Alanine 101 and Alanine 110 of the Alpha Subunit of *Pseudomonas stutzeri* OX1 Toluene-*o*-Xylene Monooxygenase Influence the Regiospecific Oxidation of Aromatics

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Abstract: Saturation mutagenesis was used to generate 10 mutants of toluene-*o*-xylene monooxygenase (ToMO) at alpha subunit (TouA) positions A101 and A110: A101G, A101I, A101M, A101VE, A101V, A110G, A110C, A110S, A110P, and A110T; by testing the substrates toluene, *o*-cresol, *m*-cresol, *p*-cresol, phenol, naphthalene, *o*-methoxyphenol, *m*-methoxyphenol, *p*-methoxyphenol, *o*-xylene, and nitrobenzene, these positions were found to influence the regiospecific oxidation of aromatics. For example, compared to wild-type ToMO, TouA variant A101V produced threefold more 3-methoxycatechol from *m*-methoxyphenol as well as produced methylhydroquinone from *o*-cresol whereas wild-type ToMO did not. Similarly, variant A110C synthesized 1.8-fold more *o*-cresol from toluene and 1.8-fold more 3-methoxycatechol from *m*-methoxyphenol, and variant A110G synthesized more *m*-nitrophenol and twofold less *p*-nitrophenol from nitrobenzene. The A101V and A110C mutations did not affect the rate of reaction with the natural substrate toluene, so the variants had high activity. This is the first report that these or analogous residues influence the catalysis with this class of enzymes. Wild-type ToMO was found to oxidize *o*-methoxyphenol to methoxyhydroquinone (60%) and 4-methoxyresorcinol (40%), *m*-methoxyphenol to 4-methoxycatechol (96%) and 3-methoxycatechol (4%), and *p*-methoxyphenol to 4-methoxycatechol (100%). © 2005 Wiley Periodicals, Inc.

Keywords: toluene monooxygenase; saturation mutagenesis cresols; naphthalene; methoxyphenol; xylene; regiospecific oxidation

INTRODUCTION

Bacterial toluene monooxygenases are known for their ability to oxidize a wide range of aromatics, degrade a variety of organic pollutants, and produce industrially important compounds (Shim et al., 2001; Tao et al., 2004b; Vardar et al., 2005). These enzymes include toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4 (Newman and Wackett, 1995; Shields et al., 1995), toluene-*para*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1 (Fishman et al., 2004b), toluene-4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1 (Yen et al., 1991), and toluene-*o*-xylene monooxygenase (ToMO) of *Pseudomonas stutzeri* OX1 (Bertoni et al., 1996). These multi-component enzymes contain a three subunit hydroxylase ($\alpha_2\beta_2\gamma_2$) with a non-heme, carboxylate-bridged, diiron center contained by the α subunit, a reductase, a mediating protein, and sometimes a ferredoxin or uncharacterized subunit (Bertoni et al., 1996; Byrne et al., 1995; Cafaro et al., 2002; Newman and Wackett, 1995; Studts et al., 2000). These enzymes differ in substrate range and regiospecificity.

Both DNA shuffling and saturation mutagenesis have been used to alter toluene monooxygenase activity, and Table I summarizes positions influencing catalysis for this class of enzymes. The ToMO crystal structure (Sazinsky et al., 2004) shows many of these beneficial positions (e.g., I100, E103, A107) are located in the TouA (alpha subunit) B-helix; but, other regions of the alpha subunit have also been shown to influence the regiospecificity of oxidation, such as positions F205 and T201 (Pikus et al., 2000; Vardar and Wood, 2004), which are located in the TouA E-helix as well as Q141 in the TouA C-helix (Pikus et al., 1997). Using DNA shuffling, TouA variants A101T/M114T and A110T/E392D were identified previously and shown to influence the rate of reaction and the regiospecificity of oxidation slightly of nitrobenzene and toluene (Vardar et al., 2005); however, due to the double mutations, it was not clear which residue was responsible for the altered catalysis. Here, saturation mutagenesis of the TouA alpha subunit of ToMO showed A101 and A110, which are also located in the TouA B-helix, influence the regiospecificity of hydroxylation of substituted aromatics.

