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# Regioselectivity of Nitroglycerin Denitration by Flavoprotein Nitroester Reductases Purified from Two *Pseudomonas* Species

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**Two species of *Pseudomonas* capable of utilizing nitroglycerin (NG) as a sole nitrogen source were isolated from NG-contaminated soil and identified as *Pseudomonas putida* II-B and *P. fluorescens* I-C. While 9 of 13 laboratory bacterial strains that presumably had no previous exposure to NG could degrade low concentrations of NG (0.44 mM), the natural isolates tolerated concentrations of NG that were toxic to the lab strains (1.76 mM and higher). Whole-cell studies revealed that the two natural isolates produced different mixtures of the isomers of dinitroglycerol (DNG) and mononitroglycerol (MNG). A monomeric, flavin mononucleotide-containing NG reductase was purified from each natural isolate. These enzymes catalyzed the NADPH-dependent denitration of NG, yielding nitrite. Apparent kinetic constants were determined for both reductases. The *P. putida* enzyme had a $K_m$ for NG of 52 ± 4 μM, a $K_m$ for NADPH of 28 ± 2 μM, and a $V_{max}$ of 124 ± 6 μM · $min^{-1}$, while the *P. fluorescens* enzyme had a $K_m$ for NG of 110 ± 10 μM, a $K_m$ for NADPH of 5 ± 1 μM, and a $V_{max}$ of 110 ± 11 μM · $min^{-1}$. Anaerobic titration experiments confirmed the stoichiometry of NADPH consumption, changes in flavin oxidation state, and multiple steps of nitrite removal from NG. The products formed during time-dependent denitration reactions were consistent with a single enzyme being responsible for the in vivo product distributions. Simulation of the product formation kinetics by numerical integration showed that the *P. putida* enzyme produced an ≈2-fold molar excess of 1,2-DNG relative to 1,3-DNG. This result could be fortuitous or could possibly be consistent with a random removal of the first nitro group from either the terminal (C-1 and C-3) positions or middle (C-2) position. However, during the denitration of 1,2-DNG, a 1.3-fold selectivity for the C-1 nitro group was determined. Comparable simulations of the product distributions from the *P. fluorescens* enzyme showed that NG was denitrated with a 4.6-fold selectivity for the C-2 position. Furthermore, a 2.4-fold selectivity for removal of the nitro group from the C-2 position of 1,2-DNG was also determined. The MNG isomers were not effectively denitrated by either purified enzyme, which suggests a reason why NG could not be used as a sole carbon source by the isolated organisms.**

Nitroglycerin (NG, glycerol trinitrate) is an aliphatic nitrate ester-containing compound that is an important component of dynamite and other propellants (36, 42). In addition, nitro-substituted compounds, including nitroaromatics, are used as explosives, agricultural chemicals, and dyes (18, 32, 39). Consequently, compounds containing nitro functional groups have become widely distributed in the environment. Since naturally occurring nitro functional groups are rare (18, 32), the molecules containing these groups have typically been classified as xenobiotics. Consistent with this classification, a number of early studies of the environmental fate of NG revealed toxicity to algae, invertebrates, and vertebrates (42) and further suggested that NG was recalcitrant to microbial degradation. However, more recently, the metabolism of NG has been demonstrated in fungal (8, 9, 28, 29, 45) and bacterial (2, 3, 16, 24, 25, 38, 40) systems.

Along with the uses described above, NG and related aliphatic nitrate esters are used as pharmaceutical agents to deliver nitric oxide (NO) for the treatment of angina (1, 4, 35). The mammalian metabolism of NG has been well characterized (31) and is catalyzed primarily by glutathione *S*-transferases (31, 35), but heme-based and other transformation pathways have been observed. Both nitrite and NO are products of mammalian metabolism.

Fungi also apparently use a similarly diverse array of enzymes to metabolize NG (29) and also produce nitrite and NO (28). As observed for mammalian metabolism, studies of fungal cultures have revealed that NG can be degraded to a mixture of dinitroglycerol (DNG) isomers with selectivity for either the C-1 and C-3 positions (9) or the C-2 position (8), depending on the organism. The DNG isomers were also slowly converted to mononitroglycerol (MNG) isomers. During fungal degradation, the MNG isomers accumulated as the final metabolites except in the case of *Penicillium corylophilum* Dierckx, which slowly degraded the MNG isomers to glycerol. A kinetic model consistent with this overall metabolism is shown in Fig. 1; this model has often been used to qualitatively describe the results of NG denitration (24, 25, 37–40). However, no systematic evaluation of the properties of purified enzyme(s) responsible for NG metabolism or of the relative magnitudes of the rate constants associated with the different product distributions have been reported.

NG was metabolized to DNG and MNG intermediates by various bacterial cultures (18), and in some cases all glycerol nitrates could be removed from the culture medium. Recently, the biodegradation of NG by *Bacillus thuringiensis*/*cereus*, and

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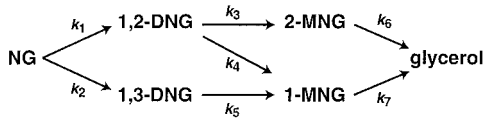


FIG. 1. Kinetic model accounting for the products observed from the denitration of NG.

*Enterobacter agglomerans* has been reported, and a hydrolytic reaction mechanism was suggested based on studies of dialyzed cell extracts (24). The denitration of NG by pure cultures of *Agrobacterium radiobacter* has also been reported (40), and in vivo nuclear magnetic resonance measurements showed that both isomers of DNG accumulated, with 1,3-DNG preferred by a roughly 8:1 ratio (corresponding to selectivity for denitration at the C-2 position). Subsequent conversion of the DNG isomers to 1-MNG and 2-MNG also occurred over a longer time scale. Cell extracts prepared from this organism used a reductive pathway in the presence of NADPH to release nitrite. A purified recombinant pentaerythritol tetranitrate (PETN) reductase (40), originally from *Enterobacter cloacae* PB2 (2), has also been shown to produce nitrite during the NADPH-dependent denitration of NG. Due to the relative insolubility of PETN, detailed steady-state kinetic studies of the reaction with NG were undertaken with the recombinant enzyme (40), but no analysis of the regioselectivity of reaction was reported.

