Purification and Characterization of Nitrobenzene Nitroreductase from *Pseudomonas pseudoalcaligenes* JS45

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Pseudomonas pseudoalcaligenes **JS45 grows on nitrobenzene as a sole source of carbon, nitrogen, and energy. The catabolic pathway involves reduction to hydroxylaminobenzene followed by rearrangement to *o*-aminophenol and ring fission (S. F. Nishino and J. C. Spain, Appl. Environ. Microbiol. 59:2520, 1993). A nitrobenzene-inducible, oxygen-insensitive nitroreductase was purified from extracts of JS45 by ammonium sulfate precipitation followed by anion-exchange and gel filtration chromatography. A single 33-kDa polypeptide was detected by denaturing gel electrophoresis. The size of the native protein was estimated to be 30 kDa by gel filtration. The enzyme is a flavoprotein with a tightly bound flavin mononucleotide cofactor in a ratio of 2 mol of flavin per mol of protein. The K_m for nitrobenzene is 5 μM at an initial NADPH concentration of 0.5 mM. The K_m for NADPH at an initial nitrobenzene concentration of 0.1 mM is 183 μM. Nitrosobenzene was not detected as an intermediate of nitrobenzene reduction, but nitrosobenzene is a substrate for the enzyme, and the specific activity for nitrosobenzene is higher than that for nitrobenzene. These results suggest that nitrosobenzene is formed but is immediately reduced to hydroxylaminobenzene. Hydroxylaminobenzene was the only product detected after incubation of the purified enzyme with nitrobenzene and NADPH. Hydroxylaminobenzene does not serve as a substrate for further reduction by this enzyme. The products and intermediates are consistent with two two-electron reductions of the parent compound. Furthermore, the low K_m and the inducible control of enzyme synthesis suggest that nitrobenzene is the physiological substrate for this enzyme.**

Nitroaromatic compounds are used in the production of dyes, plastics, high explosives, pharmaceuticals, and pesticides (6, 14, 15, 22). Nitrobenzene (NB) alone is discharged to the environment at a rate of tens of millions of pounds annually (34). In addition, nitrated polycyclic aromatic hydrocarbons are formed during a variety of combustion processes and are common environmental contaminants (27). Reduction of the nitro group is a common first step in the biotransformation of nitroaromatic compounds, whether leading to mineralization of the compound (13, 22) or to the accumulation of dead-end products, many of which are cytotoxic and/or mutagenic (1, 7, 10, 19, 20, 33).

Two types of enzymes with nitroreductase activity can be distinguished on the basis of their ability to reduce nitro groups in the presence of oxygen. Oxygen-sensitive (type II) enzymes catalyze a one-electron reduction of the nitro group which yields a nitro anion radical (26). The nitro anion radical reacts with oxygen to form superoxide and regenerate the parent nitro compound (6). Oxygen-insensitive (type I) enzymes reduce the nitro group in a series of two-electron transfers to produce the corresponding nitroso, hydroxylamino, and amino derivatives (9, 40). Even though the nitroso intermediate is so reactive that it is difficult to isolate from biochemical reactions, its role can be inferred from studies of nitro compounds reduced in controlled chemical reactions (11). The formation of the hydroxylamino intermediate is well established because it has been detected in numerous studies of nitro-group reduction (13, 18, 22, 24, 39). Because the nitro group is a facile electron acceptor, a number of enzymes can catalyze the reduction of aromatic nitro groups even though the reaction is

not their physiological role. Enzymes with nitroreductase activity include aldehyde oxidase, cytochrome *c* reductase, cytochrome P-450 reductase, glutathione reductase, hepatic cytochrome P-450s, hepatic NAD(P)H:quinone reductase, hydrogenosomal pyruvate:ferredoxin oxidoreductase, succinic dehydrogenase, xanthine dehydrogenase, and xanthine oxidase (7). Several bacterial nitroreductases have been characterized in crude extracts or purified form (1, 4, 6, 8, 13, 16, 20, 33, 35). With few exceptions (1, 13), these enzymes reduce nitro-substituted compounds to the corresponding amines. Bacterial nitroreductases, like those from eukaryotic sources, have been studied mainly to elucidate the role of reduction products in the toxicity of nitroaromatic compounds.

