NAD(P)H:Flavin Mononucleotide Oxidoreductase Inactivation during 2,4,6-Trinitrotoluene Reduction

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Bacteria readily transform 2,4,6-trinitrotoluene (TNT), a contaminant frequently found at military bases and munitions production facilities, by reduction of the nitro group substituents. In this work, the kinetics of nitroreduction were investigated by using a model nitroreductase, NAD(P)H:flavin mononucleotide (FMN) oxidoreductase. Under mediation by NAD(P)H:FMN oxidoreductase, TNT rapidly reacted with NADH to form 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene, whereas 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were not produced. Progressive loss of activity was observed during TNT reduction, indicating inactivation of the enzyme during transformation. It is likely that a nitrosodinitrotoluene intermediate reacted with the NAD(P)H:FMN oxidoreductase, leading to enzyme inactivation. A half-maximum constant with respect to NADH, K_N , of 394 μM was measured, indicating possible NADH limitation under typical cellular conditions. A mathematical model that describes the inactivation process and NADH limitation provided a good fit to TNT reduction profiles. This work represents the first step in developing a comprehensive enzyme level understanding of nitroarene biotransformation.

2,4,6-Trinitrotoluene (TNT), a widely used explosive, is a contaminant frequently found at military bases and munitions production facilities. In addition to its explosion hazard, TNT is a suspected mutagen (56). Extensive research is under way to determine methods for remediation of soil and groundwater contaminated with this pollutant. Bacteria under both aerobic and anaerobic conditions commonly reduce TNT. The nitrogen atom in a nitro group retains a net positive charge and consequently is a strong electrophile and susceptible to reduction. Nitro groups are typically reduced by the sequential addition of three electron pairs, producing a nitroso group, a hydroxylamino group, and finally an amino group (40, 55). Nitroso intermediates are extremely reactive and account for much of the toxicity associated with nitroarenes (56). Through successive reductions of the nitro groups, microorganisms sequentially transform TNT to aminodinitrotoluenes (ADNTs), diaminonitrotoluenes, and triaminotoluene (13, 24, 46, 49, 55). As electron-deficient nitrogen atoms are reduced, the molecule becomes less susceptible to reduction and reaction rates decrease, i.e., TNT is reduced faster than ADNTs and ADNTs are reduced faster than diaminonitrotoluenes (13, 33, 40, 42, 46). To date, there is no conclusive evidence that mineralization of TNT via a triaminotoluene pathway occurs.

Products other than amino compounds have been identified during bacterial nitroarene reduction. Numerous studies have reported the accumulation of hydroxylamino compounds (15, 33, 46, 48), and hydroxylamino and nitroso intermediates may abiotically condense to azoxy dimers that persist (24, 27, 28, 40). TNT is also susceptible to aerobic hydrogenation of the aromatic ring. Several research groups have reported the formation of a Meisenheimer complex and a dihydride Meisen-

heimer complex by various aerobic pure cultures (17, 23, 27, 59).

Many bacterial species are capable of nitroreduction, including *Desulfovibrio* spp. (5, 15, 25), *Methanococcus* spp. (4, 25), *Clostridium* spp. (1, 25, 33), *Escherichia* spp. (39), *Vellonella* spp. (40), nitrate-reducing bacteria (6, 35), *Pseudomonas* spp. (42), *Enterobacter cloacae* (23), and *Salmonella enterica* serovar Typhimurium (60). Enzymes that reduce nitroarenes have been purified from bacteria and typically are soluble flavoproteins that use NADH or NADPH as electron donors (10). Examples of known nitroreductases include flavomononucleotide reductases (48), hydrogenases, ferredoxin-like proteins (40), pyruvate:ferredoxin oxidoreductases, sulfite reductases, carbon monoxide dehydrogenases (46), nitrite reductases, quinone reductases (8), and xanthine oxidase (41). For these enzymes, TNT is not the physiological substrate and TNT reduction does not appear to benefit the organism. These enzymes are in contrast to an uncharacterized reductase in *Pseudomonas* sp. strain JLR11 that transfers electrons to TNT in the final step of a respiratory chain in the absence of other terminal electron acceptors (20). This organism also used TNT as a sole nitrogen source while growing anaerobically on glucose and incorporated 45% of radiolabeled TNT into its cell mass (21). Similarly, a *Desulfovibrio* sp. strain incorporated 42% of radiolabeled TNT into its cell mass during anaerobic growth (15).