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Table I. Summary of the important residues in the alpha subunits of toluene monooxygenases.

Corresponding ToMO residue	Location	Enzyme	Effect on catalysis
I100	Helix B	ToMO I100Q	Changes the regiospecific oxidation of methyl and nitro aromatics and enhances TCE degradation (Vardar and Wood, 2004, 2005b; Vardar et al., 2005)
		T4MO I100L	Enhances 3-methoxycatechol formation from guaiacol (Tao et al., 2004a)
		T4MO I100A	Enhances 4-nitrocatechol formation from nitrobenzene (Fishman et al., 2004a)
		T4MO I100C	Changes the regiospecific oxidation of butadiene (Steffan and McClay, 2000)
		TOM V106A	Gate residue; Enhances TCE and naphthalene oxidation (Canada et al., 2002)
		TOM V106F	Enhances chloroform oxidation and *p*-cresol formation from toluene (Rui et al., 2004)
I100/E103	Helix B/B	TpMO I100S/G103S	Converts para specific TpMO into a *meta* enzyme (Fishman et al., 2005)
A101	Helix B	ToMO A101T/M114T	Changes the regiospecific oxidation of toluene (Vardar et al., 2005)
E103	Helix B	T4MO G103L	Enhances *o*-cresol formation from toluene (Mitchell et al., 2002)
E103/A107	Helix B	T4MO G103S/A107T	Changes the regiospecific oxidation of methyl and methoxy aromatics (Tao et al., 2004a)
		T4MO G103L/A107G	Converts *para* specific T4MO into an *ortho* enzyme (Mitchell, 2002)
A107/E214	Helix B/E	ToMO A107T/E214A	Converts nonspecific ToMO into a *para* enzyme (Vardar et al., 2005)
A107	Helix B	TpMO A107G	Converts *para* specific TpMO into an *ortho* enzyme (Fishman et al., 2005)
		TpMO A107T	Converts TpMO into a better *para* enzyme (Fishman et al., 2005)
		TOM A113V	Alters indole oxidation to produce primarily indigo (wild-type produces isoindigo) (Rui et al., 2005)
		TOM A113I	Alters indole oxidation to produce primarily indirubin (Rui et al., 2005)
		TOM A113H	Alters indole oxidation to produce primarily isatin (Rui et al., 2005)
A110	Helix B/loop	ToMO A110T/E392D	Changes the regiospecific oxidation of toluene (Vardar et al., 2005)
Q141	Helix C	T4MO Q141C	Enhances 3-methylbenzyl alcohol formation from *m*-xylene (Pikus et al., 1997)
M180	Helix D	ToMO M180T	Enhances and changes the regiospecific oxidation of methyl and nitro aromatics (Vardar and Wood, 2004, 2005a; Vardar et al., 2005)
T201	Helix E	T4MO T201G	Enhances 2,5-dimethylphenol formation from *p*-xylene (Pikus et al., 2000)
		T4MO T201F	Enhances benzyl alcohol formation from toluene (Pikus et al., 2000)
F205	Helix E	T4MO F205I	Enhances *m*-cresol formation from toluene (Pikus et al., 1997)
		ToMO F205G	Changes the regiospecific oxidation of methyl and nitro aromatics (Vardar and Wood, 2004; Vardar et al., 2005)
E214	Helix E	ToMO E214G	Gate residue; Enhances nitro aromatic oxidation and *cis*-DCE degradation (Vardar and Wood, 2005a,b; Vardar et al., 2005)