Enzymes involved in the biodegradation of nitroaromatic compounds have received little attention. It has been generally accepted that the nitro group could be removed from the ring by either an oxygenase reaction leading to the release of nitrite or reduction to the amino derivative followed by the release of ammonia. A third mechanism, involving reduction of the ring and subsequent release of the nitro group from an intermediate Meisenheimer complex, is now known (36). Recently, novel reductive pathways were found by Groenewegen et al. (13) in a *Comamonas acidovorans* isolate capable of degrading 4-nitrobenzoate and by Nishino and Spain (22) in a strain of *Pseudomonas pseudoalcaligenes* growing on NB. In both cases, the hydroxylamino intermediate was on the degradative pathway, but the fully reduced amino derivative was not. The reductase of *C. acidovorans* was partially purified and appears to be specific for the reduction of nitrobenzoate to hydroxylaminobenzoate (13).

P. pseudoalcaligenes JS45 grows on NB as a sole source of carbon, nitrogen, and energy. NB is first reduced to nitrosobenzene (NOB), which is further reduced to hydroxylaminobenzene (HAB), but aniline is not formed from NB by whole

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cells or cell extracts. Instead, HAB is converted by a specific mutase to 2-aminophenol, which is the substrate for ring fission (22). The initial, reductive portion of the pathway could theoretically occur via a number of different mechanisms. The reduction of NB to HAB could be catalyzed by one or two enzymes. Individual nitro- and nitrosoreductase activities have been observed in bacterial preparations (28–30, 40). Alternatively, the reduction of NB to NOB could be enzymatic, and the reduction of NOB to HAB could be due to a nonenzymatic reaction (3). The enzymatic reduction(s) could occur in one- or two-electron increments. The reductase enzyme(s) involved could produce HAB as the end product of reduction, or the absence of aniline on the metabolic route could be due to rapid chemical or enzymatic removal of HAB. To determine which of these mechanisms is used and to characterize the first enzyme of NB metabolism, nitrobenzene nitroreductase was purified to homogeneity from *P. pseudoalcaligenes* JS45.

MATERIALS AND METHODS

Chemicals. NB, NOB, aniline, and protease were purchased from Sigma (St. Louis, Mo.). HAB was synthesized from NB as previously described (22). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Fast protein liquid chromatography packings, columns, and gel filtration calibration standards were from Pharmacia LKB (Piscataway, N.J.). Protein molecular weight standards for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Melville, N.Y.).

Bacteria and growth conditions. *P. pseudoalcaligenes* JS45 (22) was maintained in a MultiGen chemostat (New Brunswick Scientific, Edison, N.J.), using a nitrogen-free basal medium (BLKN [5]) with NB as the sole source of both carbon and nitrogen. Large-scale cultures of JS45 were grown in a New Brunswick MicroFerm fermentor equipped with a 14-liter vessel. The reactor contained 9.5 liters of BLKN (pH 7) and was inoculated with 2.5 liters of a culture grown overnight on NB in BLKN, and 6.0 ml of NB (4.9 mM, final concentration) was added. The reactor was stirred at 250 rpm overnight at 30°C. Air was provided at 6.0 liters/min. After 12 h, air flow was increased to 10 liters/min, and the stirring rate was increased to 400 rpm. NB was replaced as it was depleted until the A_{600} reached 0.7.

Cells were recovered by filtration across a 0.45-μm-pore-size Durapore filter cassette (Millipore Corp., Bedford, Mass.), washed with cold potassium phosphate buffer (20 mM, pH 7), and collected by centrifugation at $7,000 \times g$ for 15 min.

Enzyme purification. All subsequent steps were carried out in potassium phosphate buffer (20 mM, pH 7.0) at 0°C. The cell paste (41.5 g [wet weight]) was suspended in 180 ml of buffer, and the cells were lysed by two passes through a French pressure cell at 20,000 lb/in^2. The resulting suspension was centrifuged at $27,000 \times g$ for 20 min, and the pellet was discarded. The supernatant fluid was centrifuged at $100,000 \times g$ for 1 h, and the pellet was discarded.