Here we describe the isolation of two species of *Pseudomonas* from NG-contaminated soils using enrichment culture. One species was selected for the ability to use NG as a sole source of nitrogen, while the other was selected for the ability to tolerate high concentrations of NG in the growth medium. The enzymes responsible for NADPH-dependent NG denitrification have been purified to homogeneity from each bacterial species. Both of these enzymes are flavoproteins with biochemical properties similar to those of the class of α/β-barrel flavoprotein oxidoreductases including old yellow enzyme (14), morphinone reductase (15), PETN reductase (2), and possibly others (43, 44). In studies using purified enzyme preparations, a linkage between the reduced redox state of the flavin and the denitration of both NG and DNG isomers by a single enzyme has been established under single turnover conditions. The product distributions of the denitration reactions have also been determined and analyzed based on the kinetic model of Fig. 1. This analysis reveals that the two flavoenzymes have different selectivities for removal of the first nitro group from NG and that the selective preference of each enzyme is also partially maintained during the removal of the second nitro group from the DNG isomers.

## MATERIALS AND METHODS

**Materials.** All chemicals used were analytical grade or better. A 4.4 mM solution of NG dissolved in water was obtained from Olin Corporation (Baraboo, Wis.). Analytical reference standards of NG, 1,3-DNG, 1,2-DNG, 2-MNG, and 1-MNG were purchased from Radian International (Austin, Tex.).

**Strain isolation and identification.** Soil samples were collected at the Badger Army Ammunition Plant (Baraboo, Wis.) from sites previously contaminated with NG-manufacturing wastes. The soil samples were stored at 4°C until use. All strains were isolated and cultured in a minimal medium (33) at 30°C with gentle agitation unless otherwise noted. The NG-degrading isolates were identified based on morphological observations, the bioMerieux (Marcy l'Etoile, France) API 20E identification system for gram-negative bacteria, and additional physiological tests. Morphological observations and physiological tests were interpreted with the aid of *Bergey's Manual of Determinative Bacteriology* (20).

To isolate bacteria that tolerated NG concentrations inhibitory to other organisms, minimal medium liquid cultures were prepared as described above. Aliquots of these cultures were used to inoculate a series of minimal medium cultures containing NG at 0.13, 0.22, 0.44, 0.88, 1.1, 1.32, and 2.2 mM. Organisms that grew in liquid medium containing 2.2 mM NG were then isolated on minimal medium plates containing 1.32 mM NG and checked for purity by isolation of single colonies on nutrient agar plates.

To isolate bacteria that used NG as a sole nitrogen source, minimal medium liquid cultures supplemented with 0.44 mM NG, but lacking ammonium sulfate, were inoculated with soil. After 2 days of enrichment culture growth, NG-degrading organisms were isolated on nutrient agar plates. Isolates were reinoculated into minimal medium cultures containing NG as the sole nitrogen source, and cultures were assayed for NG degradation.

**Bacterial growth and substrate utilization.** Starting inocula grown in minimal medium supplemented with 1.32 mM NG were used to inoculate triplicate cultures of the same medium. Culture densities and NG denitration by *Pseudomonas putida* II-B and *P. fluorescens* I-C were measured initially and over a 24-h period. Cell densities were determined by using a Klett-Summerson (New York, N.Y.) photoelectric colorimeter with a red filter, and the amount of NG denitration was monitored by high-performance liquid chromatography (HPLC) as described below. To evaluate the ability of laboratory bacterial strains to degrade NG, minimal medium starter cultures were used to inoculate duplicate minimal medium cultures supplemented with either 0.44 or 1.76 mM NG. NG denitration was then measured after 24 h.

**Enzyme purification.** Stationary-phase cells grown in minimal medium in the absence of NG were harvested, washed in 100 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer (2 ml of buffer per g [wet weight] of cells). All subsequent steps were conducted at 4°C. Cells were broken by two passages through a French pressure cell (SLM Instruments, Rochester, N.Y.) at 16,000 lb/in$^2$. The lysate was centrifuged for 60 min at 25,000 × *g*, and the supernatant from the first centrifugation was then subjected to another centrifugation step for 60 min at 25,000 × *g*. The resultant supernatant, called the cell extract, was frozen in liquid nitrogen and stored at −80°C until needed.

The *P. putida* II-B cell extract was loaded onto a 2.5- by 35-cm column containing DEAE-cellulose (Sigma, St. Louis, Mo.) equilibrated with 100 mM potassium phosphate buffer (pH 7.0). The column was washed with 1 bed volume of the equilibration buffer, and NG reductase activity was eluted overnight with a 600-ml linear gradient from 0.0 to 0.8 M KCl in the same buffer. Active fractions from the DEAE column were pooled and concentrated by ultrafiltration with a YM-30 membrane (Amicon, Beverly, Mass.). The concentrated protein was diluted fourfold with 50 mM potassium phosphate buffer (pH 7.0) to reduce the ionic strength and was then loaded onto a 2.5- by 10-cm column containing Q-Sepharose (Pharmacia, Piscataway, N.J.) equilibrated with the same buffer. The column was washed with 1 bed volume of equilibration buffer, and NG reductase activity was eluted overnight with a 250-ml linear gradient from 0.0 to 0.4 M KCl in the same buffer. Active fractions from the Q-Sepharose column were concentrated to approximately 8 ml, using a Centriprep-30 (Amicon). Four 2-ml portions of the concentrated protein were individually loaded onto a 1.6- by 60-cm HiPrep Sephacryl S-100 column (Pharmacia). The column was equilibrated with 50 mM potassium phosphate (pH 7.0) containing 150 mM KCl and was eluted with the same buffer at a flow rate of 0.4 ml/min. Active fractions were pooled, frozen in liquid nitrogen, and stored at −80°C.

The *P. fluorescens* I-C cell extract was loaded onto a 1.5- by 14.5-cm column containing DEAE-Sepharose (Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The column was washed with 1 bed volume of 50 mM potassium phosphate buffer (pH 7.0), and NG reductase activity was eluted overnight with a 350-ml linear gradient from 0.0 to 1.0 M KCl in the same buffer. Active fractions from the DEAE column were pooled, diluted eightfold with 50 mM potassium phosphate buffer (pH 7.0), and concentrated by ultrafiltration with a YM-30 membrane. The concentrated protein was diluted an additional twofold with distilled, deionized water to reduce the ionic strength and loaded onto a 2.5- by 10-cm column containing Q-Sepharose equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The column was washed with 1 bed volume of equilibration buffer, and NG reductase activity was eluted overnight with a 250-ml linear gradient from 0.0 to 0.6 M KCl in the same buffer. Active fractions from the Q-Sepharose column were concentrated to approximately 7 ml by ultrafiltration and loaded onto a 1- by 93-cm column containing Sephacryl S-100. The column was equilibrated with 100 mM potassium phosphate buffer (pH 7.0) and eluted with the same buffer at a linear flow rate of 0.4 ml/min. Active fractions were pooled, frozen in liquid nitrogen, and stored at −80°C.

