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Purification and Characterization of a Bacterial Nitrophenol Oxygenase Which Converts *ortho*-Nitrophenol to Catechol and Nitrite

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A nitrophenol oxygenase which stoichiometrically converted *ortho*-nitrophenol (ONP) to catechol and nitrite was isolated from *Pseudomonas putida* B2 and purified. The substrate specificity of the enzyme was broad and included several halogen- and alkyl-substituted ONPs. The oxygenase consisted of a single polypeptide chain with a molecular weight of 58,000 (determined by gel filtration) or 65,000 (determined on a sodium dodecyl sulfate-polyacrylamide gel). The enzymatic reaction was NADPH dependent, and one molecule of oxygen was consumed per molecule of ONP converted. Enzymatic activity was stimulated by magnesium or manganese ions, whereas the addition of flavin adenine dinucleotide, flavin mononucleotide, or reducing agents had no effect. The apparent K_ms for ONP and NADPH were 8 and 140 μM, respectively. 2,4-Dinitrophenol competitively (K_i = 0.5 μM) inhibited ONP turnover. The optimal pH for enzyme stability and activity was in the range of 7.5 to 8.0. At 40°C, the enzyme was totally inactivated within 2 min; however, in the presence of 1 mM ONP, 40% of the activity was recovered, even after 10 min. Enzymatic activity was best preserved at −20°C in the presence of 50% glycerol.

Nitroaromatic compounds are the building blocks of many pesticides, dyes, explosives, and solvents, and they also serve as precursors for the production of aminoaromatic derivatives (11, 17, 21). As a consequence, nitroaromatic compounds have become pollutants in rivers, wastewaters, groundwaters, pesticide-treated soils, and the urban atmosphere (5, 7, 12, 19, 30). Some nitroaromatic compounds, such as 1-nitropyrene, are highly mutagenic (15), and several nitrophenols are potent uncouplers of oxidative and photosynthetic phosphorylation (23). *ortho*-Nitrophenol (ONP) and 2,4-dinitrophenol are listed as priority pollutants by the U.S. Environmental Protection Agency (1, 12).

Both reductive and oxidative pathways for the removal of nitro groups from nitroaromatic compounds have been described. A reduction of the nitro substituent, resulting in an aminoaromatic compound, can occur under both aerobic and anaerobic conditions and seems to be a common enzymatic mechanism in the environment (5, 9). The activity of nitroreductases has been demonstrated in cell-free systems, and some enzymes have been purified and characterized (15, 17, 24). Removal of the amino substituent, yielding ammonium, may occur after reduction (2, 4).

In the oxidative pathway, the nitro substituent is directly removed from the nitroaromatic compound and nitrite is liberated (4, 8, 22, 27, 28). To date, the activity of two oxygenases which catalyze this reaction has been demonstrated in cell-free systems. A nitrophenol oxygenase was isolated by Spain and co-workers (22; J. C. Spain, Ph.D. dissertation, University of Texas, Austin, 1979) from a *Moraxella* sp. able to grow on *para*-nitrophenol as a sole source of carbon and nitrogen. This enzyme liberated nitrite from *para*-nitrophenol, which was subsequently converted to hydroquinone. The enzyme was found to be membrane bound and was not purified. We previously described the isolation of a nitrophenol oxygenase from *Pseudomonas putida* B2 capable of growing on ONP (27, 28). This enzyme converted ONP to catechol and nitrite, and it was not membrane bound but soluble. In this publication, we report the purification and characterization of this nitrophenol oxygenase.

MATERIALS AND METHODS

Chemicals. The sources of nitrophenols, including [^{14}C]ONP, are described elsewhere (20a). 2-Nitro-6-chlorophenol was a gift from H.-J. Knackmuss, University of Stuttgart, Stuttgart, Federal Republic of Germany. Streptomycin sulfate and ammonium sulfate were obtained from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Bio-Rad Laboratories (Richmond, Calif.), and 2-mercaptoethanol was from E. Merck (Darmstadt, Federal Republic of Germany). Flavine adenine dinucleotide (FAD), flavine mononucleotide, and reduced β-NADPH were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany). Reduced β-NADH was from Sigma Chemical Co. (St. Louis, Mo.). An aqueous solution of *ortho*-benzoquinone was prepared enzymatically from catechol by the method of Dawson and Tarpley (3). Catechol (0.1 mM; Fluka) and tyrosinase (10 μg/ml; Sigma) were incubated in phosphate buffer for a few minutes at room temperature. The conversion of catechol to *ortho*-benzoquinone and the rapid reverse reaction after the addition of ascorbic acid (0.3 mM; Fluka) (3, 6) was monitored by analyzing samples with high-pressure liquid chromatography (see below).