The cleared lysate was brought to 45% saturation with ammonium sulfate, and the precipitate was removed by centrifugation and discarded. The supernatant was brought to 60% saturation with ammonium sulfate, and the precipitate was recovered by centrifugation. The pellet was dissolved in buffer and desalted by passage through a Pharmacia PD-10 desalting column. Pooled protein solutions were loaded on Q-Sepharose FF in a Pharmacia XK-50 column (5 by 25 cm) that had been preequilibrated with phosphate buffer at 4°C. The column was sequentially washed with 200 ml each of phosphate buffer and phosphate buffer plus KCl (150 mM) at a flow rate of 2.5 ml/min. The remaining proteins were eluted with a linear KCl gradient (150 to 300 mM) over 600 ml followed by 500 ml at 300 mM KCl. Fractions with nitroreductase activity were pooled and concentrated on an Amicon PM-10 membrane. The volume of the retentate was adjusted to 1.5 ml, and the remaining proteins were resolved by ascending chromatography through Sephacryl S-200 in a Pharmacia XK-16 column (1.6 by 94 cm) preequilibrated with KCl (50 mM) in phosphate buffer at 4°C. Proteins were eluted from the column with 180 ml of KCl (50 mM) in phosphate buffer at a flow rate of 1 ml/min. Active fractions were pooled, concentrated, divided into 100-μl aliquots, and stored at −20°C. Protein concentrations were determined by the bicinchoninic acid method (31). The N-terminal amino acid sequence of the purified protein was determined by the ICBR Protein Chemistry Core Laboratory at the University of Florida, Gainesville.

Enzyme assays. Reductase activity was measured spectrophotometrically as the decrease in A_{340} due to the oxidation of NADPH in a reaction mixture containing enzyme, NB (0.1 mM), NADPH (0.5 mM), and phosphate buffer (20 mM, pH 8) in a final volume of 1 ml.

Molecular mass determinations. The purified protein was examined by denaturing polyacrylamide gel electrophoresis (17). Gels were stained with Coomassie blue R-250, and the molecular mass of the reductase was determined by

comparison with a standard protein mixture. The molecular mass of the native protein was estimated by Sephacryl S-200 gel filtration chromatography and comparison with standard proteins.

Cofactor analyses. Purified enzyme was dialyzed overnight against KBr (1 M, 4°C) in phosphate buffer and then dialyzed for 9 h against phosphate buffer alone in order to dissociate the flavin cofactor (33). Samples of the dialyzed enzyme were supplemented with either flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), or riboflavin to a final concentration of 10 μM, stored on ice for 24 h, and assayed for nitroreductase activity.

In a separate experiment, the purified enzyme (0.18 mg) was digested in a volume of 1 ml with 1 mg of protease for 2 h at 25°C in the dark. The solution was centrifuged at $14,000 \times g$ for 10 min at 4°C, and the supernatant fluid was analyzed by high-pressure liquid chromatography (HPLC) for the presence of FMN or FAD (2). Flavin stoichiometry was estimated by comparison of the flavin peak from digested protein with flavin standards and by estimation of flavin concentration on the basis of the A_{450} of the native protein as described by Bryant and DeLuca (6).

Metal requirements were assayed after dialysis of the enzyme at 4°C overnight against phosphate buffer (pH 8) containing EDTA (1 mM) and 1,10-phenanthroline (1 mM). A second portion of the enzyme was dialyzed against buffer alone. The effects of chelating agents were also tested by incubation of enzyme with EDTA (5 mM) or 1,10-phenanthroline (5 mM) in phosphate buffer for 30 min on ice prior to assay of the reductase in the presence of EDTA (5 mM). The metal content of the protein was determined by plasma emission spectroscopy at the University of Minnesota Soil Testing Laboratory, Minneapolis.

Identification of intermediates and products of NB reduction. Products of the reductase reaction were determined by HPLC analyses of reaction mixtures containing NB (0.6 mM), NADPH (1.8 mM), and purified reductase (9 μg) in 1 ml of phosphate buffer. Samples (100 μl) were analyzed on a 250-mm Spherisorb C_8 column (Alltech, Deerfield, Ill.) with a solvent system of 65% trifluoroacetic acid (0.1% in H_2O)–35% acetonitrile. Products of the reactions were identified and quantified by comparison of the spectra and retention times with those of standards.