Most research to date on TNT biotransformation has focused on the identification of transformation pathways and degradation products for organisms growing in different redox environments, while attempts at mathematical modeling of the process have been modest (1). Simple kinetic models, like the traditional Michaelis-Menten model, fail to consider potentially relevant effects like substrate toxicity, product toxicity, and cometabolic energy limitations and are likely inappropriate for depicting TNT biotransformation. A greater understanding of the fundamental mechanisms that control TNT

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transformation is required to predict the aggregate transformation of TNT in the environment. Processes at the enzyme level such as enzyme inhibition, enzyme inactivation, and enzyme recovery ultimately control cometabolic reaction kinetics (18). To initiate the development of a biochemically and kinetically valid model for TNT reduction by bacterial cells, a reductionist model system was employed consisting of a single enzyme, NAD(P)H:flavin mononucleotide (FMN) oxidoreductase derived from *Photobacterium fischeri*, known to exhibit nitroarene reduction activity (63). This enzyme has been well characterized and is a member of a larger class of flavoproteins, including *Escherichia coli*, *E. cloacae*, and *S. enterica* serovar Typhimurium nitroreductases, that share over 80% amino acid sequence identity, making it a suitable representative nitroreductase (60, 63). Our objective was to develop a comprehensive depiction of the kinetics of TNT reduction by this enzyme.

MATERIALS AND METHODS

Chemicals. TNT was purchased from Chemserv (West Chester, Pa.), nicotinamide adenine dinucleotides (NADH and NAD⁺) were purchased from Sigma (St. Louis, Mo.), and riboflavin 5'-monophosphate FMN was purchased from Bio-Rad Laboratories (Hercules, Calif.). *P. fischeri* NAD(P)H:FMN oxidoreductase was purchased from Roche Diagnostics (Indianapolis, Ind.), which reports the enzyme as being >99.9% pure. 2-Hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), and 2,4-dihydroxylamino-6-nitrotoluene (24DHANT) were generously provided by Joseph Hughes (Rice University, Houston, Tex.). Actual concentrations of TNT, 2ADNT, and 4ADNT in stock solutions were verified by high-performance liquid chromatography (HPLC) analysis by using standards (in 50% methanol–50% acetone) obtained from Radian International, Inc. (Austin, Tex.). A standard for 2,2',6,6'-tetraniro-4,4'-azoxytoluene (in acetonitrile) was obtained from Accustandard, Inc. (New Haven, Conn.).

TNT was dissolved in deionized water at 121°C to create stock solutions. Spectrophotometric and HPLC analyses confirmed that no transformation occurred with this treatment. 2HADNT, 4HADNT, and 24DHANT were dissolved in methanol, because they decayed rapidly in deionized water. NADH and NAD⁺ stock solutions were prepared in 50 mM Tris(hydroxymethyl)aminomethane hydrochloride buffer at pH 7 and stored at 4°C. The concentration of reduced NADH was confirmed before each experiment by absorption measurement at 340 nm ($\epsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$). NAD(P)H:FMN oxidoreductase was dissolved in 40% (by volume) glycerol containing 1 mM EDTA, 0.1 mM dithiothreitol, and 50 mM potassium phosphate buffer at pH 7 per the manufacturer's instructions. The enzyme's reported activity was 100 U/mg of protein at 25°C and pH 7 with FMN and NADH as substrates, where 1 U is the amount of enzyme that catalyzes the reduction of 1 μmol of FMN per min.

