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Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for oxidizing nitrobenzene to 3-nitrocatechol, 4-nitrocatechol, and nitrohydroquinone

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Abstract

Toluene-*o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 was found to oxidize nitrobenzene (NB) to form *m*-nitrophenol (*m*-NP, 72%) and *p*-NP (28%) with an initial rate of 0.098 and 0.031 nmol/(min mg protein), respectively. It was also discovered that wild-type ToMO forms 4-nitrocatechol (4-NC) from *m*-NP and *p*-NP with an initial rate of 0.15 and 0.0082 nmol/(min mg protein), respectively, and 3-NC (12%) and nitrohydroquinone (NHQ, 88%) from *o*-NP with an initial rate of 0.11 and 0.8 nmol/(min mg protein), respectively. To increase the oxidation rate and alter the oxidation regiospecificity of nitro aromatics as well as to study the role of the active site residues I100, Q141, T201, and F205 of the alpha hydroxylase fragment of ToMO (TouA), DNA shuffling and saturation mutagenesis were used to generate random mutants. The mutants were initially identified by screening via a rapid agar plate assay and then were further examined by high-performance liquid chromatography (HPLC) and gas chromatography (GC). Several mutants with higher rates of activities and with different regiospecificities were identified; for example, *Escherichia coli* TG1 cells expressing either TouA mutant M180T/E284G or E214G/D312N/M399V produce 4-NC 4.5- and 20-fold faster than wild-type ToMO (0.037 and 0.16 nmol/min mg protein from *p*-NP, respectively). TouA mutant A107T/E214A had the regiospecificity of NB changed significantly from 28% to 79% *p*-NP. From 200 μM NB, TouA variants A101T/M114T, A110T/E392D, M180T/E284G, and E214G/D312N/M399V produce 4-NC whereas wild-type ToMO does not. From *m*-NP, TouA mutant I100Q produces 4-NC (37%) and NHQ (63%), whereas wild-type ToMO produces only 4-NC (100%). Variant A107T/E214A acts like a *para* enzyme and forms *p*-cresol as the major product (93%) from toluene with enhanced activity (2.3-fold), whereas wild-type ToMO forms 32%, 21%, and 47% of *o*-, *m*-, and *p*-cresol, respectively. Hence, the non-specific ToMO was converted into a regiospecific enzyme, which rivals toluene 4-monooxygenase of *P. mendocina* KR1 and toluene *o*-monooxygenase of *Burkholderia cepacia* G4 in its specificity.

Keywords: DNA shuffling; Saturation mutagenesis; Toluene-*o*-xylene monooxygenase

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1. Introduction

Nitroaromatic compounds are widely used in industry as dyes, pesticides, plasticizers, explosives, and solvents (Spain and Gibson, 1991). 3-Nitrocatechol (3-NC) is useful for high-temperature electrolytic capacitors (Yamada, 1990) and is used to increase the amplification factor of transistors; however, there is only one worldwide source of 3-NC and its price is $20,000/g (Vitas-M Laboratory, Ltd.). Nitrohydroquinone (NHQ) is mainly used as electrophotographic photoreceptor (Akiyoshi et al., 2000) and dyes (Corbett, 1967).

Dihydroxy nitroaromatics are important for medicine. Nitrocatechol derivatives have been shown to be selective inhibitors of catechol-*o*-methyltransferase and may be used in the treatment of Parkinson disease (Borgulya et al., 1989; Learmonth and Freitas, 2002; Learmonth et al., 2002). 4-NC and 3-NC were recently found to be competitive inhibitors of nitric oxide synthase and may be new leads for innovative, nitrocatechol-based pharmacophores of potential therapeutic interest (Palumbo et al., 2002). 3-NC is also essential as a building block for the production of some antihypertensive pharmaceuticals such as flesinoxan (Ennis and Ghazal, 1992; Krab-Husken, 2002). NHQ has been used to synthesize dephostatin, an inhibitor of the protein tyrosine phosphatase (Hamel and Girard, 1994), which is a candidate therapeutic agent for diabetes mellitus and neural diseases such as Alzheimer's disease and Parkinson's disease (Umezawa et al., 2003).

Direct microbial oxidation of nitrobenzene (NB) or nitrophenols (NP) for the synthesis of NC or NHQ is attractive to reduce wastes (relative to organic-based methods) and since chemical synthesis of these compounds is problematic in terms of yield and selectivity (Palumbo et al., 2002). Biocatalysis is likely to account for 30% of the chemical business by 2050 (van Beilen et al., 2003).

Activity with nitroaromatics is reported with several microorganisms. Cells of *Pseudomonas putida* F1 and *Pseudomonas* sp. strain JS150 convert NB to 3-NC (Haigler and Spain, 1991). *P. mendocina* KR1 converts NB to 4-NC via *m*-NP (10%) and *p*-NP (63%), and *P. pickettii* PKO1 converts NB to 3-NC and 4-NC via *m*-NP and *p*-NP (Haigler and Spain, 1991); we have shown that toluene 4-monooxygenase (T4MO) of *P. mendocina* KR1 is responsible for the nitro catechol formation (Fishman et al., 2004a). *P. putida* 2NP8 grown on *m*-NP has been shown to degrade NB into ammonia, nitrosobenzene, and hydroxylaminobenzene (Zhao and Ward, 2000). *o*-NP is degraded by this strain with production of nitrite, and *m*-NP resulted in the formation of ammonia. *P. pseudoalcaligenes* JS45 degrades NB to 2-aminomuconate which is also an intermediate in the metabolism of tryptophan in mammals (He and Spain, 1998). Twenty-one oxygenase-containing bacteria were screened for the ability to convert nitroaromatics into 3-NC (Kieboom et al., 2001). *Mycobacterium chelonae* strain NB01 was shown to degrade NB via a reductive degradation mechanism, which resulted with the formation of ammonia (Oh et al., 2003). *Comamonas* sp strain JS765 was shown to convert NB to an unstable nitrohydrodiol that decomposes to form catechol and nitrite via nitrobenzene 1,2-dioxygenase (Parales et al., 1997).