The formation of intermediates and products of the reduction reaction were also monitored spectrophotometrically with a Cary (Varian, Sunnyvale, Calif.) 3E spectrophotometer. Both sample and reference cuvettes contained glucose-6-phosphate (6 mM), glucose-6-phosphate dehydrogenase (5 U), NADPH (10 μM), and purified enzyme in 1 ml of phosphate buffer (pH 8.0). The sample cuvette also contained NB (100 μM), and the reactions were started in both cuvettes by the addition of the NADPH.

Reactions were assayed for the appearance of NOB by the addition of hydroxylamine (10 mM) followed by 1-naphthylamine (10 mM). Under these conditions, NOB reacts to form 2-phenylazo-1-naphthylamine (11), which is detected as a broad absorbance peak around 460 nm. The detection limit for NOB in our hands was below 10 μM.

Sequence data. The amino acid sequence data presented here has been reported to the Protein Identification Resource databank and has been assigned accession number A44682.

RESULTS AND DISCUSSION

Protein purification. The purification of nitrobenzene nitroreductase from a culture of *P. pseudoalcaligenes* JS45 grown on NB is outlined in Table 1. In contrast to results described in previous reports of bacterial nitroreductases (6, 10, 16, 33), a single peak of nitrobenzene nitroreductase activity was detected during chromatographic separations. The purified enzyme was homogeneous, as determined by examination of SDS-polyacrylamide gels stained with Coomassie blue R-250.

Solutions of the purified reductase were yellow, and the spectrum obtained for the pure protein (Fig. 1) is consistent with that of a flavoprotein. The purified enzyme had maximal activity at pH 8, although activity varied little from pH 5 to 9. Although the enzyme was relatively stable at temperatures of 40°C and below, it lost activity rapidly when stored at temperatures above 45°C.

Cofactor analyses. Purified enzyme dialyzed overnight against chelating agents retained 98% of the activity of enzyme dialyzed against phosphate buffer alone. This result suggests either that metals do not play an important role in reductase activity or that the metal cofactor is not effectively removed by dialysis. Enzyme activity was not inhibited by a relatively high concentration of EDTA (5 mM) in the reaction mixture (Table 2) but was decreased about 20% by preincubation of the enzyme with either EDTA or 1,10-phenanthroline along with

TABLE 1. Purification of nitrobenzene nitroreductase from *P. pseudoalcaligenes* JS45

Fraction	Vol (ml)	Total activity (U)[a]	Protein concn (mg/ml)	Sp act (U)[a]	Yield (%)	Purification factor
Crude lysate	180	10,454	24	2.4	100	
Cleared lysate	161	11,630	17	4.2	111	1.8
45–60% $(NH_4)_2SO_4$ cut, desalted	25	4,621	28	6.5	44	2.7
Q-Sepharose	10	3,631	6.8	53	35	22
Sephacryl S-200	6.7	2,958	1.8	245	28	102

[a] One unit is the amount (in micromoles) of NADPH oxidized per minute per milligram of protein.

inclusion of EDTA in the enzyme assay. The results could be explained by the presence of a tightly bound metal cofactor, which would be consistent with observations by Saz and Martinez (28) on a nitroreductase isolated from an aureomycin-resistant strain of *Escherichia coli*. Metal requirements have been demonstrated or postulated for a number of other bacterial nitroreductase enzymes (4, 9, 21, 29, 30). Alternatively, reduction of the nitro group to the corresponding hydroxylamine may not require a metal cofactor. Two other enzymes that reduce nitro compounds to the corresponding hydroxylamine have been described (1, 13). The activity of the enzyme isolated from *C. acidovorans* (13) was not altered by the addition of metals, and no information regarding metal requirements was reported for the *E. coli* enzyme (1). Analysis of the purified protein by plasma emission spectroscopy revealed that Fe, Mg, and Mn, the metals most often implicated in reductase activity, were not present in concentrations above those in the buffer alone. Ca and Zn were present in concentrations equivalent to 0.4 and 0.1 mol of metal ion per mol of protein, respectively. These data clearly indicate that a metal cofactor is not required for the reduction of NB to HAB.

Dialysis against KBr reduced the activity of the nitroreductase by 22%. The A_{450}/A_{274} ratio of dialyzed protein was reduced by 21% relative to that of the freshly purified reductase, a value in close agreement with the observed loss of activity. Incubation of the enzyme with FMN, FAD, or riboflavin did not restore activity. The results indicate that the flavin cofactor is tightly bound to the enzyme and that the flavin binding site is not readily accessible to exogenous flavins. Similarly, the nitroreductase from an aureomycin-resistant strain of *E. coli* contains a very tightly bound flavin cofactor (28).

