphenyl (15), dibenzofuran (4), and ortho-halobenzoates (24).

The absorption spectrum, molecular weight, and subunit composition of component A are similar to those reported for the iron-sulfur proteins (ISP) that serve as the terminal

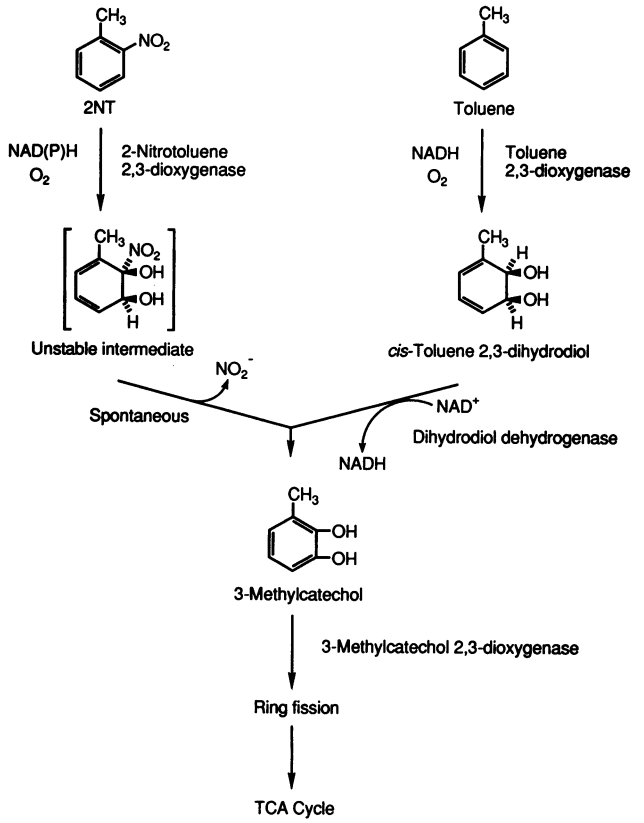

FIG. 5. Comparison of the initial reactions in the oxidation of 2NT by 2NT 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 and the oxidation of toluene by toluene 2,3-dioxygenase from *P. putida* F1. TCA, tricarboxylic acid.

oxygenase components of the dioxygenase systems cited above. Thus, component A has been given the designation ISP_{2NT}. Solutions of component B were yellow. Its absorption spectrum coupled with its ability to reduce cytochrome *c* in the presence of NAD(P)H and the stimulation of activity by FMN and FAD suggests that it may be a flavo-ISP protein similar to $reductase_{NAP}$ (11, 16) and the reductase component of methane monooxygenase (7). Component B has been designated $reductase_{2NT}$. At the present time, little is known about the properties of component C. It can be replaced by $ferredoxin_{NAP}$ (17) in the 2NT oxygenase assay, and it is essential for the anaerobic reduction of ISP_{2NT} in the presence of NAD(P)H and $reductase_{2NT}$ (data not shown). These properties suggest that component C plays an electron transfer role in 2NT oxygenase that is similar to that of the Rieske-type ferredoxins in other multicomponent enzyme systems. Component C has been given the designation $ferredoxin_{2NT}$.

The $^{18}O_2$ incorporation experiments clearly show that both atoms of dioxygen are added to the aromatic nucleus to yield 3-methylcatechol. In reactions analogous to those proposed for the dioxygenation of 2,4DNT (29), we suggest that the initial product formed would spontaneously rearomatize to 3-methylcatechol and release nitrite. Analogous reactions have been reported for 4-chlorophenyl acetate 3,4-dioxygenase (22) and 4-sulfobenzoate 3,4-dioxygenase (21), which release chloride and sulfite, respectively. In contrast, the dioxygenase systems that oxidize benzene, benzoate, toluene, naphthalene, biphenyl, and related substrates produce relatively stable *cis* dihydrodiols which are rearomatized by pyridine nucleotide-dependent dehydrogenases (13). The catechols that are formed by both types of reaction contain hydroxyl groups that are in the requisite position for subsequent fission by ring cleavage dioxygenases. These relationships for 2NT and toluene oxidation are shown in Fig. 5.

The nucleotide and deduced amino acid sequences of several multicomponent aromatic dioxygenases have been determined and have been shown to share common regions that may relate to the mechanism of action and evolutionary origin of this important group of enzymes (20). The nucleotide sequences of the genes encoding the components of 2NT 2,3-dioxygenase are currently being determined and will be published separately. Results to date with Southern blot techniques indicate significant homology with the *nah* genes of *Pseudomonas* sp. strain NCIB 9816-4 and *P. putida* G7 (25).

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