Due to its wide substrate range and malleable catalytic activity, wild-type toluene-*o*-xylene monooxygenase (ToMO) from *P. stutzeri* OX1 was investigated here for nitroaromatic oxidation. ToMO hydroxylates toluene to *o*-cresol, *m*-cresol, and *p*-cresol and 3- and 4-methylcatechol (Bertoni et al., 1996); ToMO also oxidizes *o*-xylene in both the 3 and 4 positions (Bertoni et al., 1996). ToMO oxidizes many other substrates including *m*-xylene, *p*-xylene, benzene (converting it to phenol, catechol, and 1,2,3-trihydroxybenzene (Vardar and Wood, 2004)), ethylbenzene, styrene, naphthalene (Bertoni et al., 1996), trichloroethylene (TCE) (Chauhan et al., 1998), and tetrachloroethylene (Ryoo et al., 2000).

ToMO consists of a three-component hydroxylase with two catalytic oxygen-bridged dinuclear centers ($TouA_2$ $TouB_2$ $TouE_2$), a ferredoxin (TouC), a mediating protein (TouD), and a NADH-ferredoxin oxidoreductase (TouF) (Bertoni et al., 1996; Cafaro et al., 2002). ToMO TouA (499 amino acids) contains the active site and has 68% identity to the hydroxylase (TbuA1) of toluene *p*-monooxygenase (TpMO) of *P. pickettii* PKO1 (68%) (Fishman et al., 2004b).

Using DNA shuffling (Stemmer, 1994) there is no need for a crystal structure or any information about the structure of the protein (Arnold and Moore, 1997; Arnold, 1998), and this method was used to identify the TomA1 V106A variant of toluene *o*-monooxygenase (TOM) of *Burkholderia cepacia* G4 which resulted in enhanced TCE degradation and naphthalene oxida-

tion (Canada et al., 2002) (note V106A corresponds to I100 of the alpha subunit TouA of the hydroxylase in ToMO). The importance of position I100 was corroborated via saturation mutagenesis for TmoA of T4MO (Fishman et al., 2004a; Tao et al., 2004); T4MO TmoA mutant I100L has a four-fold increase in activity for 3-methoxycatechol formation from 1 mM guaiacol, and T4MO TmoA mutant I100A formed 4-NC 16 times faster than that of wild-type T4MO. In addition, ToMO TouA variant I100Q had significantly altered hydroxylation regiospecificities for toluene, *o*-cresol, *m*-cresol, phenol, and catechol allowing for the novel formation of methylhydroquinone, hydroquinone, and 1,2,4-trihydroxybenzene (Vardar and Wood, 2004).

Alpha subunit active site positions Q141, I180, T201, and F205 for T4MO were studied previously (Pikus et al., 1997; Pikus et al., 2000), for example, TmoA mutant Q141C forms more 3-methylbenzyl alcohol from *m*-xylene (11.7% versus 2.2% for the wild-type enzyme), and the product distribution from *p*-xylene completely switched to 2,5-dimethylphenol (78%) from 4-methylbenzyl alcohol (22%). Positions T201, Q141, and F205 of TouA of ToMO have also been studied via saturation mutagenesis (Vardar and Wood, 2004), for example, TouA F205G mutation in ToMO changed the hydroxylation regiospecificities of toluene, *o*-cresol, *m*-cresol, *p*-cresol, phenol, and resorcinol, and allowed for the novel formation of methylhydroquinone, 4-methylresorcinol, hydroquinone, resorcinol, and 1,2,3-trihydroxybenzene. T4MO mutants from positions Q141, T201, and F205 were not studied previously for nitrobenzene oxidation with the exception of T4MO mutant T201G of TmoA which produced 7.9% *o*-NP whereas wild-type T4MO did not (Mitchell et al., 2002).

In this work, our goals were to evaluate the ability of wild-type ToMO to oxidize NB and NP, and to use both DNA shuffling of *touA* and saturation mutagenesis of TouA at positions I100, Q141, T201, and F205 of ToMO to enhance the synthesis rates of NCs and NHQ and to alter the regiospecific hydroxylation of nitro aromatic compounds by ToMO. It was discovered that wild-type ToMO forms *m*-NP and *p*-NP from NB as well as 4-NC, 3-NC, and NHQ from NPs (four new reactions and first report of NHQ from *o*-NP from toluene monooxygenases), and protein engineering was used to enhance significantly the synthesis rates of NC and NHQ by ToMO.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and SDS-PAGE

Escherichia coli strain TG1 (*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F′[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) (Sambrook et al., 1989) was used as the host with pBS(Kan)ToMO (Vardar and Wood, 2004) and its variants which express the *touABCDEF* genes from a constitutive *lac* promoter. Cells were initially streaked from −80 °C glycerol stocks on Luria-Bertani (LB) agar plates (Sambrook et al., 1989) containing 100 μg/mL kanamycin and incubated at 37 °C. After growth on LB agar plates, cells were cultured from a fresh single colony in LB medium (Sambrook et al., 1989) supplemented with 100 μg/mL kanamycin at 37 °C with shaking at 250 rpm (New Brunswick Scientific Co., Edison, NJ). The relative expression of the *touA* loci from *E. coli* TG1/pBS(Kan)ToMO was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1989) with a 12% Tris–HCl gel both with and without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Fisher Scientific. Co., Fairlawn, NJ).

