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ORIGINAL PAPER

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Active site residues controlling substrate specificity in 2-nitrotoluene dioxygenase from *Acidovorax* sp. strain JS42

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Abstract *Acidovorax* (formerly *Pseudomonas*) sp. strain JS42 utilizes 2-nitrotoluene as sole carbon, nitrogen, and energy source. 2-Nitrotoluene 2,3-dioxygenase (2NTDO) catalyzes the initial step in 2-nitrotoluene degradation by converting 2-nitrotoluene to 3-methylcatechol. In this study, we identified specific amino acids at the active site that control specificity. The residue at position 350 was found to be critical in determining both the enantiospecificity of 2NTDO with naphthalene and the ability to oxidize the ring of mononitrotoluenes. Substitution of Ile350 by phenylalanine resulted in an enzyme that produced 97% (+)-(1*R*, 2*S*)-*cis*-naphthalene dihydrodiol, in contrast to the wild type, which produced 72% (+)-(1*R*, 2*S*)-*cis*-naphthalene dihydrodiol. This substitution also severely reduced the ability of the enzyme to produce methylcatechols from nitrotoluenes. Instead, the methyl group of each nitrotoluene isomer was preferentially oxidized to form the corresponding nitrobenzyl alcohol. Substitution of a valine at position 258 significantly changed the enantiospecificity of 2NTDO (54% (−)-(1*S*, 2*R*)-*cis*-naphthalene dihydrodiol formed from naphthalene) and the ability of the enzyme to oxidize the aromatic ring of nitrotoluenes. Based on active site modeling using the crystal structure of nitrobenzene 1,2 dioxygenase from *Comamonas* sp. JS765, Asn258 appears to contribute to substrate specificity through hydrogen bonding to the nitro group of nitrotoluenes.

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Introduction

Nitroaromatic compounds are the direct products or by-products of various industrial activities for the manufacture of pesticides, dyes, polymers, pigments, and explosives [11]. These compounds are toxic and resistant to degradation because of the stability of the nitroaromatic ring structures. Because of widespread environmental contamination, there is a need for environmental clean up, and bioremediation may provide a cost-effective strategy. Bacteria capable of complete degradation of simple nitroarene compounds (nitrobenzene, and mono- and dinitrotoluenes) have been isolated from various contaminated samples [9, 10, 22, 36]. *Acidovorax* (formerly *Pseudomonas* [20]) sp. JS42 was isolated from nitrobenzene-contaminated soil and groundwater [9]. JS42 uses 2-nitrotoluene (2NT), a by-product of 2,4,6-trinitrotoluene (TNT) production, as sole carbon, nitrogen and energy source. The first step in 2NT degradation is catalyzed by the enzyme 2-nitrotoluene dioxygenase (2NTDO), which consumes molecular oxygen and requires NAD(P)H as a cofactor to convert 2NT to 3-methylcatechol (3-MC) and nitrite [1, 30]. 2NTDO is a member of a large family of Rieske non-heme iron oxygenases that initiate the degradation of a wide variety of aromatic substrates [8].

The *ntdAaAbAcAd* genes, which encode the three components of 2NTDO [26], are in an operon regulated by NtdR, a LysR-type regulator [20]. The products of the *ntd* genes were purified and characterized as a three component system with an iron-sulfur flavoprotein reductase, a Rieske [2Fe-2S] ferredoxin, and an oxygenase composed of α and β subunits [1, 30]. Comparisons of the deduced amino acid sequences demonstrated that the 2NTDO components are very similar in sequence to those of other three-component dioxygenase

systems (84–95% identity with naphthalene dioxygenases (NDO), nitrobenzene dioxygenase (NBDO), 2,4-dinitrotoluene dioxygenase (DNTDO) [7, 19, 26, 35, 38]). Closely related aromatic hydrocarbon dioxygenases such as NDO from *Pseudomonas* sp. NCIB 9816-4 are unable to catalyze the oxidation of nitrotoluenes with release of nitrite; instead NDO oxidizes the methyl group [38]. Similarly, toluene dioxygenase from *P. putida* F1 is capable of oxidizing the methyl groups of 2- and 3-nitrotoluenes, and the unsubstituted ring carbons of 4-nitrotoluene [34]. We have been interested in defining the features of 2NTDO that allow the oxidation of 2NT at the nitro-substituted carbon, which results in the formation of an unstable nitrohydrodiol that rearranges to form nitrite and 3-methylcatechol.