The flavin cofactor of the JS45 nitroreductase was subsequently characterized by HPLC. The analysis of digested protein revealed a single major peak that coeluted with FMN. The amount of FMN detected in the analysis corresponded to 1.8

mol of FMN per mol of protein. Analysis of the spectrum shown in Fig. 1 indicates an FMN concentration of 0.12 mM ($\varepsilon_{450} = 11.29$ mM^{-1} cm^{-1} [6]) at a protein concentration of 0.06 mM. Both the HPLC and spectral analyses suggest that the protein carries two bound flavin molecules per polypeptide. The existence of enzymes with multiple redox centers has been established, and cytochrome P-450 reductase contains both FAD and FMN (12). To our knowledge, however, this is

TABLE 2. Effects of specific inhibitors on nitrobenzene nitroreductase

Inhibitor	Concn (mM)	% Activity remaining ± SD[a]
Chelating agents[b]		
EDTA (buffer only)	5.0	108 ± 5
EDTA (preincubation)	5.0	80 ± 2
1,10-Phenanthroline (preincubation)	5.0	78 ± 2
Salicyl hydroxamate	1.0	6 ± 0
	0.1	55 ± 3
	0.01	98 ± 1
Sulfhydryl inhibitors		
N-Ethylmaleimide	1.0	77 ± 6
p-Hydroxymercuribenzoate	1.0	46 ± 2
	0.5	61 ± 5
Iodoacetamide	1.0	95 ± 0
Electron transport inhibitors		
Dicumarol	0.01	40 ± 2
	0.001	94 ± 1
Potassium cyanate	0.5	75 ± 1
Sodium azide	1.0	35 ± 1
Reducing agents		
Cysteine	1.0	87 ± 1
Dithiothreitol	1.0	81 ± 2
Glutathione	1.0	85 ± 1
Others		
CuSO$_4$	0.5	78 ± 1
	0.2	76 ± 1
Hydroxylamine	10.0	75 ± 0
	1.0	108 ± 5
Menadione	0.15	77 ± 1
	0.1	83 ± 2
	0.05	91 ± 2
Naphthylamine	1.0	31 ± 0
	0.1	94 ± 5
	0.01	98 ± 1
Neocuproin	1.0	39 ± 4
	0.1	96 ± 3
Nitrite	1.0	77 ± 1
	0.1	102 ± 1

[a] The rate of oxidation of NADPH (micromoles per minute per milligram of protein) in the presence of inhibitor expressed as a percentage of the rate observed in the absence of inhibitor.
[b] Reductase assays were performed with the addition of EDTA in the reaction mixture (buffer only) or by preincubating aliquots of the enzyme with a chelating agent followed by assay in the presence of 5 mM EDTA (preincubation).
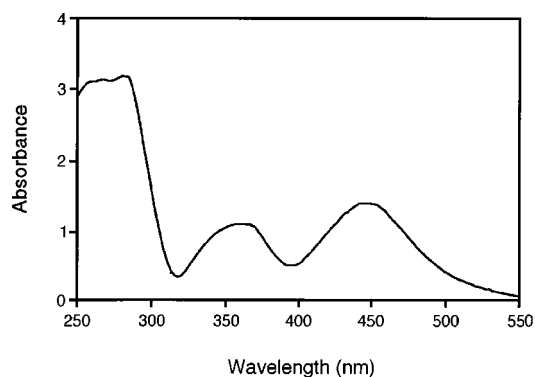

FIG. 1. Absorbance spectrum of *P. pseudoalcaligenes* nitrobenzene nitroreductase. Active fractions from size exclusion chromatography were pooled and concentrated prior to spectral analysis.

TABLE 3. Kinetic properties of nitrobenzene nitroreductase

Substrate	K_m (µmol)	V_{max} (µmol min⁻¹ mg⁻¹)	V_{max}/K_m
Nitrobenzene	5	92	18.4
Menadione	9	12	1.3
NADPH	183	144	0.8

the first report of a bacterial nitroreductase with a stoichiometry of 2 mol of flavin per mol of protein (1, 4, 6, 16, 35, 37, 41).