2.2. Chemicals

NB, toluene, *o*-xylene, and *p*-cresol were purchased from Fisher Scientific Co. (Fairlawn, NJ); *o*-cresol and *m*-cresol were purchased from Sigma Chemical CO. (St. Louis, MO); 4-NC, 2-nitroresorcinol (2-NR), *o*-NP, *m*-NP, and *p*-NP were obtained from Acros Organics (Morris Plains, NJ); NHQ was obtained from Frinton Labs (Vineland, NJ), and 3-NC was obtained from Vitas-M Laboratory, Ltd. (Moscow, Russia). All materials used were of the highest purity available and were used without further purification.

2.3. Saturation mutagenesis and DNA shuffling of ToMO

Saturation mutagenesis at positions I100, Q141, T201, and F205 of the alpha subunit (*touA*) of ToMO and DNA shuffling of 90% of *touA* of ToMO was performed as described previously (Vardar and Wood, 2004).

2.4. Colony screening for NB and p-NP

A nylon membrane plate assay was used as described previously (Vardar and Wood, 2004). The mutant libraries were first streaked from transformant plates to LB (100 μg/mL kanamycin) agar plates containing 1% (w/v) glucose. The glucose-grown colonies were then transferred to fresh LB (100 μg/mL kanamycin) plates containing 1 mM substrate (NB or *p*-NP) with a nylon membrane. After incubating for 24 h at room temperature in a chamber, the colonies were checked visually to search for those that developed a red color around the cell mass (Fishman et al., 2004a) indicating the formation of NCs or NHQ from NB or *p*-NP. The control expressing wild-type ToMO remained yellow on NB (indicates the formation of NPs only) and very light red on *p*-NP agar plates (indicates the formation of small amounts of 4-NC). The negative control expressing no monooxygenase, TG1/pBS(Kan), remained colorless on NB and *p*-NP. At least three replicates were checked before proceeding with high-performance liquid chromatography (HPLC) analysis.

2.5. Product identification and rates of formation

The possible mutants initially identified by screening via the agar plate assay were further examined by reverse-phase HPLC. Experiments were conducted with exponentially-grown cells harvested at an optical density at 600 nm (OD) of 1.0. Centrifuged cells ($6000 \times g$ for 5 min at 25 °C (JA-17 rotor in a J2 series centrifuge, Beckman, Palo Alto, CA)) were washed once with one volume 50 mM Tris–HNO_3 buffer, pH 7.0 and resuspended to an OD of 5–10.

Cell suspensions (2.5 mL) were sealed with a Teflon-coated septum and aluminum seal in 15 mL glass vials, and the substrates, NB at 200 μM and *o*-NP, *m*-NP, and *p*-NP at 500 μM were added from ethanol stock solutions. After contacting at room temperature, 1 mL of the cell suspension was centrifuged for 2–3 min, the supernatants (500 μL) were filtered with a 1 mL syringe (Becton Dickinson) coupled to a nylon membrane filter unit (Millex-HN, 0.45 μm, 4 mm), and the samples were analyzed with HPLC. A Zorbax SB-C8 column (Agilent Technologies, 5 μm, 4.6 mm × 250 mm) was used with a Waters Corporation (Milford, MA) 515 solvent delivery system coupled to a photodiode array detector (Waters 996) and injected by an autosampler (Waters 717 plus). To detect and determine the formation rates of nitro-substituted catechols, nitro-substituted resorcinols, and nitro-substituted hydroquinone, a gradient elution was performed with H_2O (0.1% formic acid) and acetonitrile (70:30, 0–8 min; 40:60, 15 min; 70:30, 20 min) as the mobile phases at a flow rate of 1 mL/min. A Supelcosil ABZ+PLUS column (Supelco Technologies, 3 μm, 15 cm × 4.6 mm) was used to separate 3-NC, NHQ, and 2-NR from *o*-NP. To detect and determine the formation rates of NHQ and 3-NC from *o*-NP, a gradient elution was performed with H_2O (0.1% formic acid) and acetonitrile (80:20, 0–20 min) as the mobile phases at a flow rate of 1 mL/min. A gradient elution was performed with H_2O (0.1% formic acid) and acetonitrile (95:05, 0–60 min) for a better separation of 2-NR and 3-NC from *o*-NP (Table 1). To confirm product identifications, the retention times and UV–vis spectra of the standard chemicals were compared with those of the mutant enzyme-derived samples (Table 1), and the enzyme products were co-eluted with authentic standards. At least two independent cultures were analyzed for each substrate and strain tested, and at least five injections were made for each substrate. The molar amount of products formed was calculated using calibration

Table 1
Retention times and maximum wavelengths (λ_{max}) of substrates and products used in the HPLC analysis

Compound	Retention time (min)	λ_{max} (nm)
Nitrobenzene	16.8[a]	264
o-Nitrophenol	15.8[a], 18.9[b], 48.4[c]	274
m-Nitrophenol	12.9[a]	272
p-Nitrophenol	11.5[a]	317
3-Nitrocatechol	8.8[a], 11.8[b], 40.5[c]	300
4-Nitrocatechol	7.4[a]	348
Nitrohydroquinone	9.1[a], 14.0[b], 44.2[c]	280
2-Nitroresorcinol	9.4[a], 12.5[b], 37.8[c]	312

[a] HPLC retention times for standards in the gradient method with the C-8 column.