Nitroarene concentrations were quantified by using reversed-phase HPLC with photodiode array detection (HPLC-DAD) (PU980 and FP920; Jasco, Inc., Easton, Md.). TNT, 2HADNT, 4HADNT, 2ADNT, 4ADNT, and 24DHANT were separated on a Betasil Cyano column (Keystone Scientific, Inc., Bellefonte, Pa.) with a methanol-deionized water mobile phase (1 ml/min) buffered with 20 mM phosphate at pH 4, starting with 30% methanol and linearly increasing to 45% methanol over the course of 15 min. 2,2',6,6'-Tetraniro-4,4'-azoxytoluene was identified by using 75% methanol for 10 min, linearly increasing the methanol to 100% over the course of 5 min, and holding it at 100% for 10 min (1.5 ml/min). Absorbance spectra at wavelengths from 200 to 400 nm were collected to permit compound identification, while quantitation was performed at a wavelength of 254 nm. NADH concentrations were determined by UV-visible-light spectrophotometry (Varian Cary Bio50 spectrophotometer) at 340 nm. At this wavelength, NADH strongly absorbs while NAD⁺ does not. Nitroarenes absorb very weakly at 340 nm, so concentrations of NADH were kept three times higher than the nitroarene concentrations. For experiments with TNT paired with NADH, less than 4% of the absorbance at 340 nm was due to interference from the initial TNT concentration. Inspection of absorbance spectra for 2HADNT and 4HADNT revealed insignificant changes in the absorbance at 340 nm produced by nitroarenes during the course of the experiments. FMN concentrations were determined spectrophotometrically as well. FMN has three stable redox forms, forms that are fully oxidized (FMN), reduced by one electron (FMNH),

and reduced by two electrons (FMNH₂). FMN has an absorbance peak at 445 nm ($\epsilon = 12,600 \text{ M}^{-1} \text{ cm}^{-1}$), while FMNH₂ does not absorb at this wavelength. FMNH has an absorbance peak at 570 nm, while neither FMN nor FMNH₂ absorbs at this wavelength. FMNH was not detected in any experiments. Concentrations of FMNH₂ were calculated with respect to the loss of initial FMN concentration.

Nitroreductase kinetic experiments. Transformation experiments were performed in 1.5-ml volumes in 3-ml sealed quartz spectroil cuvettes (Aldrich, Milwaukee, Wis.). A Tris(hydroxymethyl)aminomethane hydrochloride-buffered solution of NADH, TNT, and enzyme was prepared in a cuvette kept at 25°C via a water-jacketed cuvette holder. To maintain oxygen-free conditions, the solution was initially sparged with water vapor-saturated nitrogen gas for 5 min while nitrogen flushing of the cuvette headspace continued throughout the experiment. Cap septa were replaced on a daily basis to maintain gastight seals.

TNT reduction. TNT reduction was initiated by the addition of enzyme at 0.533 mg/liter into a solution with an NADH concentration of 500 μM and a TNT concentration of 50, 100, or 150 μM , with triplicate experiments being performed at each concentration. NADH consumption was monitored spectrophotometrically, and initial and final samples were analyzed by HPLC-DAD to determine final nitroarene concentrations and identify products. Several final samples were also analyzed for 2,2',6,6'-tetraniro-4,4'-azoxytoluene by HPLC-DAD. From the change in NADH and nitroarene concentrations, the stoichiometry of reactant consumption was determined while the NADH consumption profile was translated into a nitroarene consumption profile. Details of this method have been reported previously (48).