If not stated otherwise, the phosphate buffer used throughout this study consisted of 3.8 ml of 1 M KH_2PO_4–16.2 ml of 1 M K_2HPO_4 per 980 ml of distilled H_2O, corresponding to a concentration of 20 mM and a pH of 7.5.

Microorganism. The isolation of *P. putida* B2 from soil and its identification have been reported (27). The organism grew on 1 mM ONP as the sole source of carbon and nitrogen at pH 7.5 and 30°C, with a generation time of 5 h (27, 28).

Preparation of crude extract. *P. putida* B2 was cultivated

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in batch cultures on basal medium (26) supplemented with 1 mM ONP and 0.2% yeast extract. Yeast extract was added to increase the cell yield. As soon as the ONP was depleted, the cells were harvested by centrifugation (7,000 × *g*, 15 min), washed twice with phosphate buffer, resuspended in the same buffer, and finally broken by sonification (Sonifier cell disruptor B-30; Branson Sonic Power Co., Danbury, Conn.). The extract was then centrifuged at 20,000 × *g* for 15 min; the resulting supernatant is referred to as crude extract. All procedures were carried out at <5°C. The crude extract had a protein content of 5 to 15 mg/ml, and it was stored at −20°C. The crude extract lost <15% of its activity per month of storage. Preliminary experiments showed that the addition of 0.02% yeast extract to the medium, as done in earlier experiments (27, 28), and 0.2% yeast extract, as used in this report, resulted in the same specific activity of the nitrophenol oxygenase in the crude extract.

Purification of nitrophenol oxygenase. All steps in the purification of nitrophenol oxygenase were carried out at <5°C; they included the following.

(i) Ultracentrifugation. The crude extract was centrifuged for 2 h at 100,000 × *g* in a Beckman L5-50B ultracentrifuge to remove the membrane-bound proteins. The supernatant was retained.

(ii) Streptomycin treatment. The supernatant was treated with 1/10 volume of a 10% streptomycin sulfate solution in phosphate buffer to precipitate the DNA. The mixture was stirred for 15 min, and the supernatant obtained after centrifugation (4,500 × *g*, 15 min) was retained.

(iii) Ammonium sulfate fractionation. The supernatant was treated with an equal volume of a saturated $(NH_4)_2SO_4$ solution in phosphate buffer to yield a 50% saturation. The mixture was stirred for 1 h, and the supernatant obtained after centrifugation (4,500 × *g*, 15 min) was retained. Saturated $(NH_4)_2SO_4$ solution was then added to the supernatant to yield a 65% saturation. The solution was stirred for 1 h and centrifuged. The supernatant was discarded, and the sediment was dissolved in a small volume (1.0 to 2.5 ml) of phosphate buffer.

(iv) Gel-filtration column. The dissolved sediment was applied to a Sephadex G-150 superfine column (2 by 85 cm; Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer containing 2 mM $MgSO_4$ over a 2-day period at a flow rate of about 4.5 ml/h. Fractions (3 ml each) were taken, the activity of the nitrophenol oxygenase was determined, and the two or three fractions with the highest activity were pooled.

(v) Ion-exchange column. The pooled fractions were applied to a Trisacryl-DEAE column (1.0 by 1.5 cm; LKB, Bromma, Sweden) previously equilibrated with phosphate buffer. The applied sample was eluted with 60 ml of a linear gradient of phosphate buffer into phosphate buffer plus 300 mM KCl at a flow rate of about 30 ml/h. Fractions (3 ml each) were taken and dialyzed for 12 h against phosphate buffer. Dialysis was essential since KCl strongly inhibited nitrophenol oxygenase activity (50 to 60% inhibition in the presence of 25 mM KCl or NaCl). The enzymatic activity of the dialyzed fractions was determined, and the two or three fractions with the highest activity were pooled.