[b] HPLC retention times for standards in the isocratic method (80-20) for 3-nitrocatechol, nitrohydroquinone, and 2-nitroresorcinol separation (from *o*-nitrophenol) by using ABZ + PLUS column.

[c] HPLC retention times for standards in the isocratic method (95-05) for better separation of 3-nitrocatechol and 2-nitroresorcinol (from *o*-nitrophenol) by using ABZ + PLUS column.

curves for each product. Initial product formation rates were determined by sampling at 15 min intervals for 2 h and were quantified in nmol/(min mg protein) by converting product peak areas to concentrations using standard curves prepared at the specific absorbance wavelength (Table 1) for each product formed. Protein content was 0.22 mg protein/(mL 1 OD) for recombinant *E. coli* TG1 as determined using the Protein Assay Kit (Sigma Diagnostics Inc., St. Louis, MO).

To determine the toluene oxidation rates and regiospecificities, gas chromatography (GC) experiments were performed as described previously (Vardar and Wood, 2004). To determine *o*-xylene oxidation rates and regiospecificities, the same conditions were used as for toluene (Vardar and Wood, 2004); the retention times for *o*-xylene, 2,3-dimethylphenol (2,3-DMP), and 3,4-DMP were 6.5, 30.3, and 31.7 min, respectively.

2.6. DNA sequencing

A dideoxy chain termination technique (Sanger et al., 1977) with the ABI^{TM} Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Wellesley, MA) and PE Biosystems ABI^{TM} 373 DNA sequencer (Perkin-Elmer, Wellesley, MA) was used to determine the ToMO nucleotide sequences.

2.7. Modeling of ToMO TouA

Part of the wild-type ToMO TouA alpha subunit (amino acid residues W44-N380) was modeled using SWISS-MODEL Server (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003) and was based on the sMMO MmoX alpha subunit (polymer chain D) from *M. capsulatus* (Bath) (Rosenzweig et al., 1997). The A107T, E214G, and E214A mutations were modeled from the generated wild-type TouA ToMO model using the Swiss-Pdb Viewer program (DeepView) (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003). The program Swiss-Pdb Viewer performed the amino acid substitutions isosterically for the ToMO TouA based on residue interactions, steric hindrance, and energy minimization.

3. Results

3.1. Screening

DNA shuffling of 90% of *touA* and saturation mutagenesis at four different amino acid positions in *touA* (I100, Q141, T201, and F205) were performed to create mutations in ToMO in order to increase the rate of NC or NHQ formation from NB, *o*-NP, *m*-NP, and *p*-NP. A library of 8000 mutants was generated for saturation mutagenesis. The number of colonies in saturation mutagenesis that are needed to be screened to ensure with a 99% probability that all the possible codon substitution occurred was calculated by us as 293 (Rui et al., 2004); hence, around 500 colonies from each position (4000 colonies total) were screened with NB and *p*-NP as substrates using the high-throughput, agar-plate assay. *o*-NP and *m*-NP were not used as substrates for high-throughput screening since wild-type ToMO formed dark red halos on *o*-NP and *m*-NP agar plates. Formation of red halos indicated the presence of NCs, NHQ, or NRs; yellow halos indicated NPs were produced. For DNA shuffling of *touA*, an additional 2500 colonies were screened.

Mutants that formed dark red halos were identified with amino acid changes in TouA including I100Q, A101T/M114T, A107T/E214A, A110T/E392D, M180T/E284G, and E214G/D312N/M399V (Table 2). No NB or NP mutants that formed darker red halos than wild-type ToMO were found from saturation mutagenesis at TouA positions Q141, T201, and F205. Low-activity but regiospecific mutants of NB, those which have low initial oxidation rates, were also found in TouA including I100H, T201G, F205C, F205G, F205H, F205Y, W266R, and T281A/F290S (Table 3). Wild-type ToMO and TouA mutants were further examined by HPLC analysis, and Tables 2 and 3 summarize the products obtained from the oxidation of NB, and Table 4 summarizes the products obtained from the oxidation of *o*-NP, *m*-NP, and *p*-NP. The mutants were sequenced to identify both the beneficial and deleterious amino acid changes (Tables 2 and 3).