NAD⁺ inhibition. The effect of NAD⁺ accumulation on TNT reduction kinetics was evaluated at initial NAD⁺ concentrations of 0, 350, and 750 μM (duplicate experiments at each concentration). Enzyme concentrations were 0.533 mg/liter, and the reaction mixtures contained TNT at 100 μM and NADH at 500 μM . The potential inhibitory effect of NAD⁺ on physiological enzyme activity (FMN reduction) was also evaluated. For these experiments, NADH concentrations ranged from 14 to 153 μM while the combined NADH and NAD⁺ concentration was held constant at 370 μM , reflective of intracellular bacterial concentrations (14). In addition, physiological enzyme activity at NADH concentrations increasing from 14 to 153 μM was measured in the absence of NAD⁺. Initial enzyme concentrations were 2.67 mg/liter, FMN reduction was monitored spectrophotometrically at 445 nm, and initial FMN reduction rates were determined.

Enzyme inactivation. To distinguish between enzyme inactivation and product inhibition, experiments were performed to assess residual enzyme activity after one cycle of TNT reduction and product removal. In the first cycle, 100 μM TNT or FMN and 500 μM NADH were combined with 4.00 mg of enzyme/liter. After the completion of one reaction cycle, low-molecular mass compounds were removed from the reaction mixture by three successive ultrafiltrations (Polysulfonate MSI UltraFuge filters with a molecular weight cutoff of 10,000; Fisher, Suwanee, Ga.). Because the molecular masses of NAD(P)H:FMN oxidoreductase, TNT, and NADH are 24,450 (31), 225, and 352 Da, respectively, this method enabled the separation of enzyme from the dissolved reactants and potential products. Samples were centrifuged for 60 min at 5,000 $\times g$ and 4°C, and the retentate was resuspended to achieve twofold dilutions of the reduction products. After three treatments, 96% of the reduction products was removed from the enzyme solution. This level of removal was confirmed by analysis of the final retentates of control samples with only TNT. NADH was added at 500 μM to the final retentate, and a second cycle of activity was initiated with the recovered enzyme by FMN addition at 100 μM . FMN depletions were measured with a spectrophotometer, and initial reaction rates were determined. By comparing the residual activities in cycle 2 of enzyme aliquots that reduced TNT or FMN in cycle 1, the degree of enzyme inactivation was calculated.

Additional experiments were performed to quantify the relationship between the degree of enzyme inactivation and the amount of TNT transformed. Enzymes at fixed concentrations (2.67 mg/liter) were mixed with 300 μM NADH and increasing concentrations of TNT (20 to 100 μM). After completion of the reaction, enzyme was diluted twofold and residual enzyme activity was determined, with an NADH concentration of 300 μM and TNT addition at 100 μM . From NADH consumption profiles and HPLC-DAD analysis at reaction completion, rates of TNT reduction were calculated. The amount of enzyme inactivated was calculated as

$$\Delta E = E_i \left(1 - \frac{v_i}{v_{i,0}} \right)$$

where E_i is the initial enzyme concentration (in milligrams per liter), $v_{i,0}$ is the initial reaction rate (in micromoles per milligram per minute) of TNT removal at

FIG. 1. TNT reduction profiles by NAD(P)H:FMN oxidoreductase for three different initial concentrations. The points display experimental data, the dashed lines display best simultaneous fits to the three data sets by the Michaelis-Menten model, and the solid lines display best simultaneous fits to the three data sets by a Michaelis-Menten model that includes NADH limitation and inactivation.

100 μM without previous TNT exposure, and v_i is the initial reaction rate (in micromoles per milligram per minute) of TNT removal at 100 μM after exposure to a specified amount of TNT.