Enzyme assay. The nitrophenol oxygenase assays were carried out at room temperature in a quartz cuvette (1 cm) in a total volume of 1 ml. The standard assay contained 0.1 mM ONP, 0.4 mM NADPH, and 4 mM $MgSO_4$ plus enzyme in 20 mM phosphate buffer (pH 7.5) unless otherwise specified. The decrease in the A_{410} (corresponding to the λ_{max} of ONP [28]) was monitored on a Beckman model 25 spectrophotometer. From the molar extinction coefficient (ε = 3470 [28]) and from the protein content of the assay solution, the specific activity of the enzyme was calculated and expressed in micromoles or millimoles of ONP degraded per minute per gram of protein.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a modification of the method of Laemmli (16) by using a slab gel apparatus. Electrophoresis was carried out on a linear 10 to 18% gradient polyacrylamide gel in an SDS-Tris-glycine buffer at pH 8.2. The proteins were stained with Coomassie blue R-250 (Serva, Heidelberg, Federal Republic of Germany).

In order to correlate protein staining with enzymatic activity, 7.5% polyacrylamide slab gels were run without SDS. One part of the gel was stained with Coomassie blue; the other part was cut into 0.5-cm-wide slices, which were individually submerged in enzyme assay solution (containing ONP, NADPH, and $MgSO_4$ in phosphate buffer as previously mentioned) and incubated. The disappearance of ONP allowed localization of the enzymatic activity on the gel.

Analytical methods. Protein concentrations were determined by the method of Lowry as modified by Herbert et al. (10). Oxygen was measured with a Clark-type electrode (Rank Brothers, Cambridge, England), and nitrite concentration was measured by using the Griess-Jlosvay reaction as modified by Montgomery and Dymock (18). NADPH was quantified spectrophotometrically by measuring the extinction at 340 nm and by assuming a molar extinction coefficient (ε) of 6300 (29). Catechol, *ortho*-benzoquinone, and ONP concentrations were determined by high-pressure liquid chromatography. The instrument and the C_{12} reversed-phase column used have previously been reported (20a). The mobile phase was a mixture of water and methanol which was pumped under the following conditions: 0 to 4 min, isocratic (85/15, vol/vol); 4 to 14 min, gradient to 35/65 (vol/vol); >14 min, isocratic (35/65, vol/vol). The water-methanol mixture contained 0.1 M acetic acid, the flow rate was 1 ml/min, and a wavelength of 278 nm was used for detection. Under these conditions, catechol, *ortho*-benzoquinone, and ONP standards showed retention times of 5.7, 7.5, and 15.2 min, respectively. Quantitative data were obtained by (i) comparing the peak areas of unknowns with the peak areas of standards or by (ii) performing the enzyme assay in the presence of [^{14}C]ONP (0.5 × 10^6 dpm/ml), injecting aliquots into the high-pressure liquid chromatography column, and collecting and counting effluent samples at particular retention times, as reported previously (27).

RESULTS

Purification of the enzyme. A typical purification procedure protocol is summarized in Table 1. A 40-fold purification with a final activity recovery of >15% was routinely obtained. The ultracentrifugation and the streptomycin treatment did not result in a significant purification, but these steps were essential for efficient ammonium sulfate fractionation and gel filtration.

Electrophoresis of the purified enzyme on SDS-PAGE revealed a single band (Fig. 1). A nondenaturating gel of the purified enzyme was also run to correlate protein staining with enzymatic activity (see Materials and Methods). Again, a single but somewhat diffuse band was found, and the enzymatic activity was recovered at the same position.

Physical properties of the enzyme. The molecular weight of the purified nitrophenol oxygenase was determined by SDS-PAGE (Fig. 2A) and by gel filtration (Fig. 2B) to be 65,000

TABLE 1. Typical protocol of purification of nitrophenol oxygenase from *P. putida* B2

Purification step	Vol (ml)	Amt of protein (mg/ml)	Total activity (μmol/min per liter)	Sp act (μmol/min per g of protein)	Yield (1%)	Purification factor
Crude extract	20.0	12.9	13,150	51	100	1
Ultracentrifugation	19.3	8.6	12,490	76	95	1.5
Streptomycin precipitation	20.5	9.3	11,800	62	90	1.2
Ammonium sulfate fractionation (50–65%)	2.3	14.6	4,170	124	32	2.4
Sephadex G-150 superfine column	6.3	0.41	3,280	1,270	25	24.9
Trisacryl-DEAE column	9.0	0.11	2,100	2,000	16	39.2

and 58,000, respectively. Therefore, it can be assumed that the enzyme does not consist of subunits but of a single polypeptide chain.