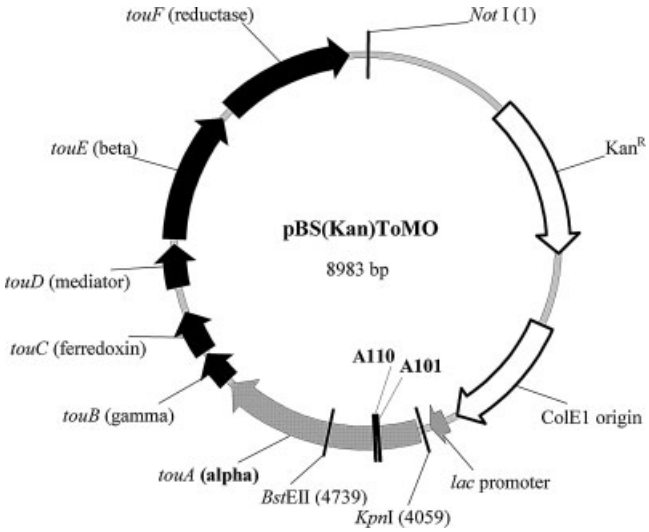

Figure 1. Vector pBS(Kan)ToMO for constitutive expression of wild-type ToMO and mutants. Kan^R is the kanamycin resistance gene.

MATERIALS AND METHODS

Escherichia coli strain TG1 (Sambrook et al., 1989) was used to express ToMO from pBS(Kan)ToMO (Fig. 1) using exponentially-growing cells (Vardar and Wood, 2004). Chemicals were from Fisher Scientific Co. (Fairlawn, NJ), Sigma Chemical CO. (St. Louis, MO), and Acros Organics (Morris Plains, NJ).

Saturation mutagenesis of TouA positions A101 and A110 was performed using the method of three PCR reactions as described previously (Vardar and Wood, 2004; Vardar et al., 2005). To introduce all amino acids at position A101 of TouA, a 473 bp DNA fragment that includes the *Kpn*I restriction site upstream of codon A101 was amplified using primers ToMO-*Kpn*I-front (5′-CCGGCTCGTATGTTGTGTGGAATTGTGAGCGG-3′) and A101-rear (5′-GCGGCGTATTCTTCAAGNNNGATCGCTCCGAAGTG-3′), and an 458 bp DNA fragment that includes the *Bst*EII restriction site downstream of codon A101 was amplified using primers A101-front (5′-GCAACTTCACTTCGGAGCGATCNNNCTTGAAGAATACGC-3′) and ToMO-*Bst*EII-rear (5′-CCAGGATCTTGAGCGACGGTCCACCTTGCTGTGCG-3′). Similarly, to introduce all amino acids at position A110 of TouA, a 507 bp DNA fragment that includes the *Kpn*I restriction site upstream of codon A110 was amplified using primers ToMO-*Kpn*I-front and A110-rear (5′-GGCGAATCGCGCCATACGGGCTTCNNNAGTGCTTGCGG-3′), and an 430 bp DNA fragment that includes the *Bst*EII restriction site downstream of codon A101 was amplified using primers A110-front (5′-GAAGAATACGCCGCAAGCACTNNNGAAGCCCGTATGGCG-3′) and ToMO-*Bst*EII-rear. The two sets of DNA fragments were combined separately in a 1:1 ratio to obtain the full-length, products (889 bp) with the ToMO-*Kpn*I-front and ToMO-*Bst*EII-rear primers; these fragments were cloned into pBS(Kan)ToMO using restriction enzymes *Kpn*I and *Bst*EII (Fig. 1).

To create the A101V/A110C TouA variant (double mutant), site directed mutagenesis was performed to add A101V to TouA using the A110C TouA variant as a PCR template. *Pfu* polymerase was used to minimize random point mutations. A 473 bp DNA fragment that includes the *Kpn*I restriction site upstream of codons A101 and A110 was amplified using primers ToMO-*Kpn*I-front and ToMO-A101V-rear (5′-GCGGCGTATTCTTCAAGGACGATCGCTCCGAAGTG-3′), and a 458 bp DNA fragment that includes the *Bst*EII restriction site downstream of codons A101 and A110 was amplified using primers ToMO-A101V-front (5′-GCAACTTCACTTCGGAGCGATCGTCCTTGAAGAATACGC-3′) and ToMO-*Bst*EII-rear. The two fragments were combined in a 1:1 ratio as templates with the ToMO-*Kpn*I-front and ToMO-*Bst*EII-rear primers to obtain the full-length, 889 bp product that introduces the A101V mutation (along with A110C) of TouA and has two unique restriction enzyme sites, *Kpn*I and *Bst*EII (Fig. 1). This PCR product was cloned into pBS(Kan)ToMO using restriction enzymes *Kpn*I and *Bst*EII. The double A101V/A110C TouA variant was confirmed via DNA sequencing of TouA. The resulting plasmid libraries were electroporated into *E. coli* TG1 competent cells using a Bio-Rad GenePulser/Pulse Controller (Hercules, CA) at 15 kV/cm, 25 μF, and 200 Ω.