Kinetic modeling. Variations of the Michaelis-Menten kinetic model were used to depict TNT reduction. The basic TNT transformation equation is

$$\frac{d[\text{TNT}]}{dt} = \frac{-k_m[E][\text{TNT}]}{[\text{TNT}] + K_s}$$

where k_m is the maximum reduction rate (in micromoles per milligram per minute) and K_s is the half-maximum reduction constant (in micromolar units) for TNT. To depict NADH limitation, the following equation was used (51):

$$\frac{d[\text{TNT}]}{dt} = \frac{-k_m[E][\text{TNT}][\text{NADH}]}{([\text{TNT}] + K_s)([\text{NADH}] + K_N)}$$

where K_N is the half-maximum constant (in micromolar units) for NADH. Enzyme inactivation was modeled with a linear transformation capacity (in micromoles per milligram), T_c , such that

$$T_c = \frac{\Delta\text{TNT}}{\Delta E}$$

$$\frac{d[E]}{dt} = \frac{1}{T_c} \left(\frac{d[\text{TNT}]}{dt} \right)$$

where ΔE is the amount of enzyme inactivated (in milligrams per liter) and ΔTNT is the amount of TNT transformed (in micromolar units) (2). Experiments that considered NAD^+ inhibition with NADH limitation were interpreted by using a noncompetitive-inhibition Michaelis-Menten kinetic model with NADH at a limiting dilution and FMN in excess (51, 57).

The preceding nonlinear ordinary differential equations were solved by using forward differences on a Microsoft Excel spreadsheet (9). Parameter estimation

was performed by minimization of the sum of the squared differences between measured and modeled data points by using the solver routine from Microsoft Excel. The conjugate gradient method with central differences was used, and the routine was started from several different points to ensure that a global minimum was obtained. When fit to several reduction profiles simultaneously, the sum of the squared differences for each profile was weighted according to the number of data points, so that the overall sum equally weighted the profiles.

RESULTS

Nitroreductase kinetic experiments. Selected TNT reduction profiles at different initial concentrations are shown in Fig. 1. HPLC analysis at the end of the NAD(P)H:FMN oxidoreductase-mediated reaction indicated the presence of TNT, 4HADNT, and 2HADNT but an absence of aminodinitrotoluenes (Table 1). In several cases, trace amounts of 24DHANT were detected. Although ongoing abiotic transformation complicated the accurate quantification of the HADNTs (11, 44), the average ratio of 4HADNT to 2HADNT was 6.25 ± 0.211 to 1 (Table 1). Even after extended incubations, 2ADNT and 4ADNT were never detected in our experiments. These results indicate that with this enzyme, HADNTs are end products rather than intermediates of TNT reduction. On average, only $65.6\% \pm 3.20\%$ of transformed TNT was recovered as 2HADNT and 4HADNT (Table 1), a result which is likely due to poor quantification of the HADNTs. No peaks were observed upon analysis for 2,2',6,6'-tetranitro-4,4'-azoxytoluene, indicating that 2,2',6,6'-tetranitro-4,4'-azoxytoluene and other azoxy dimers were likely not present.

The molar ratio of NADH oxidized to TNT reduced ($\Delta\text{NADH}/\Delta\text{TNT}$) averaged 1.66 (± 0.0648) for all nine experiments. Because four electrons and four H^+ 's are required to convert a nitro group to a hydroxylamino group, two NADH molecules are required to convert each TNT molecule. The experimentally observed stoichiometry of 1.66 suggests that only one nitro group is reduced per TNT molecule and that reduction does not proceed beyond HADNTs, a result consistent with their identification by HPLC-DAD.

The TNT depletion profiles in Fig. 1 could not be described via simple Michaelis-Menten kinetic expressions (see "Kinetic modeling" above). Reduction rates decreased much faster with time than was characterized by the Michaelis-Menten-postulated dependence on enzyme and substrate concentrations. Hence, different causes of activity inhibition or reduction were explored. A slight inhibition was found due to NAD^+ concentrations of 350 or 700 μM , though it was negligible during TNT reduction experiments performed here (47). Similarly, inhibition at NAD^+ concentrations typical of intracellular concentrations was also negligible (Fig. 2).