The absorption spectrum of an aqueous solution of the purified enzyme (0.12 mg of protein per ml of phosphate buffer) showed maxima at 280 nm (absorbance of 0.22) and at 415 nm (yellow color, absorbance of 0.08). If one assumes a molecular weight of 60,000 for the enzyme, the molar extinction coefficient at 415 nm is calculated to be ca. 40,000. The absorption maximum at 280 nm is due to the presence of aromatic amino acids in the protein, but there is no obvious explanation for the maximum at 415 nm. FAD and flavin mononucleotide are essential cofactors of many oxygenases (13, 14, 25), but their absorption maxima (determined in phosphate buffer) were at 370 and 450 nm, respectively, and the molar extinction coefficients were below 11,000. Further investigation will be necessary to determine whether the absorption maximum at 415 nm is due to adsorbed residues of ONP (absorption maximum at 410 nm, ε of 3470 [28]) which were not removed from the enzyme during the purification procedure.

Catalytic properties of the enzyme. A complete enzymatic turnover of 0.1 mM ONP required 0.1 mM molecular oxygen and 0.2 mM NADPH and resulted in the production of 0.1 mM catechol and 0.1 mM nitrite (all species were quantified as described in Materials and Methods). To determine whether the conversion of ONP to catechol was accompanied by a transient accumulation of intermediates, the enzymatic assay was incubated with [^{14}C]ONP as a substrate. Samples were frequently withdrawn during the reaction period and analyzed by high-pressure liquid chromatography as indicated in Materials and Methods. The total radioactivity collected at the retention times for ONP and catechol always amounted to 100% of the injected radioactivity, and no intermediates, in particular *ortho*-benzoquinone, were detectable.

The apparent K_ms for NADPH and ONP were found to be 140 and 8 μM, respectively, and a substrate inhibition occurred above 20 μM ONP (Fig. 3). The rate of ONP turnover was not reduced even in the presence of 1 mM catechol and 1 mM nitrite (data not shown); therefore, a product inhibition can be excluded. The enzyme had a very low affinity for NADH (apparent K_m, >2 mM), which explains the reported loss of activity after replacing NADPH with NADH (28). Throughout this study, ONP and NADPH concentrations of 0.1 and 0.4 mM, respectively, were routinely used to assay the activity of the nitrophenol oxygenase. The data presented in Fig. 3 suggest that activity is not optimal at these concentrations because of substrate inhibition by ONP and probably incomplete enzyme saturation by NADPH. The standard assay for the nitrophenol oxygenase was always performed under oxygen-saturated conditions (around 0.26 mM O_2 at room temperature); therefore, oxygen was not likely to be a limiting factor. The apparent K_m for oxygen, however, was not determined. Addition of the cofactors FAD and flavin mononucleotide or of the reducing agents DTT and 2-mercaptoethanol did not affect the specific activity of the purified enzyme towards ONP.

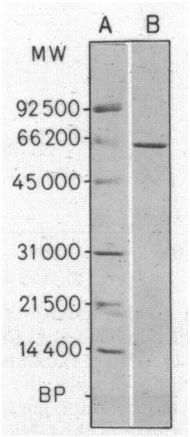

FIG. 1. SDS-PAGE of molecular weight standards (lane A) and purified nitrophenol oxygenase (lane B). Standards (described in the legend to Fig. 2) and about 30 μg of purified nitrophenol oxygenase were applied to the linear 10 to 18% gradient gel, and electrophoresis with bromophenol blue (BP) as a tracking dye was carried out as indicated in Materials and Methods.