The overall substrate specificity of 2NTDO is determined by the C-terminal region of the α subunit as demonstrated by the analysis of hybrid dioxygenases constructed with the genes encoding 2NTDO, NDO, and DNTDO [27, 28]. Using amino acid sequence comparisons between 2NTDO, NBDO, DNTDO and NDO, and the available crystal structure of NDO [15], we chose three positions (258, 314, and 350) near the proposed active site of the α subunit of the enzyme for site-directed mutagenesis. We report here the substrate specificity and enantiospecificity of five mutant forms of 2NTDO, and propose a role for specific active site residues based on a model of the 2NTDO active site.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely maintained on Luria-Bertani agar plates [4] in the presence of appropriate antibiotics (ampicillin 200 μg/ml; tetracycline 25 μg/ml). DH5α was used for subcloning, and *E. coli* JM109 was used during the mutagenesis procedure. For the biotransformation experiments, DH5α carrying pUC13 with cloned wild-type or mutant 2NTDO genes was grown at 37°C in minimal salts medium (MSB) [37] containing 10 mM glucose, 1 mM thiamine, and 200 μg/ml ampicillin.

Chemicals

Catechol (99.5 + %), 3-methylcatechol (98%), 4-methylcatechol (95 + %), 2-nitrobenzyl alcohol (97%), 3-nitrobenzyl alcohol (98%), 4-nitrobenzyl alcohol (99%), and 4-nitrotoluene (99%) were purchased from Aldrich (Milwaukee, WI, USA). Naphthalene (99%) and nitrobenzene were from Acros Organics (Morris Plains, NJ, USA), and 2- and 3-nitrotoluene (99%) were from Avocado (Heysham, Lancashire, UK). (+)-(1*R*, 2*S*)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene was prepared as previously described by Jeffrey et al. [13] using *Pseudomonas* sp. 9816/11 [18], a naphthalene *cis*-dihydrodiol dehydrogenase mutant of the naphthalene degrading strain *Pseudomonas* sp. NCIB 9816-4 that accumulates the product when incubated with naphthalene.

Site-directed mutagenesis

Site-directed mutagenesis of *ntdAc*, which encodes the α subunit of 2NTDO, was performed with the Altered Sites II in vitro Mutagenesis System (Promega Corp., Madison, WI, USA). A 1.1-kb *Kpn*I-*Bam*HI fragment carrying the 3′ half of *ntdAc* and the 5′ half of the *ntdAd* gene from pDTG850 [28] was cloned into *Kpn*I-*Bam*HI-digested pALTER-1 (Promega Corp.). The resulting plasmid,

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	Δ(*lacZYA-argF*)U169, *hdsR*17, *relA*1, *supE*44, *endA*1, *recA*1, *thi-1*, *gyrA*96, *ϕ*80d *lacZ*ΔM15	Life Technologies, Gaithersburg, MD, USA
JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hdsR*17, *relA*1, *supE*44, Δ(*lac-proAB*), *mcrA*, [F', *traD*36, *proAB*$^+$, *lacI*^{q}*Z*ΔM15]	[39]
ES1301 *mutS*	Km^r, *lacZ*53, *mutS*201::Tn*5*, *thyA*36, *rha*-5, *metB*1, *deoC*, IN(*rrnD-rrnE*)	Promega Corp.
Plasmids		
pALTER-1	Tc^R, mutagenesis vector	Promega Corp.
pDTG800	Ap^R, pUC18 carrying the *ntdAaAbAcAd* genes from *Acidovorax* sp. JS42 on a *Sac*I fragment	[26]
pDTG840	Tc^R, pALTER-1 carrying the 3′ end of *ntdAc* on a *Bam*HI-*Kpn*I fragment	This study
pDTG850	Ap^R, pUC13 carrying the *ntdAaAbAcAd* genes on a *Sac*I-*Eco*RI fragment	[28]
pDTG852	Ap^R, W314F mutation in pDTG850	This study
pDTG854	Ap^R, N258V mutation in pDTG850	This study
pDTG856	Ap^R, N258V/W314F mutations in pDTG850	This study
pDTG864	Ap^R, I350T mutation in pDTG850	This study
pDTG865	Ap^R, I350F mutation in pDTG850	This study

Ap^R ampicillin resistance; *Tc^R* tetracycline resistance

Table 2 Oligonucleotides used for site-directed mutagenesis

Mutation	Mutagenic oligonucleotide[a]	Restriction site introduced
W314F	5′-GCTACCTTCAAGGTC**TTC**AACCCGATCGATGAA-3′	*Bbs*I
N258V	5′-GACTACTACTCCGGT**GTC**TTCAGCGCTGATATG-3′	*Bbs*I
I350T	5′- GCGGCTCAGCGCAG**TACTG**GACCAGCAGGATTC-3′	*Sca*I
I350F	5′-CGCGGCTCAGCG**AAGCTT**CGGACCAGCAGG-3′	*Hin*dIII