We also investigated whether the enzyme was inactivated

TABLE 1. TNT reduction by NAD(P)H:FMN oxidoreductase at different initial concentrations^a

Initial [TNT]	Final [TNT]	[2HADNT]	[4HADNT]	$\Delta\text{NADH}/\Delta\text{TNT}$	[4HADNT]/[2HADNT]	Recovery of TNT (%) ^b
52.2 $\pm$ 0.10	5.5 $\pm$ 0.46	4.4 $\pm$ 0.21	27.5 $\pm$ 1.2	1.69 $\pm$ 0.057	6.33 $\pm$ 0.042	68.3 $\pm$ 2.4
103.0 $\pm$ 1.1	11.6 $\pm$ 1.0	8.5 $\pm$ 0.11	50.8 $\pm$ 2.1	1.60 $\pm$ 0.055	6.01 $\pm$ 0.17	64.9 $\pm$ 2.4
157.6 $\pm$ 0.82	14.3 $\pm$ 6.7	12.3 $\pm$ 0.87	78.7 $\pm$ 4.4	1.69 $\pm$ 0.049	6.41 $\pm$ 0.093	63.5 $\pm$ 3.3
Avg				1.66 $\pm$ 0.065	6.25 $\pm$ 0.21	65.5 $\pm$ 3.2

^a Concentrations are given in micromolar units, and results are average values from triplicate experiments ± 1 standard deviation.

^b Recovery is calculated as $([2\text{HADNT}] + [4\text{HADNT}]) / (\text{initial} [\text{TNT}] - \text{final} [\text{TNT}])$.

FIG. 2. Initial rates of FMN reduction by NAD(P)H:FMN oxidoreductase with increasing NADH concentrations. □, results of experiments conducted in the absence of NAD^+ ; ■, results of experiments for which $[\text{NAD}^+] + [\text{NADH}]$ was $370 \mu\text{M}$. The dashed and solid lines indicate simultaneous best fits to both data sets.

during TNT transformation. Enzyme activities retained after TNT reduction, FMN reduction, or no exposure to a substrate and after the enzymes were subjected to ultrafiltration are shown in Table 2. Prior reduction of FMN did not result in a loss of enzyme activity. However, prior reduction of TNT resulted in a significant (67.7%) loss of enzyme activity. Because of the significant loss of enzyme activity during the ultrafiltration procedure (36.7% decrease in activity with no pretreatment) (Table 2), another approach was used to quantify the ratio of enzyme inactivation per mole of TNT transformed. After various TNT preincubations, TNT was again added and initial reaction rates were computed (Table 3). Residual enzyme activity decreased with the amount of TNT previously transformed, yielding up to a 30.4% reduction in activity when the enzyme aliquot was preincubated with a $100 \mu\text{M}$ concentration of TNT. A linear relationship was inferred to exist between the amount of TNT transformed (ΔTNT) and the amount of enzyme inactivated (ΔE), yielding a T_c of $246 \pm 79 \mu\text{mol/mg}$. The decrease in TNT activity from these experiments (30.4% with a $100 \mu\text{M}$ TNT preincubation) was significantly less than the observed decrease in FMN activity in the previous enzyme isolation experiments (67.7% with a $100 \mu\text{M}$ TNT preincubation). This may be due to a disproportionate inactivation resulting from the combined effects of the ultrafiltration process and reactive TNT transformation species.

TABLE 2. Relative NAD(P)H:FMN oxidoreductase activities on FMN after pretreatment and ultrafiltration^a

Pretreatment	No. of expts	Relative activity	
		Avg	SD
None	4	0.633	0.137
FMN	6	0.613	0.168
TNT	3	0.205	0.0689

^a Relative activity is calculated as the activity of pretreated enzyme on FMN divided by the average daily activity of fresh enzyme on FMN.