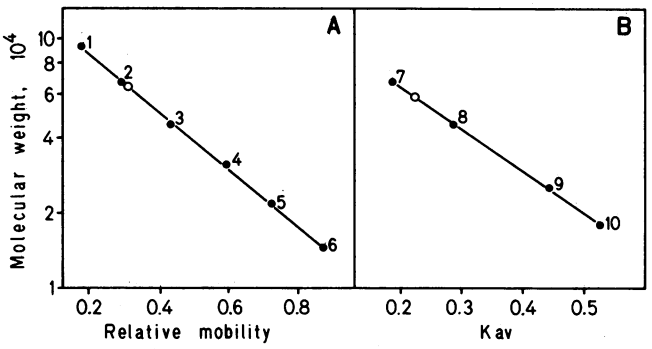

FIG. 2. Determination of the molecular weight of the nitrophenol oxygenase (○) by SDS-PAGE (A) and by gel filtration (B). SDS-PAGE was performed on a linear 10 to 18% gradient gel, and gel filtration was performed on a Sephadex G-150 superfine column as described in Materials and Methods. The standards and their molecular weights were: 1, phosphorylase B (92,500); 2, bovine serum albumin (66,200); 3, ovalbumin (45,000); 4, carbonic anhydrase (31,000); 5, soybean trypsin inhibitor (21,500); 6, lysozyme (14,400); 7, bovine serum albumin (66,200); 8, ovalbumin (45,000); 9, chymotrypsinogen (25,000); 10, myoglobin (17,800).

The substrate specificity of the nitrophenol oxygenase is broad and includes a number of alkylated and halogenated ONPs (Table 2). Most substrates carrying only alkyl groups (e.g., methyl- and phenyl- substituents) were well transformed, whereas ONPs carrying substituents with a high electron-withdrawing effect (e.g., 2,4-dinitrophenol and 4-trifluoromethyl-2-nitrophenol) were persistent. 2,4-Dinitrophenol had a high affinity towards the enzyme and competitively inhibited (K_i, 0.5 μM) ONP turnover (data not shown). Therefore, its persistence is not due to poor binding to the enzyme but is probably due to the two nitro substituents, which deactivate the aromatic ring and consequently prevent an attack by the nitrophenol oxygenase (20). Accurate correlations of specific enzymatic activities with specific characteristics of the ONPs (e.g., pK_a, size of substituent, octanol-water partition coefficient [20a]) were not found. Moreover, the specific activities depend on the concentration of the substrate (Fig. 3, Table 2), and K_m and V_{max} values have to be determined for all ONPs before any meaningful correlations can be established.

The activity of the nitrophenol oxygenase towards ONP and 4-chloro-2-nitrophenol as a function of the pH is recorded in Fig. 4. The specific activity of the enzyme towards 4-chloro-2-nitrophenol was only 25% of the activity measured towards ONP, which is in agreement with the value reported in Table 2. Optimal activity towards both substrates, however, was found between pH 7.5 and 8.0 in the presence of phosphate or Tris buffer. No enzymatic activity was detectable at a pH of <6 in the presence of acetate or succinate buffer or at a pH of >9.5 in the presence of carbonate or ammonium buffer. The pK_as of ONP and 4-chloro-2-nitrophenol differ by an order of magnitude (7.2 versus 6.4 [20a]), but the highest enzymatic activity towards both substrates was in the same pH range (Fig. 4). Therefore, it can be concluded that the pH optimum for enzymatic activity depends primarily on the characteristics of the enzyme and not on the pK_a of the substrate.

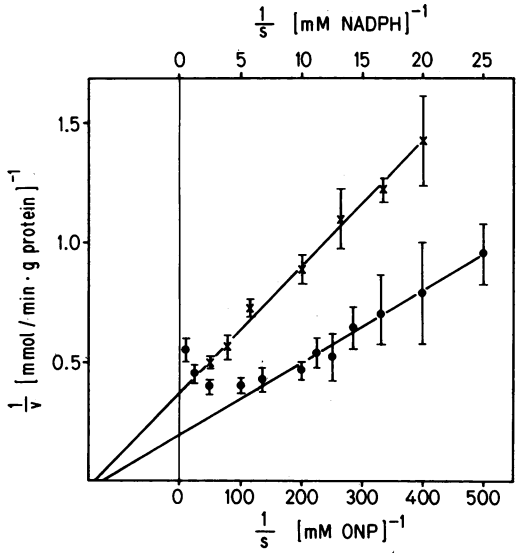

FIG. 3. Lineweaver-Burk diagram of ONP and NADPH turnover. The enzymatic assays were performed as indicated in Materials and Methods, except for the concentrations of ONP and NADPH. The protein concentration in the assays was 1.5 μg/ml. Symbols: ●, NADPH constant (0.5 mM), ONP varied (2 μM to 0.1 mM); ×, ONP constant (0.03 mM), NADPH varied (0.05 mM to 0.4 mM). The standard deviations are indicated by error bars.