Molecular mass determination. SDS-polyacrylamide gel electrophoresis of the purified nitrobenzene nitroreductase indicated a single band with a molecular mass of 33 kDa. The mass of the native protein was estimated to be 30 kDa by gel filtration. Therefore, nitrobenzene nitroreductase is active as a monomer with a molecular mass of 30 to 33 kDa. Monomeric nitroreductase enzymes in the range of 24 to 28 kDa have been reported for *Enterobacter cloacae* (6), *E. coli* (1), *Nocardia* V (35), *Salmonella typhimurium* (37, 38), and *Vibrio fischeri* (41). In contrast, the reductase isolated from *Rhodobacter capsulatus* (4) was active as a homodimer of 54 kDa.

Kinetic properties of NB reduction. Table 3 summarizes the kinetic properties of the nitrobenzene nitroreductase of JS45. The nitroreductase purified from *E. coli* (1) had K_m values of 64 µM for nitrofurazone, 80 µM for menadione, and 862 µM for 5-(aziridin-1-YL)-2,4-dinitrobenzamide. The *Enterobacter cloacae* enzyme (6) had K_m values of 56 µM for 2,4,6-trinitrotoluene, 714 µM for nitrofurazone, and 966 µM for 2,4-dinitrotoluene. The K_m values of the *P. pseudoalcaligenes* enzyme for both NB and menadione are very low by comparison. The low K_m and the fact that expression of the reductase is induced by NB (22) suggest that NB is the physiological substrate for this enzyme. The K_m for NADPH was similar to that of the *Enterobacter* nitroreductase (144 µM [6]), but much higher than that of the *E. coli* enzyme (6 µM [1]). NADH could not substitute for NADPH.

Reaction intermediates and products. Spectrophotometric analyses revealed that 1.8 ± 0.01 mol of NADPH was oxidized per mol of NB converted to product. HPLC analyses indicated that 1.1 ± 0.01 mol of HAB was formed per mol of NB reduced. Substitution of HAB for NB in reaction mixtures caused a 220-fold reduction in the rate of NADPH oxidation,

and HPLC analyses indicated no detectable formation of aniline. The low rate of NADPH oxidation was due to the reduction of NOB formed by spontaneous oxidation of HAB. The production of HAB from NB is consistent with two two-electron reductions of the parent compound, the production of NOB as an intermediate, and the observed stoichiometry of NADPH oxidation. However, NOB can be chemically reduced to HAB by NADPH (3), and there have been reports of separate nitro- and nitrosoreductase activities (25, 28–30, 40). Therefore, we determined whether NOB reduction was catalyzed by nitrobenzene nitroreductase. The reduction of NOB (100 µM) was measured both with and without enzyme at a constant concentration of NADPH (10 µM). In the absence of enzyme, NOB was reduced at a rate of 6.4 nmol min⁻¹. With enzyme (1.2 µg) the rate increased to 10.2 nmol min⁻¹, and the increase in rate was proportional to the amount of enzyme added. The difference between the nonenzymatic and enzymatic rates yields a specific activity for NOB of 3.2 µmol min⁻¹ mg of protein⁻¹. At equivalent enzyme, substrate, and NADPH concentrations, the specific activity for conversion of NB to HAB was 2.6 µmol min⁻¹ mg of protein⁻¹. The results clearly indicate that NOB is recognized as a substrate and is reduced by the purified reductase.

An NADPH-generating system and limiting initial concentrations of NADPH were used to assay for end products and intermediates of reduction (Fig. 2). The disappearance of NB (absorbance maximum at 270 nm) was accompanied by an increased absorbance at the wavelength characteristic of HAB (230 nm), and there was no indication of a NOB intermediate.

Hydroxylamine reacts rapidly with NOB to form a benzenediazonium salt, and the presence of the salt can then be detected as an orange dye upon addition of 1-naphthylamine (11). Hydroxylamine does not react with either NB or HAB. Reduction of NB in the presence of 10 mM hydroxylamine (Fig. 3) yielded less HAB, but the addition of 1-naphthylamine did not produce any detectable dye. The results provide no evidence for the production of NOB as a free intermediate of the reduction reaction but do show that high concentrations of hydroxylamine inhibit the production of HAB, perhaps by a nonspecific mechanism.