**Electrophoresis methods.** Subunit $M_r$ values were determined by denaturing gel electrophoresis in 10% polyacrylamide resolving gels, using a Tris-glycine-sodium dodecyl sulfate buffer system with β-mercaptoethanol as a reducing agent. Proteins were visualized by staining with Coomassie brilliant blue R-250. Isoelectric focusing was performed by using precast polyacrylamide gel plates (Pharmacia) with a broad-range ampholyte (pI 3.5 to 9.5) and a Pharmacia FBE-3000 flatbed apparatus. Proteins were visualized by Coomassie staining, and the isoelectric points were determined by comparison to calibration standards (Pharmacia).

**Protein characterizations.** Protein concentrations were routinely determined by using the Bio-Rad (Hercules, Calif.) protein assay, with bovine serum albumin as the standard. The native $M_r$ of the *P. putida* enzyme was determined by HPLC using a 7.8- by 300-mm Protein Pak 125 gel filtration column (Waters Chromatography, Milford, Mass.) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 400 mM KCl. Protein elution was monitored at 280 nm. The column was calibrated by using low-molecular-weight gel filtration markers

(Pharmacia). The native $M_r$ of the *P. fluorescens* enzyme was determined by velocity sedimentation in the Biophysics Instrumentation Facility at the Department of Biochemistry, University of Wisconsin—Madison. The flavin prosthetic group of each enzyme was released by denaturation with trichloroacetic acid (21) and identified by comparing the HPLC elution times of the released flavin with those of flavin mononucleotide (FMN) and flavin adenine dinucleotide standards (19). The concentration of FMN released from the denatured enzymes was determined by comparison of peak areas with those of an FMN standard curve ($\varepsilon_{445}$ = 12,400 $M^{-1}$ $cm^{-1}$). Aliquots of the same protein preparation used for flavin identification and quantitation were also subjected to amino acid analysis and triplicate Bio-Rad protein assays in order to determine protein concentration and flavin stoichiometry. Purified NG reductase samples were analyzed for metal content by inductively coupled plasma atomic emission spectrometry at the Chemical Analysis Laboratory, University of Georgia—Athens.

**NG reductase catalytic assays.** NG reductase activity was monitored during enzyme purification by measuring the rate of NADPH oxidation at 340 nm in the presence of enzyme and NG. The assay buffer was 100 mM potassium phosphate buffer (pH 7.0). A typical assay initially contained 260 μM NG and 130 μM NADPH in 700 μl of assay buffer. Reactions were initiated by the addition of enzyme and monitored for 2 min. One unit of enzyme activity was defined as the oxidation of 1 μmol of NADPH per min at room temperature in the assay buffer.

**Optical spectroscopy and reductive titrations.** Optical spectra of the purified NG reductases were measured in assay buffer, using a Hewlett-Packard 8452A diode array spectrophotometer. Reductive titrations were performed in an optical cuvette modified for anaerobic work (34). Enzyme aliquots in assay buffer were made anaerobic by repeated cycles of evacuation and flushing with $O_2$-free argon gas (12). After preparing the anaerobic enzyme solution, a catalytic amount of protocatechuate 3,4-dioxygenase and 100 μmol of protocatechuate (3,4-dihydroxybenzoate) were sequentially added as an $O_2$-scavenging system (41). Solutions of the assay buffer containing NADPH (2.1 mM) or NG (2.6 mM) were separately made anaerobic in rubber septum-sealed 6-ml reaction vials. The NADPH solution was then titrated into the anaerobic cuvette containing the NG reductase by using a gastight syringe, and the absorbance spectrum of NG reductase was measured after each addition. After conversion to the reduced form, the NG reductase was reoxidized by titration of the anaerobic NG solution into the cuvette by using a gastight syringe. Products from the single turnover reoxidation of the reduced flavoenzyme were determined by HPLC as described below.

**Product analysis.** To determine the stoichiometry and regioselectivity of NG denitration, purified enzyme from each organism was incubated at 30°C with variable amounts of NADPH and NG in a final reaction volume of 500 μl. Enzyme, NADPH, and NG amounts are noted in Table 5, Fig. 4, and Fig. 5. Each enzyme assay was conducted in triplicate. After the appropriate time, the reactions were terminated by the addition of an equal volume of a quench solution containing 0.72 mM potassium ferricyanide and 0.27 mM phenazine methosulfate (27). After quenching, the reaction samples were analyzed for nitroester-containing products by HPLC in a water-methanol gradient (6), using a Beckman (Fullerton, Calif.) instrument equipped with a 5- by 100-mm, 4-μm Nova-Pak radial compression $C_{18}$ column (Waters Chromatography). Metabolites were detected at 210 nm. NG concentrations were determined by comparison of peak areas with those of a NG standard curve, while concentrations of the DNG and MNG isomers were estimated from the NG standard curve by scaling the absorbance of each compound according to published molar absorptivity values (10, 11). Nitrite production was analyzed colorimetrically as previously described (7).

**NG denitration kinetics.** Steady-state apparent kinetic parameters for NG and NADPH utilization were calculated by nonlinear least-squares fitting (5). To determine kinetic parameters for the *P. putida* enzyme, NG concentrations were varied from 10 to 400 μM and NADPH concentrations were varied from 8 to 300 μM. To determine kinetic parameters for the *P. fluorescens* enzyme, NG concentrations were varied from 40 to 600 μM and NADPH concentrations were varied from 1 to 30 μM. The product distributions observed during NG denitration were simulated by numerical integration (13) by using the Mathematica function NDSolve (Wolfram Research, Inc., Champaign, Ill.). The inputs to this routine were the differential equations describing the change in concentration of NG and the DNG and MNG products of the denitration reactions, the initial concentrations of the metabolites, and estimates for each of the kinetic constants shown in Fig. 1. The overall quality of a given fit was tested by calculating the sum of the squared difference between the measured and calculated values for substrate and product concentrations at each experimental time point (13). A minimum summed squared difference was determined by iterative, incremental change of the initial values selected for the kinetic constants.