Tris–nitric buffer was used instead of Tris–chloride or Tris–phosphate buffer to reduce non-ToMO based reduction of the nitro group so that the formation rates of NPs from NB could be determined accurately. Aniline formation was observed with addition of NPs from NB when Tris–chloride or Tris–phosphate buffer was

Table 2
Enhanced rate and altered regiospecific oxidation of NB (200 μM) by high-activity mutants of TG1/pBS(Kan)ToMO

Enzyme	Product from NB							
	m-NP			*p*-NP			4-NC	
	Rate[a]	Relative	Mol%[b]	Rate[a]	Relative	Mol%[b]	Rate[a]	Relative
Wild-type ToMO	0.098	1.0	72	0.031	1.0	28	0	–
I100Q	0.14	1.5	61	0.090	3.0	39	0	–
A101T/M114T	0.19	2.0	84	0.036	1.2	16	0.0050	∞
A107T/E214A	0.097	1.0	22	0.31	10.0	78	0	–
A110T/E392D	0.17	1.7	78	0.046	1.5	22	0.0090	∞
M180T/E284G	0.45	4.6	95	0.030	0.97	5	0.0070	∞
E214G/D312N/M399V	0.59	6.0	73	0.19	6.2	27	0.074	∞

[a] Initial rate determined by HPLC in nmol/(min mg protein).
[b] Mol% shown for first hydroxylation (does not include 4-NC).

used. The Tris–nitric buffer suppressed this side reaction (NB to aniline) without affecting activity of ToMO (Fishman et al., 2004a).

3.2. Oxidation of NB by wild-type ToMO and high-activity mutants

The pathways for the oxidation of NB to NPs, NC, and NHQ with wild-type ToMO and high-activity TouA variants (I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V) are shown in Tables 2 and 4. There is no previous report about the hydroxylation of NB or NPs by ToMO. Here, it was discovered that *E. coli* TG1/pBS(Kan)ToMO expressing wild-type ToMO performs two different hydroxylations and forms *m*-NP (72%) and *p*-NP (28%) with an initial rate of 0.098 and 0.031 nmol/(min mg protein) from 200 μM NB (Table 2). Interestingly, ToMO does not form *o*-NP from NB. No 4-NC peak was observed from 200 μM NB with TG1 expressing wild-type ToMO. Here, it was also discovered that ToMO hydroxylates *m*-NP and *p*-NP and forms 4-NC (0.15 and 0.0082 nmol/(min mg protein), respectively). Also, from 500 μM *o*-NP, ToMO forms 3-NC (12%) and NHQ (88%) with an initial rate of 0.11 and 0.8 nmol/(min mg protein), respectively. The negative control, TG1 expressing pBS(Kan), did not form NPs or NCs; therefore, ToMO is responsible for the formation of NP, NC, and NHQ. However, the nitroaromatic oxidation rates are 5- to 10-fold lower with wild-type ToMO compared to wild-type T4MO (Fishman et al., 2004a).

Table 3
Altered regiospecific oxidation of NB (200 μM) by low-activity mutants of TG1/pBS(Kan)ToMO

Enzyme	Product	Mol%	Rate, (nmol/min mg protein)	Relative
Wild-type ToMO	*m*-NP	72	0.098	1
	p-NP	28	0.031	1
I100H	*p*-NP	100	0.0025	1/12
T201G	None	0	0	–
F205H	*m*-NP	100	0.0037	1/26
F205Y	*p*-NP	100	0.0023	1/13
F205C	None	0	0	–
F205G	*m*-NP	65	0.00167	1/59
	p-NP	35	0.0009	1/34
W266R	None	0	0	–
T281A/F290S	None	0	0	–

All the substrate (NB, *o*-NP, *m*-NP, and *p*-NP) disappearance rates were similar with the overall product (*m*-NP, *p*-NP, 4-NC, 3-NC, and NHQ) appearance rates with wild-type ToMO and the TouA mutants. For example, there was a good agreement between the formation rate of *m*-NP and *p*-NP from NB and the disappearance rate of NB with wild-type ToMO (0.15 nmol NB react/min mg protein versus 0.13 nmol *m*-NP formed and *p*-NP formed/min mg protein).

All of the high-activity TouA variants (I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V) hydroxylate NB and form *m*-NP and *p*-NP at elevated rates compared to wild-type ToMO (overall 1.7- to 6-fold faster) (Table 2). Different from wild-type ToMO, mutants A101T/M114T, A110T/E392D, M180T/E284G, and E214G/D312N/M399V form

Table 4
Enhanced rate and altered regiospecific oxidation of *o*-NP, *m*-NP, and *p*-NP by TG1/pBS(Kan)ToMO expressing wild-type ToMO and TouA variants I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V

Enzyme	Substrate											
	o-NP				*m*-NP				*p*-NP			
	Product	Rate[a]	Relative	Mol%	Product	Rate[a]	Relative	Mol%	Product	Rate[a]	Relative	Mol%
Wild-type ToMO	3-NC	0.11	1.0	18	4-NC	0.15	1.0	100	4-NC	0.0082 ± 0.0002	1.0	100
	NHQ	0.8	1.0	82								
I100Q	3-NC	0.076	0.7	16	4-NC	0.09	1.6	37	4-NC	0.0137 ± 0.0001	1.7	100
	NHQ	0.48	0.6	84	NHQ	0.15	∞	63				
A107T/E214A	3-NC	0.10	0.9	8	4-NC	0.49	3.3	100	4-NC	0.017 ± 0.001	2.0	100
	NHQ	1.2	1.5	92								
M180T/E284G	3-NC	0.095	0.9	9	4-NC	0.17	1.2	100	4-NC	0.0365 ± 0.0005	4.5	100
	NHQ	1.3	1.6	91								
E214G/D312N/M399V	3-NC	0.28	2.6	14	4-NC	0.53	3.6	100	4-NC	0.164 ± 0.002	20.0	100
	NHQ	1.8	2.3	86								

[a] Initial rate @ 500 μM determined by HPLC in nmol/(min mg protein).