On the basis of the results presented above, we propose that NB is reduced to NOB by a two-electron reduction and that NOB is immediately reduced to HAB by a second two-electron
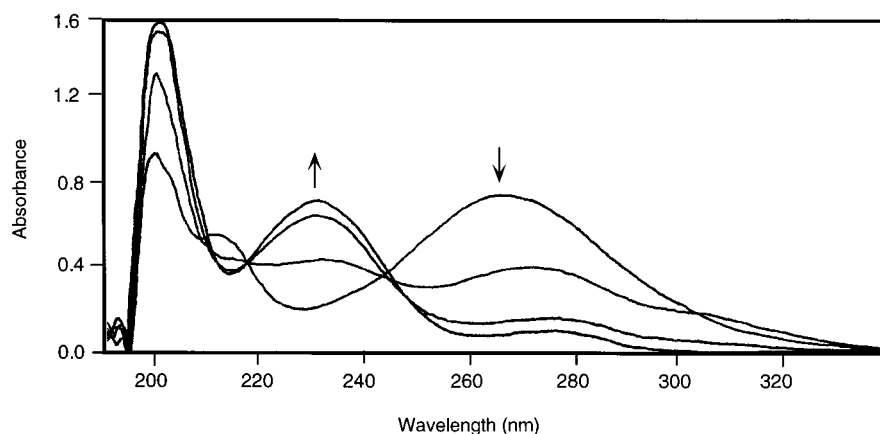

FIG. 2. Spectral changes associated with the reduction of NB by the nitroreductase of *P. pseudoalcaligenes* JS45. The sample cuvette contained NB (100 µM), NADPH (10 µM), glucose-6-phosphate (6 mM), glucose-6-phosphate dehydrogenase (5 U), and purified enzyme (2 µg) in 1 ml of phosphate buffer (pH 8). The reference cuvette did not contain NB. Reactions were started by the addition of NADPH, and spectra were recorded every 10 min. Arrows indicate the decrease in A_{270} concomitant with the disappearance of NB and the increase in A_{230} indicating the accumulation of HAB.
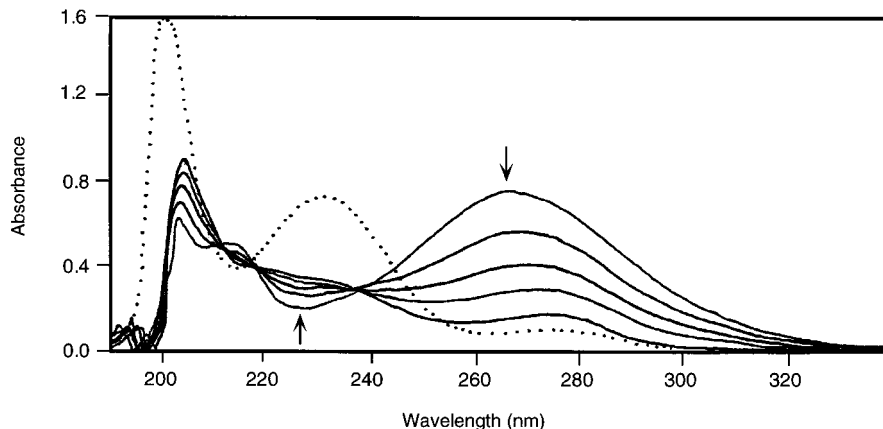
FIG. 3. Spectral changes associated with the reduction of NB by the nitroreductase of *P. pseudoalcaligenes* JS45 in the presence of hydroxylamine. Reaction conditions were as described in the legend to Fig. 2 with the addition of hydroxylamine (10 mM) to both the reference and sample cuvettes. Spectra were recorded every 10 min. The dotted line shows the accumulation of HAB after 50 min in the absence of hydroxylamine.

transfer. The high specific activity of the enzyme for NOB causes the NOB intermediate to be extremely short-lived, which would explain why it was not detected.

Inhibitors of enzyme activity. The JS45 reductase was clearly inhibited by dicumarol, an inhibitor of diaphorase (1) and menadione reductases (33) (Table 2). It showed similar sensitivity to nitroreductase I of *Bacteroides fragilis* (16) and was much more resistant than nitroreductase enzymes of *S. typhimurium* TA100 (33) and *E. coli* B/r (10), and nitroreductases II through IV of *B. fragilis* (16).