[a]Underlined bases indicate the position of the introduced restriction site. Base changes are in bold

pDTG840, was used as the template for mutagenesis. Mutagenic oligonucleotides were designed with a silent mutation that introduced a new restriction site within the gene to facilitate screening for clones carrying the desired mutation (Table 2). Phosphorylated oligonucleotides were synthesized by MWG Biotech Inc. (Greensboro, NC, USA). *E. coli* ES1301 *mut*S was made competent according to the protocol provided with the Altered Sites kit (Promega Corp.). The nucleotide sequences of the insertions in pALTER-1 were determined for each mutant by fluorescent-automated DNA sequencing with an Applied Biosystems 3730 automated DNA sequencer (University of California, Davis DNA sequencing facility). After verifying the sequences, 0.8-kb *Kpn*I-*Mfe*I fragments carrying a specific mutation were individually cloned into *Kpn*I-*Mfe*I-digested pDTG850 to regenerate the complete *ntd* operon. This subcloning step was followed by restriction digestion to verify the presence of the mutations. The resulting plasmids were introduced into DH5α for expression studies.

Biotransformation reactions

For biotransformation reactions, DH5α carrying plasmids encoding mutant forms of 2NTDO were grown in 5 ml MSB containing 10 mM glucose, 1 mM thiamine, and 200 μg/ml ampicillin at 37°C in a shaking incubator overnight. The overnight cultures were inoculated into 250 ml of the same medium and grown until the turbidity at 660 nm was 1.0–1.4. To these cultures, 10 ml of phosphate buffer solution (0.1 M, pH 7.4) and 10 mM glucose were added. The cultures were split into 30 ml in 125 ml Fernbach flasks with 0.1% substrate (final concentration). After 5 h of incubation at 30°C in a shaking incubator, the cells were harvested by centrifugation (6,000*g*, 4°C for 10 min). The supernatants were extracted three times with 25 ml of NaOH-washed ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness by rotary evaporation.

Analytical methods

Gas chromatography-mass spectrometry (GC-MS) analyses were performed with an Agilent 6890N gas chromatograph equipped with a Supelco Equity-1 capillary column (30.0 m×250 μm×25.0 μm), 5973N mass selective detector, and 7683 Series auto sampler and injector (Agilent Technologies, Palo Alto, CA, USA) as described previously [33]. For GC-MS analysis, dried samples were dissolved in 0.1 ml of anhydrous Na_2SO_4-dried acetonitrile. Aliquots of 25 μl were added to the same volume of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, IL, USA) for trimethylsilyl (TMS) derivatization. The derivatized samples (1 μl) were injected for GC-MS analysis.

Reverse phase high-performance liquid chromatography (HPLC) analyses were carried out with a Beckman Coulter system (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a System GOLD 125 solvent module and System GOLD 168 photo diode array (PDA) detector operated by 32 Karat software (ver. 5.0). Separations were carried out on a Zorbax SB-C18 column (250×4.6 mm; Agilent Technologies) as described previously as method 2 [27]. Under these conditions, the retention times of 2NT, 3-MC, and 2-nitrobenzyl alcohol (2-NBA) were 72.0, 36.9, and 40.5 min, respectively. Biotransformation samples (1 ml each) were withdrawn every hour for HPLC analysis and cells were removed by centrifugation. The supernatants were filtered and 10 μl of each was injected for HPLC analysis. The chromatograms were monitored at 270 nm. To quantify metabolites, calibration curves were prepared with standard compounds. The calibration curves were linear with $R^2 > 0.999$ and the concentrations of all of the metabolites analyzed were within the concentration ranges of the calibration curves.

Chiral stationary phase HPLC analysis was performed to evaluate the enantiospecificity of wild-type and mutant forms of 2NTDO with naphthalene. The Beckman Coulter system was equipped with a Chiralcel OJ column (250×4.6 mm ID, Chiral Technologies Inc., Exton, PA, USA) linked to an Ultrasphere C-18 guard column (7.5×4.6 mm, 5 μm particle size, Alltech Associates, Inc., Deerfield, IL, USA). Naphthalene *cis*-dihydrodiol was purified as previously described [28]. Isocratic separation was carried out at a flow rate of 0.7 ml min^{-1} with *n*-hexane:isopropanol (9:1, v/v). The retention times were 21.2 and 24.7 min for (+)-*cis*-(1*R*, 2*S*)- and (−)-*cis*-(1*S*, 2*R*)-naphthalene dihydrodiol, respectively.

Gel electrophoresis and Western blot analyses.