For saturation mutagenesis, 500 colonies were screened on *o*-cresol, toluene and nitrobenzene to ensure with a 99.96% probability that all possible substitutions were screened (Rui et al., 2004) using a colony-based agar plate assay that detected secreted, altered dihydroxy products (Vardar and Wood, 2004; Vardar et al., 2005). The interesting variants were examined further with various substrates (Tables II and III) using reverse-phase high performance liquid chromatography (HPLC) and by gas chromatography (GC) as described previously (Vardar and Wood, 2004; Vardar et al., 2005). A Zorbax SB-C8 column (Agilent Technologies, Palo Alto, CA, 5 μm, 4.6 × 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). For the methoxy substrates, gradient elution (80:20 (H_2O-0.1% formic acid:acetonitrile) for 0–17 min, 50:50 for 22 min, and 80:20 for 35 min) was used. The retention times for *m*-methoxyphenol, *o*-methoxyphenol, *p*-methoxyphenol, 3-methoxycatechol, 2-methoxyresorcinol, 4-methoxyresorcinol, and methoxyhydroquinone were 29.9, 28.8, 26.2, 16.7, 12.9, 11.3, and 8.8 min, respectively. To confirm product identifications, the retention times and UV-visible spectra of the standard chemicals were compared with those of the mutant-enzyme-derived samples (Tables II and III), and the enzyme products were co-eluted with authentic standards (except for 4-methoxycatechol).

RESULTS AND DISCUSSION

TouA A101 is closer to the Fe_A site of the diiron center than Fe_B, whereas TouA A110 is closer to Fe_B diiron site (Fig. 2).

Table II. Regiospecific oxidation of toluene and *o*-cresol by the TouA A101 and A110 variants (% regiospecificity indicated in parenthesis). Products determined by GC and HPLC.

	Enzyme										
	Wild-type ToMO	A101G	A101I	A101M	A101E	A101V	A110G	A110C	A110S	A110P	A110T
Substrate											
Toluene	*o*-cresol (33)	*o*-cresol (37)	*o*-cresol (36)	*o*-cresol (45)	*o*-cresol (46)	*o*-cresol (46)	*o*-cresol (37)	*o*-cresol (58)	*o*-cresol (37)	*o*-cresol (33)	*o*-cresol (46)
	m-cresol (20)	*m*-cresol (19)	*m*-cresol (20)	*m*-cresol (21)	*m*-cresol (17)	*m*-cresol (24)	*m*-cresol (18)	*m*-cresol (11)	*m*-cresol (17)	*m*-cresol (20)	*m*-cresol (16)
	p-cresol (47)	*p*-cresol (44)	*p*-cresol (44)	*p*-cresol (34)	*p*-cresol (37)	*p*-cresol (31)	*p*-cresol (45)	*p*-cresol (31)	*p*-cresol (46)	*p*-cresol (47)	*p*-cresol (38)
o-cresol	3-MC (100)	3-MC (98) MHQ (2)	3-MC (96) MHQ (4)	nm[1]	nm	3-MC (93) MHQ (7)	3-MC (99) MHQ (1)	3-MC (97) MHQ (3)	3-MC (98) MHQ (2)	3-MC (96) MHQ (4)	3-MC (97) MHQ (3)

nm[1], not measured; MC, methylcatechols; MHQ, methylhydroquinone.

Table III. Regiospecific oxidation of aromatic substrates by TouA variants A101V, A110C, and A101V/A110C. Products (indicated by % in parenthesis) determined by GC and HPLC. Since 4-MXC could not be purchased commercially, its concentration is based on the calibration curve of the 3-MXC isomer.