TABLE 2. Substrate specificity of nitrophenol oxygenase[a]

Substrate	ε at 410 nm (1,000 cm²/mol)	Relative sp act (%)	
		0.1 mM substrate	0.2 mM substrate
ONP	3,470	100[b]	70
5-CH_3-	3,595	130	150
3-CH_3-	1,015	120	95
4-CH_3-	2,795	105	105
5-F-	5,070	75	90
6-Cl-	4,475	55	55
4-Phenyl-	2,625	40	20
4-OCH_3-	2,810	35	30
4-Cl-	4,210	25	20
4-*sec*-Butyl-	2,490	25[c]	15[c]
4-Cl-5-CH_3-	4,370	25	30
4-CHO-	4,705	10	5
4-CF_3-	4,095	<5	<5
4-NO_2-6-CH_3-	11,850	<5	<5
5-NO_2-	3,210	<5	<5
4-NO_2-	12,430	<5	<5

[a] The enzymatic assays were performed as described in Materials and Methods for ONP, but two different substrate concentrations were used. The appropriate molar extinction coefficients (ε) were determined for all substrates as previously reported (28) and were used to calculate the specific activities. The relative standard deviation was <10% for all values.

[b] The specific activity towards 0.1 mM ONP was considered to be 100%.

[c] The relative specific activity towards 4-*sec*-butyl-ONP was initially high (25 and 15%, respectively) and dropped to <5% after a few minutes of incubation.

Stability of the enzyme. The nitrophenol oxygenase was very labile at elevated temperatures. At 40°C, a complete loss of activity was observed within 2 min. The presence of ONP, however, prevented rapid heat inactivation. In the presence of 0.2 mM ONP, 30% of the initial activity could be

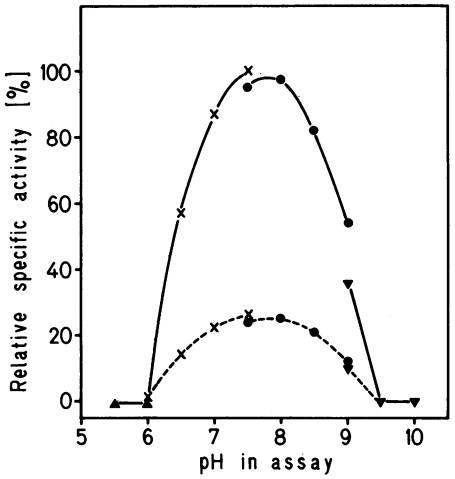

FIG. 4. Effect of pH on the activity of the nitrophenol oxygenase. The highest specific activity found towards ONP was considered to be 100%. Since the molar extinction coefficient of ONP at 410 nm varies considerably with pH, the assays to determine the enzymatic activity were based on the measurement of the NADPH turnover at 340 nm rather than of the ONP turnover at 410 nm. The buffers were prepared by dissolving the corresponding salt in water and adjusting the pH with NaOH or H_2SO_4. Buffers: ▲, 50 mM succinate (pH 5.5 to 6.0) or 50 mM acetate (pH 5.5 to 6.0); ×, 20 mM phosphate (pH 6.0 to 7.5); ●, 50 mM Tris (pH 7.5 to 9.0); ▼, 50 mM ammonium (pH 9.0 to 10.0) or 50 mM carbonate (pH 9.0 to 10.0). Substrates: ——, ONP; - - - -, 4-chloro-2-nitrophenol.

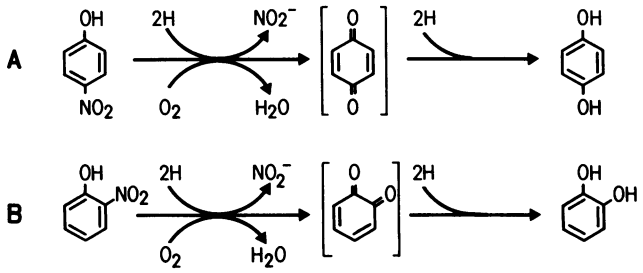

FIG. 5. Postulated pathways of nitrophenol degradation. (A) Degradation of *para*-nitrophenol via *para*-benzoquinone to hydroquinone by an enzyme extract isolated from a *Moraxella* sp. (22). (B) Degradation of ONP via *ortho*-benzoquinone to catechol by a nitrophenol oxygenase isolated from *P. putida* B2 (26, 28, this study).

recovered after 4 min at 40°C, and in the presence of 1.0 mM ONP 40% of the initial activity could be recovered, even after 10 min at 40°C. The enzyme-substrate complex was obviously more resistant to denaturation than was the enzyme alone. It is noteworthy that intermediate ONP concentrations (around 0.02 mM) inhibited the catalytic activity of the enzyme (Fig. 3) but that high concentrations (>0.2 mM) resulted in enhanced heat resistance.