Cell pellets (from 1-ml culture suspensions grown for biotransformation experiments) were resuspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) sample-loading buffer [2], and boiled for 10 min. Samples (10 µl each) were separated by SDS-PAGE (12% polyacrylamide) [2]. Gels were subjected to Western blotting as described previously using a monoclonal antibody against the α subunit of NDO that cross reacts with the α subunit of 2NTDO [2, 28]. Antigens were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce).

Protein determinations

Protein concentrations were determined by the method of Bradford [3] after boiling cell pellets for 10 min in 0.1 N NaOH. Bovine serum albumin was used as the standard.

Homology modeling of the active site of 2NTDO

A homology model of the active site of 2NTDO was made in O based on the structure of NBDO as previously described [6, 14]. Models of the active site of the N258V and I350F mutants were made in O using the appropriate portion of NDO (pdb code 1NDO) as a template [15]. 2NT was modeled in the active site based on the structure of NBDO in complex with 3NT and nitrobenzene (pdb codes 2BMR and 2BMQ). Models are available upon request from the authors.

Results

2NTDO site-directed mutagenesis

Three residues at the active site in the α subunit of 2NTDO were chosen for site-directed mutagenesis based on amino acid sequence comparisons of NDO, 2NTDO, NBDO, and DNTDO [7, 19, 26, 35, 38]. The wild-type and all of the mutant enzymes were expressed from pUC13-based clones in *E. coli* DH5α. Similar levels of dioxygenase proteins were produced by the various clones based on SDS-PAGE and Western blot analysis using a monoclonal antibody made against NDO (Fig. 1).

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*SDS-PAGE*) and Western blot analysis of wild-type and mutant forms of 2-nitrotoluene 2,3-dioxygenase (*2NTDO*) produced by *E. coli*. **a** 12% SDS gel stained with Coomassie blue. *Arrows* indicate the α and β subunits of 2NTDO. **b** Western blot of a duplicate 12% gel using anti-naphthalene dioxygenase (*anti-NDO*) α subunit monoclonal antibody. Lanes: *M* Precision plus protein standards, BioRad Laboratories, sizes are indicated in kD; *lane 1* DH5α carrying pUC13 (vector control); *lane 2* pDTG850 (wild type); *lane 3* pDTG852 (W314F); *lane 4* pDTG854 (N258V); *lane 5* pDTG856 (W314F/N258V); *lane 6* pDTG864 (I350T); and *lane 7* pDTG865 (I350F)

Substrate specificity of 2NTDO mutants with mononitrotoluenes

We tested the ability of the mutant forms of 2NTDO to oxidize 2-, 3-, and 4NT and compared the results with those of wild-type 2NTDO. All of the mutant forms of 2NTDO were able to catalyze the oxidation of the three isomers of nitrotoluene. However, major differences in product ratios obtained with nitrotoluenes as substrates were caused by changes at the active site (Table 3). The W314F and I350T mutations had little or no effect on the ratios of product formed from 2NT, but the enzyme with the W314F substitution appeared to be more efficient at 2NT oxidation than the wild-type enzyme based on comparison of the areas of the product peaks by GC-MS. In contrast, the N258V, N258V/W314F, and I350F mutants were severely defective in the ability to oxidize the ring of 2NT (Table 3). In fact, 2-NBA was the only product formed from 2NT by the N258V mutant based on GC-MS (Table 3).

Based on the preliminary evidence of improved product formation by the W314F mutant and the severely altered specificity of the N258V mutant, we carried out analysis of 2NT depletion and product formation rates by these two enzymes compared to wild-type 2NTDO (Fig. 2). The W314F mutant showed an increased rate of 3-MC formation (26 nmol/min/mg protein) compared to wild type (14 nmol/min/mg protein), as well as an increased rate of 2-NBA formation (2.9 nmol/min/mg protein vs. 1.8 nmol/min/mg protein, respectively) from 2NT (Fig. 2a,b). Although the N258V mutant formed no 3-MC, the rate of 2-NBA formation was approximately twice that of wild type (3.7 nmol/min/mg protein vs. 1.8 nmol/min/mg protein, respectively) (Fig. 2c). 2NT biotransformations with the negative control strain, DH5α(pUC18) resulted in a net loss of approximately 13% of the initial 2NT with no detectable products formed (data not shown). Total