	Substrate								
	o-cresol	*m*-cresol	*p*-cresol	phenol	naphthalene	*o*-methoxyphenol	*m*-methoxyphenol	*p*-methoxyphenol	*o*-xylene
Enzyme									
Wild-type ToMO	3-MC (100)	4-MC (96) 3-MC (4)	4-MC (100)	C (100%)	1-N (88%) 2-N (12%)	MXHQ (60) 4-MXR (40)	4-MXC (96) 3-MXC (4)	4-MXC (100)	2,3-DMP (18) 3,4-DMP (82)
A101V	3-MC (93) MHQ (7)	4-MC (91) 3-MC (9)	4-MC (100)	C (100%)	1-N (93%) 2-N (7%)	MXHQ (81) 4-MXR (19)	4-MXC (87) 3-MXC (13)	4-MXC (100)	2,3-DMP (20) 3,4-DMP (80)
A110C	3-MC (97) MHQ (3)	4-MC (89) 3-MC (9) MHQ (2)	4-MC (100)	C (100%)	1-N (93%) 2-N (7%)	MXHQ (68) 4-MXR (32)	4-MXC (93) 3-MXC (7)	4-MXC (100)	2,3-DMP (25) 3,4-DMP (75)
A101V/ A110C	nm[1]	nm	nm	nm	1-N (95%) 2-N (5%)	MXHQ (85) 4-MXR (15)	4-MXC (91) 3-MXC (9)	nm	nm

MC, methylcatechols; MHQ, methylhydroquinone; C, catechol; N, naphthol; MXHQ, methoxyhydroquinone; MXR, methoxyresorcinol; MXC, methoxycatechol; DMP, dimethylphenol; nm[1], not measured.

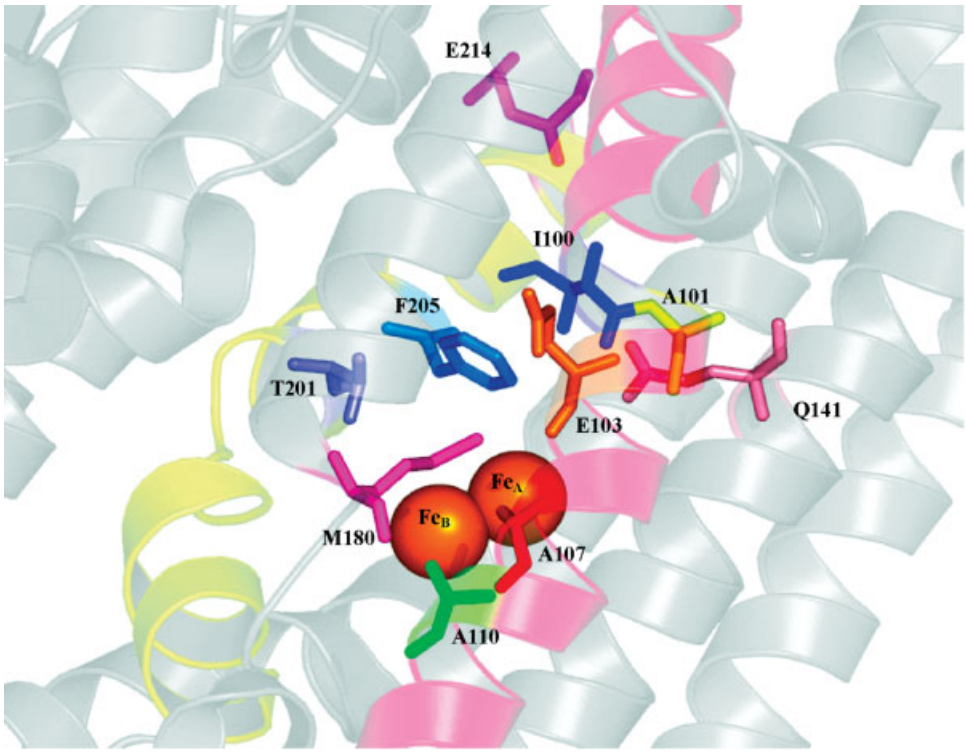

Figure 2. Important hydroxylase residues of toluene monooxygenases. The wild-type ToMO hydroxylase (Protein Data Bank accession code 1t0q (Sazinsky et al., 2004)) was visualized using Swiss-Pdb Viewer program (DeepView) (Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003) and Pymol. The diiron center is shown in orange. Many of the beneficial positions such as I100 (blue), A101 (yellow), E103 (orange), A107 (red), and A110 (green) are located on the TouA B-helix (highlighted in red). Other beneficial positions such as T201 (purple), F205 (light blue), and E214 (pink) are located in the TouA E-helix (highlighted in yellow). Position Q141 and M180 are shown in light pink and dark pink, respectively.

