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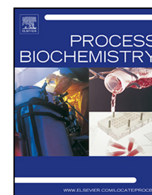

Transformation pathway of 2,4,6-trinitrotoluene by *Escherichia coli* nitroreductases and improvement of activity using structure-based mutagenesis

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ABSTRACT

2,4,6-Trinitrotoluene (TNT), the most widely used explosive, causes serious health and environment problems and is recalcitrant to degradation. Herein, we investigated the metabolic pathway of TNT catalyzed by purified nitroreductases NfsA and NfsB from *Escherichia coli*, and enhanced the transformation activity by structure-based mutagenesis. The two nitroreductases play similar roles in the transformation of TNT, which is converted initially to 4-hydroxylamino-2,6-dinitrotoluene and then 2,4-dihydroxylamino-6-nitrotoluene without production of any amine products. Of the mutants constructed, F123A and F124W mutants dramatically enhanced the catalytic activity towards TNT and its derivatives, suggesting that these two adjacent phenylalanines displayed different effects on the catalytic activity. Substitutions with Ala or Asn at Phe124 greatly impaired the reduction activity against TNT, while the mutation of Phe to Trp resulted in a substantially reduced apparent K_m and improved activity, implying the importance of aromatic residues at position 124 for substrate binding. In contrast, Phe at position 123 caused adverse effects on the activity, may be due to the steric constraint limiting movement of Phe124.

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1. Introduction

2,4,6-Trinitrotoluene (TNT), the most widely used military explosive and widespread environmental contaminant, has caused serious health and environmental issues because of its acute toxicity and mutagenicity [1,2]. Many attempts have been made to explore bacteria or enzymes to remediate and/or remove it. However, the presence of three electron-withdrawing nitro-groups prevents the oxidative transformation of the aromatic ring. Thus, TNT along with other multiple nitrated aromatic compounds are recalcitrant and resistant to degradation.

Microbes are economically viable and environmentally friendly, and have been considered for use in the remediation of nitroaromatic compounds [3–5]. Many microbes, including *Escherichia coli* [6–8], *Enterobacter cloacae* [9], *Bacillus cereus* [10], *Clostridium acetobutylicum* [11], *Clostridium thermoaceticum* [12], *Klebsiella* sp. C1 [13], *Phanerochaete chrysosporium* [14], *Pseudomonas* sp. [15–17] and *Actinomycetes* [18] have been identified for their tolerance and activity to TNT. However, microorganisms do not possess sufficient biomass due to non-optimal growth conditions or low growth rate at contaminated sites, thus the degradation rate of TNT by microorganisms is limited [4]. Alternatively, a variety of TNT metabolic-related enzymes from microbes were constitutively expressed in plants, markedly enhancing phyto-tolerance and transformation capability to TNT [19–21]. Thus, interest in enzymes with TNT transforming activity has arisen, and a better understanding of the roles of TNT metabolic-related enzymes will facilitate the optimization of the enzymes and development of effective applications for the cleanup of TNT.

Enzymes characterized to be capable of transforming TNT include nitroreductases, old yellow enzymes, and some hydrogenases [4]. Among these enzymes, nitroreductases have received much attention because of their rapid transformation activity and ubiquitous existence [22]. Nitroreductases are a family of flavoproteins that catalyze the NAD(P)H-dependent reduction of nitro groups to hydroxylamino and/or amino groups on various nitro-substituted compounds. To date, several studies have associated nitroreductases with the ability to transform TNT, but with varying metabolic end products, suggesting that different metabolic routes exist. PnrA, isolated from the TNT-degrading strain *Pseudomonas putida* JLR11, uses NADPH to reduce TNT to 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) [23]. However,

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two NADPH-dependent nitroreductases (nitroreductase I and II) from *Klebsiella* sp. C1 [24,25], as well as NitA and NitB from *Clostridium acetobutylicum* [26], are able to transform TNT via 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) to form 2-amino-4,6-dinitrotoluene (2ADNT) as the end product.

E. coli, a typically facultative anaerobe, is able to use TNT as sole nitrogen source. Recent studies indicated that the strains have multiple enzymes, including NfsA, NfsB, and NemA, that attack TNT and release nitrogen for growth. The *E. coli* double-mutant strain lacking the nitroreductases NfsA and NfsB lost almost 70% of the capacity in consuming TNT [27], thus, NfsA and NfsB may exert dominant effects in the whole-cell transformation of TNT. However, the roles that NfsA and NfsB play in the transformation of TNT are unclear.

The purpose of this study was to investigate the roles and catalytic ability of the *E. coli* nitroreductases NfsA and NfsB in the reduction of TNT. The reductive products of TNT under both anaerobic and aerobic conditions were identified, and its reductive pathway was proposed. Through a combination of multiple sequence alignments, structural analysis, and site-directed mutagenesis, mutant enzymes were constructed to improve the transformation activity against TNT.

2. Materials and methods

2.1. Chemicals and reagents

NADH (Purity > 98%) and NADPH (Purity > 95%) were purchased from Sigma–Aldrich (Shanghai, China). 2,4,6-trinitrotoluene (TNT), 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) were obtained from J&K (Beijing, China). Unless otherwise stated, other chemicals and reagents were of analytical grade. Chromatographic grade methanol was got from J&K (Beijing, China). Oligonucleotide primers were purchased from Takara (Dalian, China).