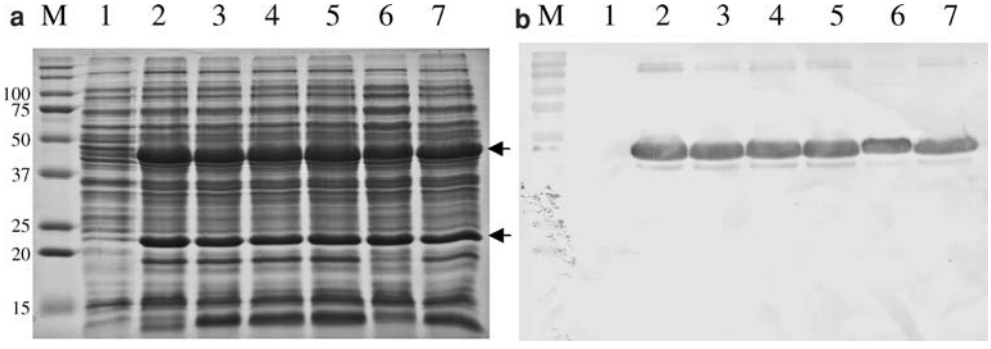

Table 3 Substrate specificity of mutant forms of 2-nitrotoluene 2,3-dioxygenase (2NTDO) with mononitrotoluenes

Substrate	Metabolite	Products[a] (%) formed by 2NTDO enzymes:					
		Wild type	N258V	W314F	N258V/W314F	I350F	I350T
2NT							
	3-MC	84	ND	80	1	7	72
	2-NBA	16	>99	20	99	93	28
3NT							
	3-MC	14	3	49	7	3	50
	4-MC	60	16	24	9	10	20
	3-NBA	26	81	27	84	87	30
4NT							
	4-MC	97	82	88	31	3	15
	4-NBA	3	18	12	69	97	85

[a]Product ratios were determined from integration of the Gas chromatography-mass spectrometry (GC-MS) total ion current chromatograms. Results reported are the averages of at least three independent experiments, and standard deviations were less than 10%

NT nitrotoluene; *MC* methylcatechol; *NBA* nitrobenzyl alcohol; *ND* not detected

product recoveries (including residual 2NT) were 66%, 70%, and 71% for wild type, W314F, and N258V, respectively. Observed losses may have been due to volatilization, formation of undetected reduction products, instability of methylcatechols, or association of products with cell material.

The W314F and I350T mutations caused a change in the ratio of 3-and 4-MC produced from 3NT, but the majority of the products were the result of ring attack rather than methyl group oxidation (Table 3). In contrast, N258V, N258V/W314F, and I350F mutants preferentially oxidized the methyl group, forming 3-NBA as the major product along with small amounts of 3- and 4-MC (Table 3).

With 4NT as the substrate, the N258V and W314F mutants produced slightly less 4-MC relative to 4-NBA than wild type, but the N258V/W314F double mutant produced a significantly altered product ratio (Table 3). Both substitutions at position 350 resulted in a strong preference for methyl group oxidation with 4NT (Table 3).

Enantiospecificity of wild-type and mutant 2NTDO enzymes

All of the mutant forms of 2NTDO were able to catalyze the conversion of naphthalene to naphthalene *cis*-1,2-dihydrodiol based on GC-MS analysis. Chiral HPLC analysis revealed differences in the ratios of naphthalene *cis*-dihydrodiol enantiomers. Wild-type 2NTDO produced 72% (+)-*cis*-(1*R*, 2*S*)-naphthalene dihydrodiol. Substitution of the asparagine by valine at position 258 in the α subunit caused a major change in the enantiospecificity of the enzyme; this mutation led to the production of 54% (−)-*cis*-(1*S*, 2*R*)-naphthalene dihydrodiol (Table 4). In contrast, the W314F mutant showed slightly increased (86%) production of (+)-*cis*-(1*R*, 2*S*)-naphthalene dihydrodiol relative to (−)-*cis*-(1*S*, 2*R*)-naphthalene dihydrodiol compared to wild-type 2NTDO (Table 4). The enzyme with the double mutation, N258V/W314F, produced a significantly lower relative ratio of (+)- to (−)-*cis*-(1*R*, 2*S*)-naphthalene dihydrodiol compared to both the wild-type enzyme and the W314F mutant, suggesting that the N258V mutation is dominant over W314F. The I350T mutation had no significant effect on the enantiospecificity of 2NTDO. In contrast, the substitution I350F resulted in the production of 97% (+)-*cis*-(1*R*, 2*S*)-naphthalene dihydrodiol.

Discussion

Models of the active site of 2NTDO with and without 2NT bound (Fig. 3) were generated based on the crystal structure of the closely related NBDO (95% amino acid sequence identity of α and β subunits). The crystal structures of NDO and NBDO show that the residues at positions 258 and 350 are located in the active site pocket of each enzyme, and the residue at position 314 is in the second coordination shell surrounding the active site, adjacent to the residues at positions 258 and 350 [6, 15].