TABLE 3. Loss of NAD(P)H:FMN oxidoreductase activity on TNT due to pretreatment with TNT

Pretreatment [TNT] (μM)	Initial reaction rate ($\mu\text{M}/\text{min}$)	ΔE (mg/l)	$\Delta E/\Delta\text{TNT}$ (mg/ μmol)
0	6.80	0	
20	6.24	0.114	175
40	5.79	0.204	196
60	5.50	0.263	228
80	5.72	0.219	365
100	4.73	0.421	238

Kinetic modeling. Initial FMN degradation rates at different NADH and NAD^+ concentrations were fit to quantify the effects of NADH limitation and NAD^+ inhibition (Fig. 2). Two data sets were simultaneously fit, yielding the following best-fit kinetic parameter estimates: a k_m of $157 \mu\text{mol/mg}/\text{min}$, a K_N of $394 \mu\text{M}$, and an NAD^+ inhibition constant of $4,330 \mu\text{M}$. A relatively high K_N value was estimated, indicating significant NADH limitation in these experiments. The inferred K_N value was consistent, however, with previously measured NADH half-saturation coefficients for this enzyme: $80 \mu\text{M}$ (16), $220 \mu\text{M}$ (57), and $270 \mu\text{M}$ (64).

Michaelis-Menten models with and without NADH limitation and enzyme inactivation were examined to describe the complete TNT biotransformation profiles. Results of simultaneous fits to the three TNT reduction profiles from Fig. 1 are shown in Table 4. Weighted sum of squared errors (WSSE) values indicate that first-order, simple Michaelis-Menten models poorly fit the experimental TNT profiles (WSSE = 181), while the enzyme inactivation model resulted in improved profile fits (WSSE = 29.6). Incorporation of NADH limitation had little effect on model fits, but it was included in the final model form, because NADH limitation was measured in independent experiments. When K_N was fit rather than set at the experimentally measured concentration of $394 \mu\text{M}$, the solutions diverged and a unique parameter set could not be determined. The best-fit estimate of T_c , $398 \mu\text{mol/mg}$, was close to the experimentally determined value of $246 \pm 79 \mu\text{mol/mg}$. However, the enzyme inactivation model was extremely sensitive to this parameter, and when T_c was fixed at $246 \mu\text{mol/mg}$, a good fit was not possible (Table 4). The overall best fit, considering enzyme inactivation and NADH limitation, is shown in Fig. 1 along with the best fit found by using simple Michaelis-Menten kinetics for comparison. By using a single parameter set, the model fit the three data sets well, except for the end of the profile in the experiment in which the initial TNT concentration was $100 \mu\text{M}$. The ability of this model form

TABLE 4. Michaelis-Menten model-based fits of profiles of TNT reduction by NAD(P)H:FMN oxidoreductase

Basic model	k_m ($\mu\text{mol}/\text{mg}/\text{min}$)	K_s (μM)	K_N (μM)	T_c ($\mu\text{mol}/\text{mg}$)	WSSE
Simple Michaelis-Menten	221	426			181
NADH limitation	391	374	394^a		118
Inactivation	172	187		347	29.6
Inactivation	198	146		246^a	270
NADH limitation and inactivation	319	187	394^a	398	27.1

^a This value was fixed and not fit during the parameter estimation.

to fit the experimental data, even when an independently measured NADH limitation coefficient was used, corroborates the experimental evidence of enzyme inactivation and NADH limitation as significant processes.

DISCUSSION

Numerous studies examining the reduction of TNT by intact bacterial cells report either transformation to ADNTs without the accumulation of any intermediates (6, 7, 24, 27, 35, 42, 46, 49) or the transient accumulation of HADNTs with subsequent transformation to ADNTs (13, 15, 28, 38, 58, 59). However, experiments using single enzymes to transform TNT and other nitroarenes produce hydroxylaminoarenes exclusively, without any production of aminoarenes (22, 43, 45, 50, 54). Also, when we employed NAD(P)H:FMN oxidoreductase in our experiments, HADNTs accumulated without production of ADNTs, even over prolonged incubations. These results suggest that typical nitroreductases may be unable to reduce hydroxylaminoarenes and that other enzymes are required to complete the conversion to an aminoarene, as has been observed in whole-cell studies (37, 50). Two whole-cell studies using *Clostridium acetobutylicum* (30) and *Clostridium thermacetivum* (29) reported the formation of HADNTs from TNT without the formation of any ADNTs. These strains may lack enzymes capable of HADNT reduction.