## RESULTS

**Strain isolation and characterization of in vivo NG denitration.** Six gram-negative, rod-shaped bacteria with differentiable colony morphologies were isolated from NG-contaminated soil, and two of these isolates were chosen for the following studies. *P. putida* II-B was isolated based on its ability to tolerate NG concentrations inhibitory to other soil microorganisms, while *P. fluorescens* I-C was isolated based on the ability to grow in minimal medium containing NG as the sole nitrogen source. Selected test results that identified these isolates as fluorescent pseudomonads included the observations that both organisms were motile, arginine dihydrolase-positive, strict aerobes that produced fluorescent pigments under iron-deficient conditions. The isolates differed in that *P. fluorescens* I-C was lecithinase positive and hydrolyzed gelatin, while *P. putida* II-B was negative for these two tests. Both isolates grew at 4°C but not at 42°C. Throughout the remainder of this work, the isolates will be referred to by their genus and species names only.

TABLE 1. Percentage denitration and cell growth by various bacterial strains exposed to NG

| Source | % NG degraded | | Culture density[a] | |
|---|---|---|---|---|
| | 0.44 mM | 1.76 mM | 0.44 mM | 1.76 mM |
| Isolates from NG-contaminated soil | | | | |
| *P. putida* II-B | 100 | 100 | 393 | 368 |
| *P. fluorescens* I-C | 100 | 99 | 291 | 289 |
| Strain collection, Department of Bacteriology, University of Wisconsin | | | | |
| *P. aeruginosa* JB2 | 100 | 89 | 399 | 330 |
| *Escherichia coli* 8008 | 100 | 85 | 357 | 272 |
| *Klebsiella oxytoca* 8701 | 100 | 77 | 387 | 202 |
| *K. planticola* | 100 | 58 | 373 | 66 |
| *E. coli* 8101 | 100 | 25 | 370 | 103 |
| *P. fluorescens* | 99 | 60 | 425 | 370 |
| *K. oxytoca* 8408 | 98 | 66 | 335 | 178 |
| *P. aerofaciens* | 94 | 33 | 414 | 176 |
| *P. aerofaciens* C16 | 94 | 23 | 430 | 185 |
| *P. fluorescens* 2-79 | 81 | 3 | 334 | 12 |
| *P. syringae* | 47 | 1 | 196 | 17 |
| *K. pneumoniae* 99 | 43 | 21 | 104 | 64 |
| *Pseudomonas* strain D | 6 | 1 | 111 | 30 |

[a] Culture densities observed 24 h after exposure to NG are reported in Klett units.

In the absence of ammonium sulfate, both *P. putida* and *P. fluorescens* utilized NG as a sole nitrogen source. Neither organism fixed $N_2$, as a culture medium lacking a nongaseous nitrogen source did not support growth. Moreover, when glucose was omitted from minimal medium containing NG, the organisms did not utilize NG as a carbon source. To verify that NG denitration was enzymatic, sterile culture medium containing NG was incubated under normal culture conditions, and no decrease in NG concentration was observed over a 24-h period at 30°C. Furthermore, NG was not denitrated in a cell-free reaction containing medium prepared from the NG-degrading cultures by filtration through a 0.45-μm-pore-size sterile filter, implying that extracellular enzymes did not catalyze NG metabolism.

To determine if the ability to denitrate NG was limited to bacteria isolated from NG-contaminated soil, bacterial strains from the collection of the Department of Bacteriology, University of Wisconsin, were tested for the ability to degrade NG (Table 1). Although these laboratory strains presumably had no previous exposure to NG, 9 of 13 strains tested were capable of degrading 90% or more of low concentrations of NG (0.44 mM). This observation suggests that denitration may be a relatively common, adventitious bacterial capability and would be consistent with the ability of a wide variety of redox

TABLE 2. Purification of NG reductase from two soil pseudomonads

| Step | Total protein (mg) | Total activity (U)[a] | Sp act (U/mg) | Fold purification | Recovery (%) |
|---|---|---|---|---|---|
| *P. putida* | | | | | |
| Cell extract | 1,660 | 1,000 | 0.6 | 1.0 | 100 |
| DEAE-cellulose | 720 | 790 | 1.1 | 1.8 | 79 |
| Q-Sepharose | 150 | 680 | 4.5 | 7.5 | 68 |
| Sephacryl S-100 | 80 | 330 | 4.1 | 6.8 | 33 |
| *P. fluorescens* | | | | | |
| Cell extract | 820 | 570 | 0.7 | 1.0 | 100 |
| DEAE-Sepharose | 120 | 500 | 4.2 | 6.0 | 88 |
| Q-Sepharose | 60 | 280 | 4.7 | 6.7 | 49 |
| Sephacryl S-100 | 30 | 350 | 11.5 | 16.4 | 61 |

[a] One unit of activity is defined as 1 μmol of NADPH oxidized per min.

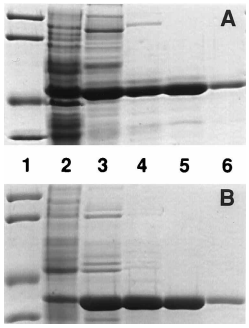


FIG. 2. Denaturing gel electrophoretic analysis of NG reductase purifications. (A) *P. putida* II-B; (B) *P. fluorescens* I-C. Lane 1, molecular mass standards of 94, 67, 43, and 30 kDa; lane 2, 30 μg of protein from the cell extract; lane 3, 30 μg of protein from DEAE chromatography; lane 4, 10 μg of protein from Q-Sepharose chromatography; lane 5, 10 μg of protein from Sephacryl S-100 chromatography; lane 6, 2 μg of protein from the sample of lane 5.

enzymes (including flavoproteins) to fortuitously transform nitro groups (30). However, in the presence of 1.76 mM NG, which was not inhibitory to either of the *Pseudomonas* species isolated from NG-contaminated soils, both the percentages of NG degraded and the final culture densities decreased for most of the organisms obtained from the strain collection.

Time course measurements of the changes in concentrations of NG and the denitration products 1,3-DNG and 1,2-DNG revealed that *P. putida* produced a ≈2-fold excess of 1,2-DNG relative to 1,3-DNG, while in contrast, *P. fluorescens* produced a ≈6-fold excess of 1,3-DNG relative to 1,2-DNG. Since the basis for the regioselectivity of NG metabolism has not been established, we were interested in characterizing the properties of the enzyme(s) responsible for this reactivity.

**Enzyme purification.** With the exception of *A. radiobacter* (40), which exhibited an ≈160-fold increase in NG reductase activity after exposure to NG, only relatively modest increases in specific activity have been observed upon exposure to NG from a wide range of other NG-metabolizing organisms (2, 24, 29). Therefore, the specific activities of the NG reductase in cell extracts prepared from cells grown in minimal medium containing or lacking 1 mM NG were measured to investigate whether NG reductase expression was induced by the presence of NG or metabolites derived from this compound. The specific activities of cell extracts prepared from *P. putida* and *P. fluorescens* grown in the absence of NG were 0.9 and 0.4 U/mg, respectively. When grown in medium containing 1 mM NG, cell extracts prepared from *P. putida* and from *P. fluorescens* had specific activities of 2.0 and 0.6 U/mg, respectively.