In 2NTDO, the I350F substitution made the enzyme more like NDO in sequence and specificity, and presumably in active site structure. The introduction of the larger residue at position 350 most likely reduced the size of the active site (Fig. 3) and forced the binding of naphthalene in a position that resulted in oxidation from primarily one face of the molecule, and resulting in the preferential formation of the (+)-enantiomer. In addition, the I350F mutant preferentially oxidized the methyl groups of all three nitrotoluenes, producing predominantly nitrobenzyl alcohols (Table 3). Based on the model of the 2NTDO active site, substitution of Ile350 to Phe suggests that the bulky aromatic ring of phenylalanine interferes with appropriate positioning of nitrotoluenes in the active site to yield the corresponding catechols (Fig. 3). Accordingly, the specificity changes caused by the I350T mutation (side chains of approxi-

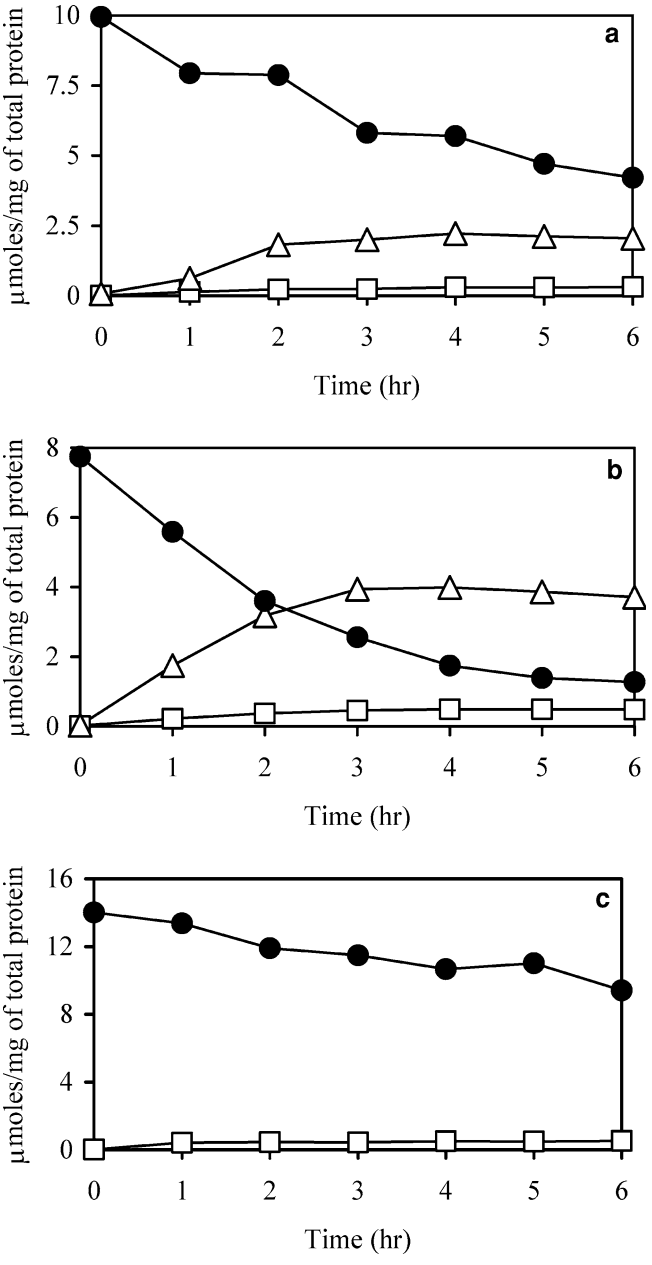

Fig. 2 2-Nitrotoluene (*2NT*) biotransformation time course by **a** DH5α(pDTG850) carrying wild-type 2NTDO, **b** DH5α(pDTG852) carrying 2NTDO-W314F, and **c** DH5α(pDTG854) carrying 2NTDO-N258V. Activities were normalized to total protein concentrations in each sample. *Closed circles* decrease in the substrate 2NT; *open triangles* production of 3-methylcatechol (*3-MC*); and *open squares* production of 2-nitrobenzyl alcohol (*2-NBA*). The vector control, DH5α(pUC18), lost approximately 13% of the 2NT in 6 h, but no products were detected (data not shown)

mately equal size) were not as drastic as that with I350F. These results confirm the importance of the residue corresponding to Phe352 in NDO in determining the regio- and enantioselectivity of these enzyme systems. In addition to the documented role of Phe352 in NDO from *Pseudomonas* sp. NCIB 9816-4 [29, 31], substitution of the corresponding residue (Phe350) in NDO from *Ralstonia* sp. U2 with Thr resulted in an enzyme with the