Inhibition by *p*-hydroxymercuribenzoate indicated that sulfhydryl groups are critical to the activity or stability of the enzyme. Inhibition by *p*-hydroxymercuribenzoate was much less than that of the nitroreductases of *Nocardia erythropolis* CA4 (9) and *E. coli* B/r (10) and was similar to that of the nitroreductase enzymes of *S. typhimurium* TA100 (33) and *R. capsulatus* E1F1 (4). The lack of inhibition by iodoacetamide argues against the presence of sulfhydryl groups at the active site.

Slight inhibition was caused by preincubating the enzyme with chelating agents, but extensive dialysis of the enzyme against both EDTA and 1,10-phenanthroline caused no inhibition. The latter observation is supported by the metal analyses which indicate that purified nitrobenzene nitroreductase has no metal cofactor. The inhibition caused by salicyl hydroxamate, a non-heme iron reagent (4), can perhaps be attributed to interaction with the active site of the enzyme.

Menadione inhibition was reversible upon the addition of increased concentrations of NB. This finding, and the fact that menadione is a good substrate for the enzyme (Table 3), suggests that menadione acts as a competitive inhibitor, but a K_i for the inhibition has not been determined.

In summary, our results indicate that the purified nitroreductase specifically catalyzes the reduction of NB to HAB. Aniline is not formed in the reaction, and HAB is not a substrate for the enzyme. NOB is reduced to HAB by the enzyme. The stoichiometric reduction of NB to HAB with concomitant oxidation of 2 mol of NADPH in the presence of oxygen indicates that this is an oxygen-insensitive (type I) nitroreductase (6, 26).

The phylogenetic relationships among bacterial nitroreductases have not been explored, although a comparison of the *E. coli* (1), *Enterobacter* (6), and *Salmonella* (38) enzymes shows a high degree of sequence similarity (1), indicating that classical

nitroreductase is conserved among the enteric bacteria. Terminal amino acid sequence data from the nitrobenzene nitroreductase (Fig. 4) do not show significant sequence similarity with the classical nitroreductase enzymes (data not shown) and do not support the inclusion of the *Pseudomonas* enzyme in the enteric family of nitroreductases.

The nitrobenzene nitroreductase of *P. pseudoalcaligenes* JS45 is well adapted to function in the degradation of NB in the environment. The higher specific activity for NOB than NB would be a sound strategy for the cell, given the reactive nature of nitroso compounds. The reduction of NB to HAB rather than aniline at first seems counterproductive given the cytotoxic and mutagenic nature of hydroxylaminoaromatic compounds. However, an examination of the oxygen requirements and energetics of NB degradation by alternative pathways (32) indicates that this partial reductive pathway is likely to be favored over either reduction to aniline or an initial oxidative removal of the nitro group under oxygen-limited conditions. This hypothesis is supported by the observation that the predominant phenotype in an NB-contaminated environment was the partial reductive pathway (23). The cell avoids the potential deleterious effects of HAB by the production of the mutase at a high specific activity (22), which ensures that no HAB accumulates.

The nitrobenzene nitroreductase of *P. pseudoalcaligenes* JS45 may be well suited to immobilized enzyme biocatalysis. The purified enzyme is relatively stable below 40°C, retains activity over a broad pH range, is active in the presence of a number of inhibitor compounds, and has a tightly bound flavin cofactor. Furthermore, preliminary experiments indicate that it catalyzes the reduction of a variety of nitroaromatic compounds (data not shown). The products formed from those compounds and the potential uses of the enzyme will be the subjects of future research.

$$\begin{array}{ccccccccccccccccccccccccccccccc} 1 & & & 5 & & & & & 10 & & & & & 15 & & & & & 20 & & & & & 25 & & & & & 30 \\ P & T & S & P & F & I & D & D & L & I & R & D & R & R & A & K & R & G & F & L & D & Q & P & V & [N] & [I] & [E] & [M] & [V] & [K] \end{array}$$

FIG. 4. Partial amino acid sequence derived from the nitrobenzene nitroreductase of *P. pseudoalcaligenes* JS45. The sequence determination yielded a positive identification of 24 residues. Amino acid residues identified in brackets (positions 25 to 30) are considered probable.

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