4-NC from 200 μM NB. TouA mutants I100Q, A101T/M114T, A107T/E214A, and M180T/E284G form *m*-NP and *p*-NP from NB with different regiospecificities (Table 2). For example, TouA mutant A107T/E214A hydroxylates NB like a *para* enzyme, and forms 22% *m*-NP and 78% *p*-NP, whereas wild-type ToMO forms 72% *m*-NP and 28% *p*-NP (Table 2). In contrast, variant M180T/E284G forms 95% *m*-NP and 5% *p*-NP.

Substrate inhibition was observed with NB with all strains. For example, mutant I100Q forms *m*-NP and *p*-NP with an initial rate of 0.37 and 0.24 nmol/(min mg protein), respectively at 100 μM NB, but these rates are reduced by 62% and 63%, respectively, at 200 μM NB, and 80 and 75%, at 800 μM NB. Similar behavior was observed when NPs were used as substrates.

3.3. Oxidation of NB by low-activity mutants of ToMO

The oxidation of NB with wild-type ToMO and low-activity TouA variants are shown in Table 3; the rate of NP formation is decreased 12- to 59-fold compared to that by the wild-type ToMO but there were interesting changes in oxidation regiospecificity. TouA saturation mutagenesis mutants I100H and F205Y oxidize 200 μM NB to form only *p*-NP (100%) unlike wild-type ToMO (72% *m*-NP and 28% *p*-NP) but with lower rates (Table 3). A substantial shift for the hydroxylation of NB is also observed with TouA saturation mutagenesis mutant F205H which oxidizes NB to form only *m*-NP (100%) unlike wild-type ToMO (Table 3). TouA saturation mutagenesis mutant F205G hydroxylates NB and forms *m*-NP (65%) and *p*-NP (35%) similar to wild-type ToMO but with lower rates (Table 3). No products with 200 μM NB are observed with TouA DNA shuffling mutants W266R and T281A/F290S and for TouA saturation mutagenesis mutants T201G and F205C.

3.4. Oxidation of NPs by wild-type ToMO and TouA mutants

Table 4 summarizes the products obtained from whole-cell oxidation of *o*-NP, *m*-NP, and *p*-NP with wild-type ToMO and TouA mutants I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V. For *o*-NP, all mutants produce mixtures of NHQ and 3-NC, but with different rates. Wild-type ToMO forms NHQ (88%) and 3-NC (12%) from *o*-NP; however, mutant E214G/D312N/M399V forms NHQ (86%) 2.3-fold faster and 3-NC (14%) 2.6-fold faster than wild-type ToMO. Both mutant A107T/E214A and M180T/E284G yield a regiospecific change by forming more NHQ (92% and 91%, respectively) and less 3-NC (8% and 9%, respectively) from *o*-NP; these mutants form NHQ 1.5- to 1.6-fold faster than wild-type ToMO. Mutant I100Q forms NHQ (86%) and 3-NC (14%) from *o*-NP like wild-type ToMO.

For *m*-NP, mutant I100Q has a different regiospecificity for the hydroxylation of *m*-NP. Wild-type ToMO forms only 4-NC (100%) from *m*-NP; however, mutant I100Q forms NHQ (63%) and 4-NC (37%). Like wild-type ToMO, mutants A107T/E214A, M180T/E284G, and E214G/D312N/M399V form 4-NC (100%) but they do so at an elevated rate (3.3-, 1.2- and 3.6-fold faster than wild-type ToMO, respectively).

The data presented in Table 4 also show the biochemical pathways for the oxidation of *p*-NP. Wild-type ToMO forms 4-NC from *p*-NP. Like wild-type ToMO, TouA mutants I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V form 4-NC from *p*-NP, but with higher initial formation rates (1.7-, 2-, 4.5-, and 20-fold, respectively). The activity of NP oxidation with TouA mutants A101T/M114T and A110T/E392D (high-activity NB mutants) did not increase, and the oxidation regiospecificity was unchanged (data not shown).

3.5. Oxidation of toluene and o-xylene by wild-type ToMO and TouA mutants

Oxidation rates and regiospecificity for the natural substrate toluene by wild-type ToMO and mutants I100Q, F205G, and M180T/E284G were reported previously by us (Vardar and Wood, 2004). The I100Q mutation caused a shift in product distribution for the first hydroxylation of toluene and made 22% *o*-cresol, 44% *m*-cresol, and 34% *p*-cresol whereas wild-type ToMO made 32% *o*-cresol, 21% *m*-cresol, and 47% *p*-cresol which agreed with Bertoni et al. (1996). Mutant M180T/E284G gave no substantial shift in the product distribution for the first hydroxylation but the rate of toluene oxidation increased slightly (1.2-fold) (Vardar and Wood, 2004). Here, we report the toluene activity of mutants I100H, A101T/M114T, A107T/E214A, A110T/E392D, T201G, T201S, F205H, F205Y, F205C, E214G/D312N/M399V, W266R, and T281A/F290S (Table 5). Mutant A107T/E214A had enhanced rate during toluene oxidation (2.3-fold) and increased regiospecificity for *para* hydroxylation (2% *o*-cresol, 5% *m*-cresol, and 93% *p*-cresol). Mutant E214G/D312N/M399V gave no substantial shift in the product distribution (35% *o*-cresol, 22% *m*-cresol, and 43% *p*-cresol) and oxidized toluene (91 μM according to Henry's law) with an initial rate slightly lower than wild-type ToMO. The I100H and T201G mutations caused a shift in product distribution for the first hydroxylation of toluene (60% *p*-cresol formed for I100H and 53% *o*-cresol for T201G) and reduced the rate of