Table 4 Enantiospecificity of mutant forms of 2NTDO

Enzyme	Products[a] (%) formed from naphthalene	
	(+)-diol	(−)-diol
Wild-type 2NTDO	72	28
2NTDO-N258V	46	54
2NTDO-W314F	86	14
2NTDO-N258V/W314F	59	41
2NTDO-I350F	97	3
2NTDO-I350T	74	26
Wild-type NDO	>99	ND

[a]Enantiomeric composition was determined by chiral-stationary phase HPLC as described in Materials and methods. Results reported are the averages of at least three independent experiments, and standard deviations were less than 6%
(+)- and (−)-diol are the (+) and (−) enantiomers of naphthalene *cis*-dihydrodiol
ND none detected

ability to remove nitrite from 2,3- and 2,6-dinitrotoluene, but not 2,4- dinitrotoluene [17]. Similarly, substitution of Val350 with either Phe or Met in the *Burkholderia cepacia* R34 DNTDO resulted in improved activities with substituted phenols [16]. Substitution of an alanine for Phe378 (corresponding to Phe350 in 2NTDO) in the *B. xenovorans* LB400 biphenyl dioxygenase (BPDO) α subunit resulted in a reduction in overall activity and a change in the regiospecificity with chlorobiphenyl substrates [41]. Phe366 (corresponding to I350 in 2NTDO) in the active site of the tetrachlorobenzene dioxygenase from *Ralstonia* sp. PC12 (TecA) was found to control regioselectivity in TecA: when a smaller residue (leucine) was substituted, the enzyme had increased preference for methyl group monooxygenation with mono- and dichlorotoluenes [32]. In contrast, this enzyme had a slightly increased preference for ring attack on 2,4,5-trichlorotoluene [32]. Substitution of a tyrosine or tryptophan at this position in either TecA or NDO resulted in inactive enzymes [29, 32].

The residues at position 258 in 2NTDO and NBDO are asparagines, while the residues at the corresponding positions in NDO and DNTDO are valines (Table 5). These differences at the active site correlate with the differences in regioselectivity of the enzymes with mononitrotoluenes: 2NTDO and NBDO are capable of oxidizing the ring with release of nitrite [19, 26], while NDO and DNTDO oxidize only the methyl groups [38]. The crystal structure of NBDO with nitrobenzene or 3NT bound at the active site revealed the presence of a hydrogen bond between Asn258 and the nitro group of either substrate. This bond was proposed to position the substrate for oxidation at the nitro-substituted carbon [6], and modeling of the 2NTDO active site suggests that the same type of positioning occurs in this enzyme (Fig. 3). Consistent with this idea is the observation that the N258V substitution in 2NTDO eliminated the ability of the enzyme to oxidize the ring of 2NT and severely reduced oxidation of the ring of 3NT (Table 3). In the α subunit of NDO, most amino acid substitutions at the

Table 5 Comparison of amino acids at the active sites of four dioxygenase α subunits

Position[a]	Amino acid			
	$NDO_{9816\text{-}4}$	$2NTDO_{JS42}$	$NBDO_{JS765}$	$DNTDO_{DNT}$
260/258/263	Val	Asn	Asn	Val
316/314/319	Trp	Trp	Trp	Phe
352/350/355	Phe	Ile	Ile	Thr

[a]The first number indicates the amino acid positions in naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB9816-4, the second in 2NTDO from *Acidovorax* sp. strain JS42 and NBDO from *Comamonas* sp. strain JS765, and the third, in 2,4-dinitrotoluene dioxygenase (*DNTDO*) from *Burkholderia* sp. strain DNT

active site, including changes at position 260 (corresponding to 258 in 2NTDO) had no effect on the ability of the enzyme to form enantiomerically pure (+)-*cis*-(1*R*, 2*S*)-naphthalene dihydrodiol [31]. In contrast, the N258V substitution in 2NTDO resulted in the formation of naphthalene *cis*-dihydrodiol of opposite stereochemistry (Table 4). This result was surprising since the opposite change in NDO (V260N) had no effect on enantioselectivity [31], and a NDO double mutant (F352I/V260N) behaved the same as the single NDO F352I mutant [40]. The role of the residue at the position corresponding to Asn258 in 2NTDO (Val260 in NDO) has not been widely studied in other enzymes. However, substitution of this residue in TecA (Leu272) with larger residues (tryptophan or phenylalanine) did not have a major effect on regiospecificity, but resulted in increased product formation rates with chlorinated toluene substrates [32].