Table 2 summarizes the purification of the NG reductases from *P. putida* and *P. fluorescens*. For each bacterium, a single enzyme was associated with all detectable NG denitration activity. As judged by denaturing gel electrophoresis (Fig. 2), both enzymes were purified to near homogeneity by using similar protocols consisting of three chromatographic steps. The *P. putida* reductase was purified ≈7-fold, suggesting that this enzyme accounted for ≈14% of the soluble protein, while the *P. fluorescens* enzyme was purified ≈16-fold, suggesting that this enzyme accounted for ≈6% of the soluble protein. For both enzymes, the assessment of fold purification based on the increase in specific activity was consistent with the relative intensity of the corresponding protein bands in the cell-free extracts prepared from these two organisms (Fig. 2).

**Enzyme characterization.** The properties of both purified reductases are summarized in Table 3. The *P. putida* NG reductase had a subunit $M_r$ of 46,000 as determined by denaturing gel electrophoresis and a native $M_r$ of 52,000 based on gel filtration chromatography, indicating that the native enzyme was monomeric. The subunit $M_r$ of the NG reductase from *P. fluorescens* was estimated to be 39,000 by denaturing gel electrophoresis, while sedimentation velocity measurements revealed a native $M_r$ of 37,000. Therefore, the *P. fluorescens* enzyme was also monomeric. The observed isoelectric points of the reductases from *P. putida* and from *P. fluorescens* were 4.5 and 5.0, respectively, while the pH optima for activity were 6.0 and 7.8, respectively.

**Steady-state kinetic analysis.** The apparent steady-state kinetic constants determined for the purified NG reductases are summarized in Table 4 and below. The *P. putida* enzyme had a $K_m$ for NG of 52 ± 4 μM, a $K_m$ for NADPH of 28 ± 2 μM, and a $V_{max}$ of 124 ± 6 μM · $min^{-1}$. The *P. fluorescens* enzyme had a $K_m$ for NG of 110 ± 10 μM, a $K_m$ for NADPH of 5 ± 1 μM, and a $V_{max}$ of 110 ± 11 μM · $min^{-1}$. These values are similar to those determined for the PETN reductase from *E. cloacae* PB2 with NG as a substrate (2, 16) and for cell extracts of *P. chrysosporium* metabolizing NG (29).

**Cofactor content and redox cycle.** Figure 3, spectrum 1, shows the optical spectrum of the as-isolated *P. putida* enzyme. This spectrum is typical for a flavin-containing enzyme and has maxima at 380 and 460 nm with well-resolved shoulders at 430 and 490 nm (22). A nearly identical optical spectrum and titration behavior (see below) were obtained with the *P. fluorescens* enzyme; these data have not been presented for the sake of brevity. Upon denaturation with trichloroacetic acid, the flavin was released from each purified enzyme and identified by HPLC analysis to be FMN. By comparing the FMN concentrations determined by HPLC with the protein concentrations determined by quantitative amino acid analysis, both NG reductases were found to bind ≈1 mol of FMN per mol of protein monomer (Table 3). Quantitative metal analyses re-

TABLE 3. Biochemical properties of NG reductase from two pseudomonads

| Strain | $M_r$ ($10^3$) | | Isoelectric point | pH optimum | $A_{280}/A_{460}$ | Prosthetic group | Mean mol of FMN/mol of protein ± SD |
|---|---|---|---|---|---|---|---|
| | Denatured | Native | | | | | |
| *P. putida* | 46 | 52 | 4.5 | 6.0 | 9.1 | FMN | 1.0 ± 0.2 |
| *P. fluorescens* | 39 | 37 | 5.0 | 7.8 | 6.8 | FMN | 1.1 ± 0.4 |

TABLE 4. Apparent kinetic constants of NG reductase from two pseudomonads

| Strain | Mean $K_m$ (μM)[a] ± SD | | Mean $V_{max}$ (μM · min$^{-1}$)[a] ± SD | $V_{max}/K_m$, NG (min$^{-1}$)[b] | Rate constant (min$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NG | NADPH | | | $k'$[c] | $k_1$[d] | $k_2$[d] | $k_3$[d] | $k_4$[d] | $k_5$[d] |
| *P. putida* | 52 ± 4 | 28 ± 2 | 124 ± 6 | 2.4 | 2.1 | 1.4 | 0.7 | 0.04 | 0.03 | 0.04 |
| *P. fluorescens* | 110 ± 10 | 5 ± 1 | 110 ± 11 | 1.0 | 1.4 | 0.25 | 1.15 | 0.003 | 0.008 | 0.005 |

[a] Apparent steady-state kinetic parameters determined as described in Materials and Methods.
[b] First-order rate constant calculated from apparent steady-state kinetic parameters.
[c] Pseudo-first-order rate constant determined by nonlinear least-squares fitting of the equation $[NG] = [NG]_0 \exp(k' t)$, where $[NG]_0$ is the initial concentration of NG and $t$ is time in minutes.
[d] Determined by simulation of the experimental data by numerical integration of the differential equations corresponding to the kinetic model of Fig. 1.

vealed that neither enzyme contained appreciable quantities of Ca, Co, Cu, Fe, Mg, Mn, Mo, Ni, or Zn.

Since nearly identical activities were observed during either aerobic or anaerobic assays, a flavoprotein oxygenase activity was not responsible for the catalytic activity. However, initial reductive titration experiments performed in the absence of NG revealed that both reduced enzymes reacted slowly in aerobic buffers (≈10- to 100-fold slower than NG denitration but significantly faster than the experimental protocol could be completed) and were stoichiometrically converted to the oxidized state. Therefore, anaerobic titration experiments were used to establish the reaction stoichiometry and to confirm the linkage between changes in redox state of the flavin cofactor and the denitration of NG.

Figure 3 shows the optical spectra obtained during a reductive titration and NG-dependent reoxidation. During the reductive half of the reaction, 17 nmol of NADPH was required to convert 17 nmol of the oxidized flavin (Fig. 3, spectrum 1) to the dihydroflavin state (Fig. 3, spectrum 5). No evidence for the formation of either a blue neutral or red anionic semiquinone state (23) was observed during intermediate stages of the reductive titration (e.g., Fig. 3, spectrum 3). Upon the anaerobic titration of NG into the reduced enzyme preparation, a reoxidation of the flavin was observed (Fig. 3, spectra 2 and 4), and the final spectrum obtained from the addition of 11 nmol of NG was indistinguishable from the starting spectrum. As for the reductive half reaction, no evidence for a stable semiquinone intermediate was observed.