Table 5
Toluene oxidation rate and regiospecificity by TG1/pBS(Kan)ToMO expressing wild-type ToMO and TouA variants via GC

Enzyme	Oxidation rate (nmol/min mg protein)	Regiospecificity (%)		
		o-Cresol	*m*-Cresol	*p*-Cresol
Wild-type ToMO	2.6	32	21	47
I100Q	1.5	22	44	34
I100H	0.19	24	14	61
A101T/M114T	0.45	39	20	40
A107T/E214A	6.0	2	5	93
A110T/E392D	1.1	48	14	38
M180T/E284G	3.1	32	26	42
T201G	0.25	53	12	35
T201S	nm[a]	31	18	51
F205G	1.1	24	33	43
F205H	0.79	28	32	40
F205Y	0.34	34	34	32
F205C	0.070	21	35	44
E214G/D312N/M399V	2.3	35	22	43
T281A/F290S	0	0	0	0
W266R	0	0	0	0

Initial toluene concentration was 91 μM based on Henry's law (250 μM if all the volatile organic was in the liquid phase).
[a] nm: not measured.

toluene oxidation compare to wild-type ToMO. The regiospecificity of toluene is slightly changed with mutants A101T/M114T, A110T/E392D, F205H, and F205Y (Table 5). No products are observed with TouA mutants W266R, T281A/F290S from 90 μM toluene.

The oxidation rates and regiospecificity for the second natural substrate, *o*-xylene, by wild-type ToMO and mutants I100Q and I100H were also measured. Wild-type ToMO oxidizes *o*-xylene at 106 μM (according to Henry's law) with an initial rate of 1.78 nmol/(min mg protein) and makes 82% 3,4-dimethylphenol and 18% 2,3-DMP which agreed with the results Bertoni et al. (1996). TouA variants I100Q and I100H gave a slight shift in the product distribution for the hydroxylation of *o*-xylene (76%/24% and 88%/12% for 3,4-DMP/2,3-DMP for I100Q and I100H, respectively) and the rates were 1.6 and 0.17 nmol/(min mg protein), respectively.

3.6. Enzyme expression level

The expression level of TouA variant E214G/D312N/M399V remained approximately the same as that of wild-type ToMO. The expression levels of TouA variants I100Q, F205G, and M180T/E284G were reported previously; (Vardar and Wood, 2004) both TouA variants I100Q and F205G are expression down mutants as evidenced by SDS-PAGE with a single nucleotide change in one codon leading to less-elevated protein expression (1.5- to 2-fold). The expression level of variant M180T/E284G remained approximately the same as that of wild-type ToMO. Hence, the increase in the activity of mutants M180T/E284G and E214G/D312N/M399V derives from the amino acid substitutions rather than protein expression level changes; for TouA mutants I100Q and F205G, the activity might be two-fold more than it is reported.

3.7. ToMO TouA modeling

Although there are limitations to homology modeling (Guex et al., 1999; Schwede et al., 2003), the model did help to visualize the positions of the side chains for the variants A107T/E214A and E214G/D312N/M399V. The substantial increase in the oxidation rate of nitroaromatics by variants A107T/E214A and E214G/D312N/M399V suggests these substrates dock in the active site in an altered manner when these residues are altered; however, the advantage of directed evolution is that mutations like these may be identified even though their impact is through subtle, longer range interactions (Joo et al., 1999).

The accuracy of the wild-type ToMO TouA alpha subunit model was judged by the conservation of the spatial positions of the diiron-coordinating residues in ToMO (E104, E134, H137, E197, E231, and H234) compared to those of sMMO (E114, E144, H147, E209, E243, and H246 (Rosenzweig et al., 1997)). The average distance between the C_α carbons of the target ToMO model relative to the sMMO template for the metal binding residues was 0.075 Å. To allow for a better fit between the template and target sequence, different portions of the target DNA were modeled, and W44-N380 of TouA (337 amino acid) resulted with the highest amino acid identity (27.4%). When 304 residues (out of 337) of sMMO and ToMO alpha subunit were superimposed upon each other, the root-mean-square between pairs of C_α atoms were found to be 0.07 Å. Since proteins which have 50% amino acid sequence identity deviate by ∼1 Å and since identical proteins solved by NMR can deviate by more than 1 Å (Guex et al., 1999), the ToMO TouA model generated by SWISS-MODEL should be reliable.

4. Discussion

In this paper it is clearly shown that wild-type ToMO forms *m*-NP and *p*-NP from NB as well as 4-NC, 3-NC, and NHQ from NPs (Tables 2–4). Further, the hydroxylation rate of substrates NB, *o*-NP, *m*-NP, and *p*-NP may be enhanced to produce NP, NC, or NHQ by introducing the mutations I100Q, A107T/E214A, M180T/E284G, and E214G/D312N/M399V in ToMO TouA (Table 4). Also, the regiospecific hydroxylation of substrate NB may be changed by introducing the mutations I100H, I100Q, A107T/E214A, F205Y, F205H, and M180T/E284G in ToMO TouA (Tables 2 and 3), and the regiospecific hydroxylation of *m*-NP may be changed by the mutation I100Q in ToMO TouA (Table 4). The activity of the enzyme is lost by introducing mutations W266R and T281A/F290S in ToMO TouA (no oxidation of NB and the natural substrate toluene are observed, Tables 2 and 5).