The change at position 314 in the second shell surrounding the active site of 2NTDO had a slight effect on enantioselectivity, and the results with the double mutant (N258V/W314F) indicate that the substitution at position 258 has a larger effect than that at 314 (Table 4). Overall, the substitution of an amino acid (W314F) in the second shell surrounding the active site had less of an effect on substrate specificity than substitution of active site pocket residues (Tables 3, 4). A significant difference in product formation was seen with 3NT, however, indicating that residues that do not

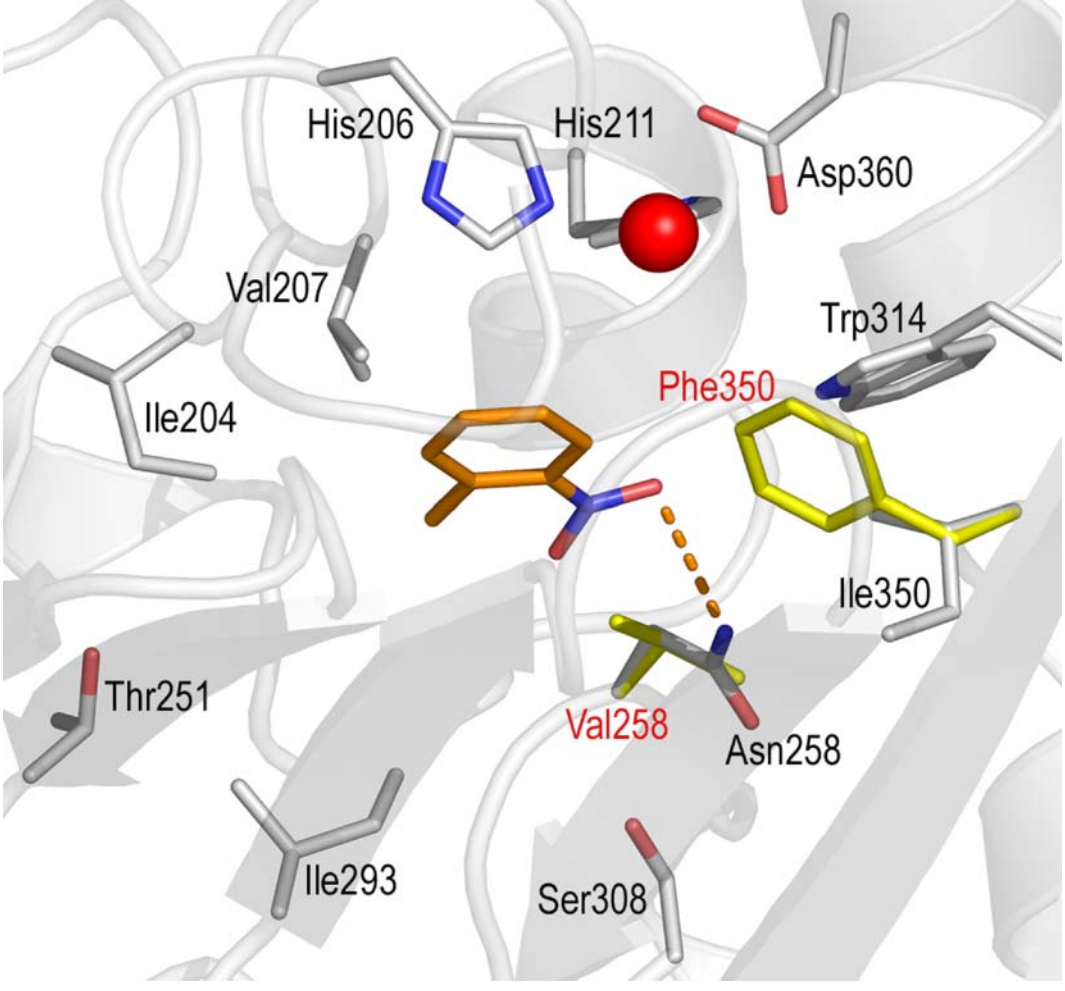

Fig. 3 Homology model of the active site of 2NTDO with 2NT (*center*) modeled in the active site. The mononuclear iron is shown in CPK model representation (*sphere*). The side chain residues coordinating the mononuclear iron, non-conserved residues in the active site of 2NTDO and Trp314 in the second coordination shell are represented in *stick format*. The side chains of the N258V and I350F mutants are shown in addition to the wild-type residues. The figure was prepared using PyMOL [5]

contact substrate directly can have an effect on substrate specificity as seen in other enzymes [12, 21, 25]. More importantly, the W314F mutant had a higher rate of 2NT oxidation than wild type (Fig. 2). In contrast, most of the other mutants had reduced efficiencies with the substrates tested (data not shown).

This study demonstrates the importance of specific residues at the active site of 2NTDO in the efficiency of 2NT oxidation and the substrate specificity with naphthalene and mononitrotoluenes. In practical terms, engineering of nitroarene dioxygenases may prove useful for generating bacterial strains with enhanced biodegradation abilities, or proteins with industrially useful catalytic activities.

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