In contrast to the closely matched stoichiometry calculated for the reductive half reaction, a substoichiometric amount of NG (11 nmol) was required to reoxidize the enzyme. However, upon quantitation of both the DNG (4 nmol) and MNG (6 nmol) isomers present in the reaction mixture, a closely matched stoichiometry of reducing equivalents added (17 nmol of NADPH added, 17 nmol of FMN reduced) and total denitration products (16 nmol) was revealed. Thus, the ability of a single, reduced flavoenzyme to catalyze reactions with NG and with both isomers of DNG was established.

**Regioselectivity of the denitration reaction.** During reaction of each purified enzyme in the presence of limiting amounts of NADPH (Table 5), nitrite was one product of the denitration reaction. Under these limiting conditions, a close correspondence between NG utilized and nitrite produced was observed. In the presence of excess NADPH, larger amounts of nitrite were released, and the stoichiometry approached a limit of 2 mol of nitrite released per mol of NG utilized.

The products detected during a time course of the denitration of NG in the presence of excess NADPH are shown in Fig. 4 (*P. putida* enzyme) and 5 (*P. fluorescens* enzyme). Several important characteristics of the denitration reaction are evident upon visual inspection of these figures: (i) NG was rapidly depleted by the purified enzyme systems, (ii) 1,2-DNG was the major product of the *P. putida* enzyme (Fig. 4), (iii) 1,3-DNG was the major product of the *P. fluorescens* enzyme (Fig. 5), (iv) the ratios of the DNG isomers produced by the two enzymes differed substantially, and (v) the conversion of DNG to the MNG isomers was significantly slower than the accumulation of the DNG isomers. The MNG isomers were not converted to glycerol by the purified enzyme preparations in the time scale of the standard aerobic assay.

The time-dependent changes in the concentration of NG and each denitration product were simulated by numerical integration of the differential equations corresponding to the kinetic model shown in Fig. 1. The results of these simulations are the lines shown in Fig. 4 and 5, and the best-fit values for the individual kinetic constants are summarized in Table 4. The following conditions pertain to this analysis: (i) the denitration reactions were irreversible (ii) NG was rapidly converted to a mixture of the DNG isomers and only slowly converted to a mixture of MNG isomers, and (iii) the MNG isomers were not further converted in the time scale of these experiments. Furthermore, the experiments of Fig. 4 and 5 were performed under conditions that approximated half-order reactivity ($[S] \approx K_m$) in the early stages of NG removal and that became first order ($[S]) \ll K_m$) during the later stages of the reaction. Therefore, the $k'$ values determined by nonlinear least-squares fitting of $[NG] = [NG]_0 \exp(-k' t)$ were an approximation of a first-order rate constant. A comparison of

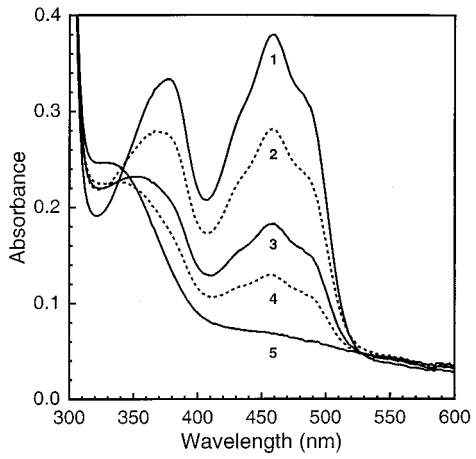


FIG. 3. Optical spectra obtained during an NADPH reduction and NG oxidation of the *P. putida* NG reductase (17 nmol). The solid lines are spectra obtained during the reductive titration with NADPH: 1, oxidized enzyme; 3, after addition of 11 nmol of NADPH; 5, after addition of 17 nmol of NADPH. The dashed lines are spectra obtained after anaerobic addition of NG to the reduced enzyme: 4, after addition of 5 nmol of NG; 2, after addition of 8 nmol of NG. A spectrum identical to spectrum 1 was obtained after addition of 11 nmol of NG.

TABLE 5. Stoichiometry of nitrite production during NADPH-limited denitration of NG[a]

| Time (min) | Amt (nmol) | | | |
|---|---|---|---|---|
| | *P. putida* | | *P. fluorescens* | |
| | NG utilized | Nitrite produced | NG utilized | Nitrite produced |
| 0 | 0 | 0 | 0 | 0 |
| 0.5 | 11 | 14 | 8 | 7 |
| 2 | 18 | 22 | 19 | 18 |
| 6 | 19 | 22 | 20 | 20 |

[a] Assays included 40 nmol of NG, 20 nmol of NADPH, and 0.1 nmol of enzyme.

$V_{max}/K_m$, NG and $k'$ values presented in Table 4 indicated that the kinetic parameters determined by the two different experiments were in reasonable agreement for both enzymes. For the assay compositions described here, the $k'$ value for the *P. putida* enzyme was overall ≈1.5-fold higher than that for the *P. fluorescens* enzyme for the utilization of NG (Table 4).

The relative contributions of the $k_1$ and $k_2$ pathways to the overall rate of NG denitration differed markedly for the two enzymes, as was indicated by the maximal amounts of DNG isomers accumulated in the experiments of Fig. 4 and 5. Equations 1 through 4 give expressions for the product fluxes of each intermediate shown in Fig. 1 and are useful in evaluating the potential for selectivity of the denitration reaction.

$$[1,2\text{-DNG}] = [\text{NG}]_0\,[k_1/(k_1 + k_2)] \quad (1)$$

$$[1,3\text{-DNG}] = [\text{NG}]_0\,[k_2/(k_1 + k_2)] \quad (2)$$

$$[1\text{-MNG}] = [1,3\text{-DNG}](k_5) + \{[1,2\text{-DNG}]\,[k_4/(k_3 + k_4)]\} \quad (3)$$

$$[2\text{-MNG}] = \{[1,2\text{-DNG}][k_3/(k_3 + k_4)]\} \quad (4)$$

Values for the kinetic constants of Fig. 1 were determined from the simulation by matching the amplitude and time course of the appearance and disappearance of the DNG isomers while maintaining the restriction $k' = (k_1 + k_2)$. The close match of the simulations with the experimental data confirms the conclusion from the titration experiments of Fig. 3 that the product distributions observed during NG denitration arise from the catalytic turnover of a single enzyme.