Compared with previous studies, a relatively high K_N value, 394 μ M, was estimated for NAD(P)H:FMN oxidoreductase in these experiments. Assuming a cell density of 0.59 mg/ μ l (32), total NAD pools ([NAD⁺] plus [NADH]) in bacteria have been reported to occur at concentrations from 1.8 to 3.5 mM (14), 1.5 to 2.0 mM (3), 1 to 3 mM (52), and 6.9 to 13 mM (26). NADH/NAD⁺ ratios vary from 0.038 under aerobic conditions to 0.71 under anaerobic conditions (14), yielding intracellular NADH concentrations on the order of 100 μ M under aerobic conditions and 1,000 μ M under anaerobic conditions. With these values, the measured half-saturation coefficient for NADH indicates that the NAD(P)H:FMN oxidoreductase is typically NADH limited, operating at between 20 and 70% of the maximum transformation rate under normal conditions. Further, this finding suggests that NAD(P)H:FMN oxidoreductase activity may be regulated by the intracellular NADH/NAD⁺ ratio. NADH/NAD⁺ regulation has been observed with enzymes involved in poly(3-hydroxybutyrate) production (36) and pyruvate catabolism (14, 53). The question of whether other nitroreductases are regulated in a similar fashion requires further investigation.

In our assays, 1.66 ± 0.065 mol of NADH was consumed per mol of TNT, a value lower than the theoretically expected value of 2 for stoichiometric reduction of a nitro substituent to a hydroxylamino substituent. A similarly low yield of 1.8 ± 0.01 was observed with a nitroreductase from *Pseudomonas pseudocaligenes* JS45, which reduced nitrobenzene to hydroxylaminobenzene (54). This low yield suggests that a portion of the TNT was not transformed to HADNTs, possibly due to enzyme inactivation. It has also been shown that products from nitroreduction form covalent bonds with DNA and proteins (11, 34, 37), and nitrosodinitrotoluenes have been implicated as the reactive species (37). On the whole-cell level, greater mutagenicity has been observed with TNT than with 2HADNT,

4HADNT, 2ADNT, or 4ADNT, due to the more rapid generation of nitroso compounds (44, 56, 61). Hence, because of the chemical reactivities of nitrosoarenes, inactivation likely proceeds via covalent bonding of nitrosodinitrotoluenes to the enzyme, which also would reduce the demand for reducing equivalents (NADH/TNT < 2).

Cometabolic transformations are complex processes. At a molecular level, enzyme-substrate interactions control the rate of transformation through processes including enzyme limitation, substrate inhibition, cofactor limitation, enzyme inactivation, and enzyme recovery (18). Frequently, these processes are described on the cellular level by the more generic terms of toxicity and cometabolic yield (2). While modeling these systems on the cellular level is useful, the limited scope of these models prevents the accurate depiction of cometabolic processes under certain circumstances. For example, in an investigation of TNT reduction by a mixed culture undergoing fermentation, only when intracellular kinetics were considered did the model adequately predict TNT reduction (12). The benefit of enzyme level models of cometabolism has already been explored in the area of trichloroethene biodegradation, where the availability of intracellular electrons plays a key role (18, 19, 52, 62, 65). This work represents the first step in developing a comprehensive enzyme level understanding of nitroarene biotransformation. Of course, enzyme inactivation as observed in this study would be manifested differently in whole-cell experiments than in enzyme experiments; e.g., cells would likely regenerate the nitroreductases as the enzymes were inactivated, while the radical intermediates might oxidize other macromolecules in the cell. Nevertheless, to understand the aggregate effect of these enzymatic processes, these fundamental enzyme level processes must first be elucidated.

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