The enzyme was stable at 4°C for several hours in 20 mM phosphate buffer at pH 7.5 or in 50 mM Tris buffer at pH 7.5 or 8, whereas 20 to 25% of the activity was lost within 1 h in 20 mM succinate buffer at pH 6 or in 20 mM carbonate buffer at pH 10. Long-term storage of the purified enzyme in phosphate or Tris buffer (0.1 mg of protein per ml) was optimal at 4°C in the presence of 4 mM DTT (<35% loss of activity within 5 days) or at −20°C in the presence of 50% glycerol (<25% loss of activity within 5 days). Storage at −20°C without glycerol resulted in rapid inactivation of the enzyme. Addition of DTT was not required to preserve the activity.

DISCUSSION

This paper describes a nitrophenol oxygenase active towards ONPs. The enzyme was soluble, and its activity was stimulated by magnesium and manganese ions but not by FAD. Spain and co-workers (22; Spain, Ph.D. dissertation) characterized an oxygenase active towards *para*-nitrophenol, but this enzyme was membrane-bound and its activity was inhibited by magnesium and manganese ions and stimulated by FAD. However, several catalytic properties of the two nitrophenol oxygenases were homologous. The two enzymes had similar apparent K_ms for their substrates (ONP, 8 μM; *para*-nitrophenol, 6 μM), both showed a substrate inhibition at concentrations above 20 μM nitrophenol, both required NADPH as a cofactor, and for both the optimal activity was found between pH 7.5 and 8.0. Stoichiometric determinations showed that the conversions of 1 mol of ONP to catechol and of 1 mol of *para*-nitrophenol to hydroquinone, respectively, were coupled with the consumption of 1 mol of oxygen and 2 mol of NADPH and with the liberation of 1 mol of nitrite. This stoichiometry is in agreement with the mass balances presented in Fig. 5.

Spain and co-workers (22; Spain, Ph.D. dissertation) postulated *para*-benzoquinone as an intermediate in the conversion of *para*-nitrophenol to hydroquinone (Fig. 5). This assumption was based on the observation that crude enzyme extracts able to degrade *para*-nitrophenol were also capable of reducing *para*-benzoquinone in the presence of NADPH; however, accumulation of *para*-benzoquinone was not detected. Similarly, we postulate *ortho*-benzoquinone as an intermediate in the enzymatic conversion of ONP to catechol (Fig. 5). However, ONP was always directly converted to catechol, and no significant accumulation of any intermediate was detected in the assay mixture. It is noteworthy, though, that the enzymatic liberation of nitrite from ONP was a rather slow reaction and required the presence of the reducing agent NADPH in the assay mixture. Since it is known that the reducing agent ascorbic acid converts *ortho*-benzoquinone rapidly to catechol in a spontaneous chemical reaction (3, 6), we also determined the action of NADPH on *ortho*-benzoquinone. We found that 0.1 mM *ortho*-benzoquinone in 20 mM phosphate buffer (pH 7.5) was completely reduced to catechol in a nonenzymatic reaction within seconds after the addition of 0.4 mM NADPH (data not shown). Therefore, in the turnover of ONP to catechol, the enzymatic conversion of ONP to *ortho*-benzoquinone rather than the spontaneous reduction of *ortho*-benzoquinone to catechol appears to be the rate-limiting step, and an accumulation of *ortho*-benzoquinone cannot be expected. It is possible that *ortho*-benzoquinone is reduced to catechol without leaving the active site of the nitrophenol oxygenase.

The nitrophenol oxygenases active towards ONPs and *para*-nitrophenol are fundamentally different from nitroalkane oxygenases isolated from *Hansenula mrakii* (14) and *Fusarium oxysporum* (13). These enzymes catalyze the degradation of nitroalkanes such as 1-nitropropane, 2-nitropropane, and nitroethane to the corresponding ketone derivatives. The nitroalkane oxygenases do not require NADPH, but they either produce superoxide (13) or convert two molecules of substrate simultaneously (14).

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