To our knowledge, there is no previous data about the formation of NHQ formation from *o*-NP by any toluene monooxygenases (here, wild-type ToMO produces NHQ and 3-NC from *o*-NP). ToMO has not been characterized previously with nitro aromatics. *Rhodococcus* sp. strain PN1 degraded *m*-NP and *p*-NP easily but not *o*-NP (Abe et al., 2002). 4-NC was an intermediate of *p*-NP degradation, and 4-NC and NHQ were detected as intermediates of *m*-NP. Toluene-grown *P. putida* F1 has been shown to convert *m*-NP to 3-NC, but the other nitrophenol isomers (*o*-NP or *p*-NP) were not oxidized. *P. putida* B2 was shown to convert *o*-NP to nitrite and catechol by a nitrophenol oxygenase (Zeyer et al., 1986). Formation of 2-aminophenol from *o*-NP was shown by a non-acclimated methanogenic mixed culture using an anaerobic model-soil column (El Fantroussi et al., 1998) and by using sodium acetate and sodium nitrate as electron donor and electron acceptor, respectively, in another study (Karim and Gupta, 2003). Recently, *P. stutzeri* DSM 6538, *P. putida* DSM 6521, and *Pseudomonas* sp. JS6 were shown to produce 3-NC (less than 0.05 mM) from *o*-NP (Kieboom et al., 2001).

The oxidation rate of nitro aromatics increased by the mutant E214G/D312N/M399V between 4 and 20 times compared to wild-type ToMO; hence, positions E214/D312/M399 of the alpha subunit TouA of ToMO (499 amino acids) may help to increase the oxidation of nitro aromatics, such as NB, *o*-NP, *m*-NP, and *p*-NP. The significance of each of the positions E214G, D312N, and M399V has not been studied previously for aromatic monooxygenases. Variant A107T/E214A acted like a *para* enzyme and formed *p*-cresol as the major product (93%) from toluene. Variant A107T/E214A also formed *p*-NP (79%) as the major product during NB oxidation and 4-methylresorcinol (43%) as the primary product during *o*-cresol oxidation (data not shown). Previously, in T4MO, the alpha subunit residue A107 has been shown to control the regiospecificity of toluene oxidation (Mitchell et al., 2002) and the corresponding residue in TOM (A113) has been shown to control oxidation of indole forming a rainbow of colors (Rui et al., in press). We just recently discovered that TmoA G103S/A107T formed 100% *p*-cresol from toluene, making T4MO a better *para*-hydroxylating enzyme (Tao et al., 2004). Since TouA E214 is ∼22 Å and A107 is 5.5 Å away from the active site (calculated via Swiss-PDB viewer), position E214 would not be predicted as an important residue, which controls the catalysis for regiospecificity. As a conclusion, A107T is most likely the amino acid responsible for making ToMO a *para* enzyme. It is also interesting that alanine to threonine mutations were found (A101T, A107T, A110T) that lead to enhanced NB oxidation (Table 2).

Pikus et al. (1997) studied one substitution of the related enzyme T4MO, TmoA I180F, but this change gave no change in product distributions for the oxidation of toluene, *m*-xylene, and *p*-xylene. Previously, we reported that TouA M180 of ToMO, which corresponds to T4MO TmoA I180 and which was identified through DNA shuffling, might be playing an important role in the regiospecificity of the oxidation of *o*-cresol, *m*-cresol, *p*-cresol, phenol, catechol, and resorcinol in TouA mutant M180T/E284G (Vardar and Wood, 2004). Here, we show that TouA M180 might also be important in the oxidation of nitro aromatics (rates increased two to five times for NB, *o*-NP, and *p*-NP oxidation with mutant M180T/E284G). Mutant M180T/E284G from DNA shuffling has a second amino acid change at position TouA E284, which may also help to increase the oxidation of nitro aromatics by this mutant. The significance of position E284 has also not been indicated before.

Previously we showed that methyl substitution on the benzene ring did not affect the regiospecificity of oxidation by wild-type ToMO and mutants I100Q, F205G, and M180T/E284G (Vardar and Wood, 2004), for example, TouA mutant I100Q produced catechol and hydroquinone (major peak) from benzene and methylcatechols and methylhydroquinone (major peak) from toluene whereas wild-type ToMO produced only catechol from benzene and methylcatechols from toluene. Here, we show that nitro substitution on the benzene ring did not alter the regiospecificity of oxidation since wild-type ToMO produced only 4-NC from *m*-NP and TouA I100Q produced both 4-NC (37%) and NHQ (63%). These results also indicate that position I100 of the alpha subunit TouA of ToMO controls the regiospecific oxidation of nitroaromatics. The I100Q mutation also increased TCE degradation with ToMO (data not shown) as was found for TomA1 variant V106A (corresponds to position TouA I100 in ToMO) of *B. cepacia* G4 (Canada et al., 2002). Hence, all the results corroborate that position I100 is a key amino acid in the alpha subunit of toluene monooxygenases.

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