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. 59:2530, 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 nitrosobenzene 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, 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 blastocyst.
cells or cell extracts. Instead, HAB is converted by a specific mutase to 2-aminophenol, which is the substrate for ring fission (2). 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 nitroreductase 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, 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 (Mylville, N.J.).

Bacteria and growth conditions. P. pseudoalcaligenes JS45 (22) was maintained in a MultiGen fermentor equipped with a 14-liter vessel. The reactor was stirred at 250 rpm overnight at 30°C. The formation of intermediates and products of the reduction reaction were determined by HPLC analyses of reaction mixtures containing NB (0.6 mM), NADPH (1.8 mM), and purified reductase (9 mg) in 1 ml of phosphate buffer. Samples (100 μl) were analyzed on a 250-μm Spherosorb C8 column (Alltech, Deerfield, Ill.) with a solvent system of 65% trifluoroacetic acid (0.1% in H2O)-35% acetonitrile. Products of the reactions were identified and quantified by comparison of the spectra and retention times with those of standards.

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 the amino acid composition on the basis of the amino acid sequence at a present here has been reported to the Protein Identification Resource databank and has been assigned accession number A44682.

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 spectrosocopy 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-μm Spherosorb C8 column (Alltech, Deerfield, Ill.) with a solvent system of 65% trifluoroacetic acid (0.1% in H2O)-35% acetonitrile. Products of the reactions were identified and quantified by comparison of the spectra and retention times with those of standards.

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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 (ε450 = 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 not readily accessible to exogenous flavins. Similarly, the flavin binding site 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).

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### Table 1. Purification of nitrobenzene nitroreductase from *P. pseudoalcaligenes* JS45

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Total activity (Sp act)</th>
<th>Protein concn (mg/ml)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>180</td>
<td>10,454</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Cleared lysate</td>
<td>161</td>
<td>11,630</td>
<td>17</td>
<td>111</td>
</tr>
<tr>
<td>45–60% (NH₄)₂SO₄ cut, desalted</td>
<td>25</td>
<td>4,621</td>
<td>6.8</td>
<td>28</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>10</td>
<td>3,631</td>
<td>6.1</td>
<td>100</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>6.7</td>
<td>2,958</td>
<td>1.8</td>
<td>100</td>
</tr>
</tbody>
</table>

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

### Table 2. Effects of specific inhibitors on nitrobenzene nitroreductase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concn (mM)</th>
<th>% Activity remaining ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chelating agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA (buffer only)</td>
<td>5.0</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>EDTA (preincubation)</td>
<td>5.0</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>1,10-Phenanthroline (preincubation)</td>
<td>5.0</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>Salicyl hydroxamate</td>
<td>1.0</td>
<td>60 ± 0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.1</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.001</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Sulphydryl inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>1.0</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>4-Hydroxynonenal</td>
<td>0.5</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1.0</td>
<td>95 ± 0</td>
</tr>
<tr>
<td>Electron transport inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neocuproin</td>
<td>1.0</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Dibutylstilbestrol</td>
<td>1.0</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.0</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.5</td>
<td>78 ± 1</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.2</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.1</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.01</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.001</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>Naphthylamine</td>
<td>1.0</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.15</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.1</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>p-Hydroxynonenal</td>
<td>0.05</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.0</td>
<td>31 ± 0</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>0.1</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.01</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.0</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>Neocuproin</td>
<td>0.1</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.0</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.1</td>
<td>102 ± 1</td>
</tr>
</tbody>
</table>

* 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.

* 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).
TABLE 3. Kinetic properties of nitrobenzene nitroreductase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μmol)</th>
<th>$V_{max}$ (μmol min$^{-1}$ mg$^{-1}$)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrobenzene</td>
<td>5</td>
<td>92</td>
<td>18.4</td>
</tr>
<tr>
<td>Menadione</td>
<td>9</td>
<td>12</td>
<td>1.3</td>
</tr>
<tr>
<td>NADPH</td>
<td>183</td>
<td>144</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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 *E. coli* enzyme (6 μM [1]), 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 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$^{-1}$ 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$^{-1}$ 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 benzamidazone 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$_{230}$ concomitant with the disappearance of NB and the increase in A$_{270}$ indicating the accumulation of HAB.](http://jb.asm.org/Downloaded_from)
In this text, the author discusses the nitrobenzene nitroreductase from *P. pseudoalcaligenes* JS45 in the presence of hydroxylamine. They explore the enzyme's activity, inhibition, and role in the degradation of nitroaromatic compounds. The enzyme is shown to be active in the presence of oxygen, and its inhibition by various compounds is examined. The study also includes a comparison with other nitroreductases, highlighting the enzyme's specificity and potential for use in biocatalysis.

**Fig. 3** shows the spectral changes associated with the reduction of NB by the nitroreductase of *P. pseudoalcaligenes* JS45 in the presence of hydroxylamine. The dotted line represents the accumulation of HAB after 50 min in the absence of hydroxylamine. The graph illustrates how the enzyme activity is monitored through absorbance measurements.

**Fig. 4** presents a 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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REFERENCES