2.2. Cloning, expression and purification of two nitroreductases and the mutants

The *nfsA* (Gene ID: 945483) and *nfsB* gene (Gene ID: 945778) were amplified from genomic DNA of *E. coli* K12 and cloned into pET28a vector (Novagen, Merck, Germany). All the mutated genes of NfsB were generated utilizing an overlap extension-PCR method using the corresponding primers (Table S1). The genes were treated with *Nde* I/*EcoR* I restriction enzymes and ligated into pET28a vector. All the correct constructs were transformed into *E. coli* BL21 (DE3) cells (Novagen, Merck, Germany) for protein expression.

Recombinant wild-type NfsA and NfsB and the mutant proteins were expressed as N-terminal His_6 enzymes. Overnight culture of a single colony was used to inoculate LB medium at 37 °C. The cells were grown to early exponential phase and induced with 0.3 mM isopropyl-β-thiogalactopyranoside (IPTG) at 30 °C for 5 h. The resulting cell pellets, harvested via centrifugation at 6000 × *g* for 10 min, were suspended in 20 mM sodium phosphate buffer (pH 7.4, containing 500 mM NaCl and 20 mM imidazole) and lysed by high pressure homogenization (1000 bar) using a nano homogenize machine (ATS Engineering inc., Canada) at 4 °C. Recombinant proteins were purified from the supernatant using a HiTrap^{TM} Chelating HP column (GE Healthcare, USA). For kinetic studies, the eluted fractions were supplemented with pure FMN and incubated at 4 °C for at least 30 min before buffer-exchange. Protein samples were more than 95% pure on SDS-PAGE. Protein concentrations were determined from Bradford assays calibrated against bovine serum albumin (BSA).

2.3. Enzymatic transformation of TNT and its derivatives

The products generated following the nitroreductase-catalyzed reduction of TNT were isolated using analytical reverse phase HPLC employing an Agilent 1200 system. A reaction mixture of 100 μl containing 100 μM TNT, 200 μM NADH and 100 nM nitroreductases in 20 mM Tris-HCl (pH 7.0) were incubated at 37 °C. Samples were removed every 10 min and terminated by heating the mixture at 100 °C for 5 min. The 10 μl of sample, after centrifugation at 12,000 × *g* for 10 min, was injected onto a Hypersil^{TM} C18 5 μm 4.6 × 250 mm column (Thermo Fisher Scientific, Waltham, USA). Reactants and products were eluted using a 30–100% (v/v) methanol linear gradient over a 30-min period, at a flow rate of 0.8 ml/min. The column eluate was monitored spectrophotometrically at 230 nm throughout each separation. HPLC-MS to identify TNT metabolites were performed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer fronted by a Hewlett Packard 1100 Series HPLC system. The mass spectrometer was equipped with an atmospheric pressure ionization (API) interface and an ESI ion source in positive or negative ion mode.

2.4. Enzymes kinetics

Steady-state enzyme kinetic studies with purified enzymes for TNT were determined spectrophotometrically by monitoring the initial rate of oxidation of NADH at 340 nm. All reactions were performed in 20 mM Tris-HCl buffer (pH 7.0) containing 60 μM NADH and a series of substrates concentrations, initiated by the addition of 20 nM purified recombinant enzymes. The temperature of each reaction was maintained at 37 ± 1 °C, and all solutions were thermally equilibrated to 37 ± 1 °C prior to measurement. Changes in absorbance were measured every 15 s for 5 min (during linearity), using a scanning spectrophotometer (Varioskan, Thermo Fisher Scientific, USA). All kinetic data were collected at least three times and analyzed by non-linear regression using Origin 8.5 software.

2.5. Molecular docking

The solved crystal structures for NfsA (PDB code 1F5 V) and NfsB (PDB code 1DS7) were used to docking studies. To predict the orientations of TNT or other amino-derivatives within NfsA, NfsB or the mutants created for this study, we dock the ligands to the enzyme active site using AutoDock 4.2 [28]. The GridBox parameters for docking the ligands to proteins were determined; for NfsA, the grid center coordinates were $x = 2.103$, $y = -14.394$, and $z = 0.138$, and the size coordinates were $x = 28$, $y = 28$, and $z = 28$; for NfsB, the grid center coordinates were $x = -5.926$, $y = -14.324$, and $z = 50.247$, and the size coordinates were $x = 24$, $y = 24$, and $z = 42$. The docked ligand output files were viewed, and the orientations and the interactions were analyzed using PyMOL version 1.5.0.4 [29].

3. Results

3.1. In vitro kinetics of NfsA and NfsB with TNT and its derivatives

To investigate the roles of NfsA and NfsB in TNT metabolism, the activities of these two enzymes against TNT and its derivatives were determined. The purified NfsA and NfsB were capable of reducing TNT and its monoamino derivatives 2ADNT and 4ADNT (Table 1), but they did not transform the diamino derivatives (24DANT and 26DANT) (Fig. S1).

Kinetic studies showed that the highest $k_{cat(app)}/K_{m(app)}$ values were obtained with TNT for the two nitroreductases, which were approximately 50-fold and 340–1200-fold higher than towards 2ADNT and 4ADNT, respectively (Table 1). The two nitroreductases, and in particular NfsA, exhibited a lower specific activity for 4ADNT

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