For the *P. putida* enzyme, the product fluxes for the conversion to DNG isomers are given by equations 1 and 2. The sum of the rate constants for conversion of NG ($k_1$ and $k_2$) and for subsequent conversion of the DNG isomers ($k_3$, $k_4$, and $k_5$) differed by ≈20-fold, which permitted the accumulation of the DNG isomers. Substitution of the values for the appropriate kinetic constants from Table 4 revealed that [1,3-DNG]/[1,2-DNG] = $k_2/k_1$ = 0.5, implying that 67% of the flux of NG metabolism proceeded through the 1,2-DNG pathway. The value $k_2/k_1$ = 0.5 could be fortuitous. Alternatively, as discussed elsewhere (39), this value could also be consistent with a random mechanism for removal of the first nitro group from either the terminal (C-1 or C-3) or the middle (C-2) position of NG by the *P. putida* enzyme. For the fraction of the total products converted to 1,3-DNG, no further information regarding the regioselectivity could be obtained. However, since 1,2-DNG could undergo either random or regioselective removal of a nitro group, further insight into the regioselectivity of the enzyme reaction could be obtained by considering the product fluxes from 1,2-DNG to the MNG isomers. In terms of the kinetic constants of Fig. 1, the amounts of 1-MNG and 2-MNG arising from 1,2-DNG denitration were given by the terms containing $k_3$ and $k_4$ in equations 3 and 4. If the removal of a nitro group from 1,2-DNG were random, the ratio [1-MNG]/[2-MNG] = $[k_4/k_3]$ would be unity. However, substitution of the values from Table 4 gave $k_4/k_3$ = 0.74, implying that the second nitro group was removed by the *P. putida* enzyme with a preference for the terminal position. The $k_4/k_3$ ratio of 1-MNG and 2-MNG estimated by evaluation of equations 3 and 4 was 1.35. This value corresponded to the initial slopes of the curves for appearance these two compounds in Fig. 4 and to the final proportion of MNG isomers observed after conversion to MNG isomers in the presence or excess enzyme over a 24-h period (21 nmol of 1-MNG and 16 nmol of 2-MNG; ratio of 1.31).

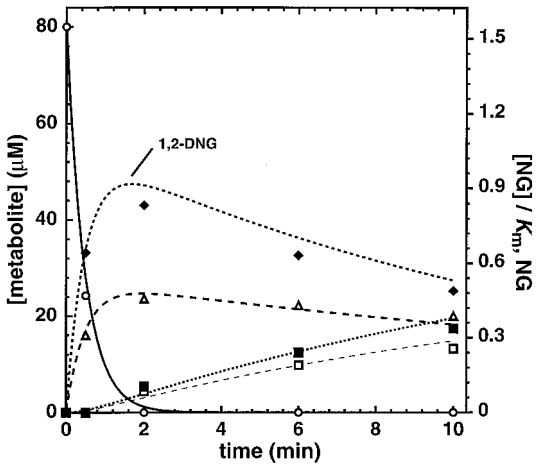


FIG. 4. Time course of the denitration of NG by the purified *P. putida* NG reductase. Assays included 40 nmol of NG, 80 nmol of NADPH, and 0.2 nmol of enzyme. The $K_m$ for NG was 52 μM. Products were determined by HPLC as described in Materials and Methods. ○, NG; ◆, 1,2-DNG; △, 1,3-DNG; □, 1-MNG; ■, 2-MNG. The lines through the experimental data points are simulations calculated by numerical integration as described in the text.

For the *P. fluorescens* enzyme, the C-2 nitro group was preferentially removed from NG, as the $k_2/k_1$ ratio was 4.6. This ratio corresponded to ≈82% of the flux of NG metabolism proceeding through the 1,3-DNG pathway. Since the rate constants for conversion of NG ($k_1$ and $k_2$) and for conversion of

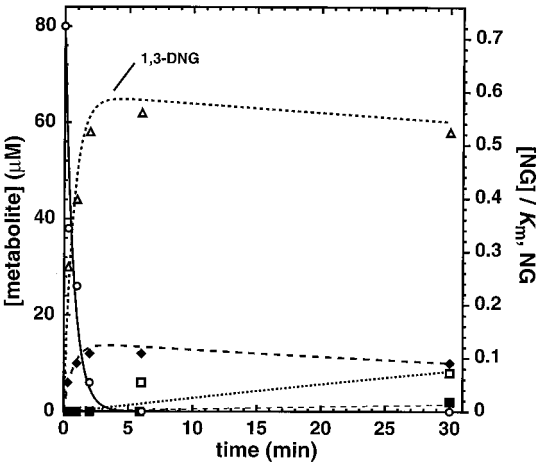


FIG. 5. Time course of the denitration of NG by the purified *P. fluorescens* NG reductase. The $K_m$ for NG was 110 μM. ○, NG; ◆, 1,2-DNG; △, 1,3-DNG; □, 1-MNG; ■, 2-MNG. Other details were as described for Fig. 4.

the DNG isomers ($k_3$, $k_4$, and $k_5$) differed by ≈100-fold, a high level of the DNG isomers (primarily 1,3-DNG) accumulated before significant conversion to the MNG isomers. Furthermore, the ratio of [1-MNG]/[2-MNG] given by $k_4/k_3$ was ≈2.5, implying that the C-2 nitro group also was preferentially removed from 1,2-DNG by the *P. fluorescens* enzyme.

During aerobic denitration reactions such as those shown in Fig. 4 and 5, no evidence for the disappearance of the MNG isomers was obtained. However, upon incubation of large quantities the *P. fluorescens* enzyme with excess NADPH for 24 h under anaerobic conditions, a decrease in the amounts of the MNG isomers was observed, demonstrating that the purified enzyme could in principle convert NG completely to nitrite and glycerol. However, these measurements also suggest that $k_6$ and $k_7$ (Fig. 1, corresponding to conversion of the MNG isomers to glycerol) must be 100- to 1,000-fold lower than the sum of $k_3$, $k_4$, and $k_5$.

## DISCUSSION

Although aliphatic nitro esters such as those in NG are rarely found in nature, man-made compounds containing these functional groups are now widely distributed in the environment. Despite early concerns that NG would not be biodegradable, the ability of microorganisms to degrade NG and other nitro-containing compounds has been recently demonstrated (2, 3, 9, 16, 24, 29, 40, 45). To more clearly understand the mechanism of reductive denitration, and to further elucidate the ways by which microbes can respond to the challenges presented by xenobiotics, we have undertaken this study of NG metabolism, which uses bacteria isolated from the NG-contaminated soil of a munitions manufacturing plant.

**Bacterial transformation of NG.** Although many species of bacteria apparently contain redox enzymes that catalyze the adventitious denitration of NG at low concentrations (Table 1), the organisms isolated from the munitions plant were able to tolerate elevated concentrations of NG and could use the nitro groups liberated from NG as a sole source of nitrogen. However, these isolates were unable to use NG as a carbon source, which is consistent with the observation that the MNG isomers were denitrated by the purified enzyme at a rate ≈4 orders of magnitude lower than the rate for NG. If other organisms utilize similar flavoproteins for NG denitration to MNG isomers, another enzyme may be required to account for the observation that the MNG isomers could be converted to glycerol in vivo (24, 45). Surprisingly, the specific activity of the NG reductase increased only slightly upon exposure to NG, although this has been observed in other organisms capable of NG denitration (2, 24, 29). This observation is consistent with the relatively high abundance of the enzymes in the bacterial cell, even without exposure to NG (Fig. 2).

**Properties of the enzymes responsible for regioselectivity of denitration.** A single enzyme of relatively high abundance was purified from each of the two natural isolates by using a similar combination of chromatographic steps. Both of these enzymes were monomeric flavoproteins containing 1 mol of FMN, and a comparison of their other biochemical, kinetic, and physical properties suggested that these enzymes were overall quite similar (Table 3). The initial rates of NG denitration ($k_1 + k_2$) for the two enzymes were also similar, differing by only 1.5-fold (Table 4). However, these enzymes were markedly different in their selectivity for removal of the nitro groups. With reference to the kinetic model of Fig. 1, the *P. putida* enzyme had a $k_2/k_1$ ratio of 0.5, which would be consistent with random removal of the first nitro group, but a $k_4/k_3$ ratio of 0.74 for removal of the second nitro group from 1,2-DNG, which would be consistent with partial regioselectivity for removal of the terminal nitro group. Assuming the $k_2/k_1$ ratio of 0.5 to be fortuitous, the *P. putida* enzyme would have partial selectivity for the terminal nitro group. In contrast, the *P. fluorescens* enzyme exhibited marked selectivity for removal of the C-2 nitro group from both NG ($k_2/k_1$ of 4.6) and 1,2-DNG ($k_4/k_3$ of 2.5). The *P. fluorescens* enzyme was also 10-fold slower in removing the second nitro group from either position than the *P. putida* enzyme (Table 4).

During the denitration of NG by whole cells of both bacteria and fungi, a distribution of DNG and MNG isomers has been found, with the composition of these distributions dependent on the organism. The titration studies (Fig. 3) and product distribution studies (Table 4, Fig. 4, and Fig. 5) presented here suggest that a single enzyme could produce the previously described product distributions for NG denitration by minor variations in the magnitudes of the kinetic constants shown in Fig. 1. The experiments summarized in Table 4, Fig. 4, and Fig. 5 also clarify several other aspects observed in the in vivo denitration studies. Notably, accumulation of the DNG isomers was 20- to 200-fold faster than the accumulation of the MNG isomers in the purified enzyme systems. It can also be concluded from the present studies that the denitration reaction involved a successive binding of NG and release of the DNG intermediate from the active site, followed by rebinding of DNG and release of MNG, as a ≈200-fold excess of the DNG intermediates was observed relative to the amount of the enzyme in the reaction mixtures.

**Role of flavoenzymes in denitration.** For both purified enzymes, the stoichiometry of NADPH utilized, flavin content, and total denitrified products observed during reductive and oxidative titrations established a linkage between $2e^-$ changes in the redox state of the flavin and the denitration reaction. Since NADPH is a $2e^-$ donor, the absence of semiquinone formation during reductive titrations suggested that the oxidized and reduced forms of the enzyme could not undergo intermolecular disproportionation reactions. Moreover, the absence of semiquinone formation during the reductive titration distinguishes the NG reductases from old yellow enzyme and many other flavoprotein oxidoreductases, where a $1e^-$ reduced state has been observed (23).

Flavoproteins could potentially initiate reductive elimination reactions by the formation of an flavin adduct or by the formation of a charge transfer complex followed by electron transfer. The flavoenzyme D-amino acid oxidase catalyzed a redox-dependent denitration of nitroethane anion, and this reaction most likely proceeded via formation of an oxidized flavin adduct, as an $N^5$-aminoacetonitrile flavin was isolated after turnover-based inactivation of the enzyme in the presence of cyanide (26). It is highly unlikely that NG could form a similar adduct as part of the denitration reaction, particularly in light of the demonstration that NG reacts with the reduced flavin. In contrast, the zwitterionic nature of the electron-deficient nitroester functional group could allow formation of a charge transfer intermediate with an electron-rich reduced flavin, such as has been recently discussed for flavoprotein NADH oxidoreductases (17). This charge transfer complex could then permit electron transfers and the subsequent cleavage of the nitroester. It has also been previously noted that NG has polarity, solubility, and extent of solvation in aqueous solution most similar to those of halogenated aliphatic compounds (39). As the nitro groups are successively removed, the remaining DNG and MNG intermediates would increase in polarity, solubility, and solvation due to the exposure of hydroxyl groups. These changes may alter the $K_m$ values of the DNG and MNG isomers such that the observed rates of deni-

tration would decrease at a fixed substrate concentration. However, it is also possible that other aspects of the binding or the chemical mechanism of the reductive denitration could change as the number of nitrate esters was decreased.

**Relationship to other enzyme systems.** The NG reductases described here share many physical characteristics with the FMN-containing PETN reductase recently cloned and purified from *E. cloacae* PB2 (2). Moreover, the DNG isomer distributions observed from *A. radiobacter* by in vivo nuclear magnetic resonance spectroscopy (40) and from the purified *P. fluorescens* enzyme are remarkably similar. The amino acid sequence of PETN reductase has substantial similarity to that of morphinone reductase, a bacterial enzyme that catalyzes the reduction of morphinone and codeinone to hydromorphone and hydrocodone, respectively (15). Both PETN reductase and morphinone reductase have been suggested to be members of a bacterial subgroup of the family of α/β-barrel flavoprotein oxidoreductases (16). This family is best exemplified by the yeast old yellow enzyme, a structurally well-characterized protein (14) whose physiological function is still unknown. Based on the similarities in physical and catalytic characteristics described here, the pseudomonad NG reductases may also belong to this newly emerging group of bacterial flavoproteins.

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The bacterial strains described in this report were isolated and identified by K. Becker.

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