All toluene monooxygenases have alanine at the position analogous to the A101 of ToMO, except for TOM, which has the larger subunit threonine (Fishman et al., 2005). ToMO and TpMO have alanine at the position analogous to the A110 of ToMO, whereas T4MO and TOM have the smallest amino acid, glycine (Fishman et al., 2005). Both ToMO positions are located in the TouA B-helix and are ~10.3 angstroms away from the Fe_A site of the diiron center (calculated via Swiss-PDB viewer (Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003)).

There is a large channel (~30–35 Å in length and 6–10 Å in width) within the alpha subunit of ToMO connecting the diiron center to the surface of the protein (Sazinsky et al., 2004). The channel starts from the diiron center, traverses the four-helix bundle between helices E and B, and passes through cavities created by helices D, E, B, G, and H (Sazinsky et al., 2004). Recent structure-mechanism studies are providing insights for substrate docking and product egress though this channel (Sazinsky and Lippard, 2005).

Ten saturation mutagenesis variants were identified using toluene, *o*-cresol, and nitrobenzene based on differences in the secreted oxidized products produced on agar plates compared to the wild-type enzyme. The regiospecific products of the oxidized toluene and *o*-cresol are shown in Table II. The variants giving the most significant shift in the product distribution were variants TouA A101V and TouA A110C; for example, from *o*-cresol, variants A101V and

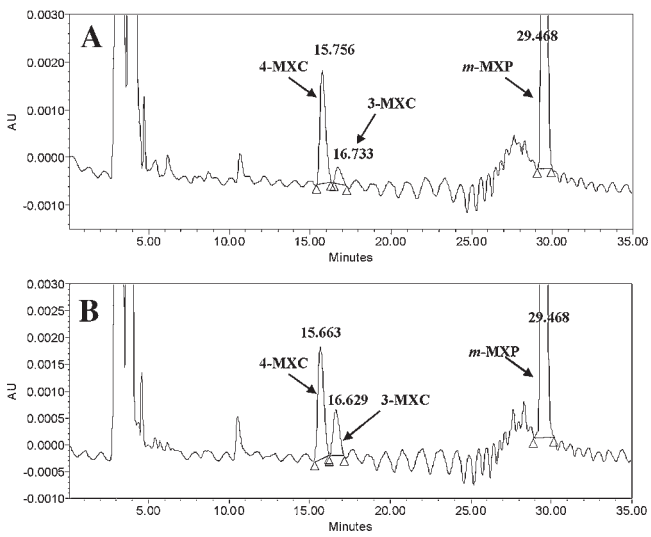

Figure 3. HPLC chromatograms (presented at a wavelength of 265 nm) of *m*-methoxyphenol (*m*-MXP) oxidation by TG1 cells expressing wild-type ToMO (**A**) and TouA A101V (**B**). Initial *m*-MXP concentration was 500 μM and the contact period was 10 min. The calibration curves and analysis of each compound were obtained at the maximum wavelength for each compound except for 4-methoxycatechol (4-MXC). Since all the possible compounds except for 4-MXC (Kolanczyk et al., 1999) that can be produced from *m*- and *p*-methoxyphenol were purchased, the peak at ~15.7 min represents 4-MXC (maximum wavelength of 286 nm).

Table IV. Substrate utilization rates by TG1/pBS(Kan)ToMO expressing wild-type ToMO and TouA variants A101V, A110C, and A101V/A110C.

	Rate (nmol/min · mg protein)				
Enzyme	Toluene	*o*-cresol	*o*-methoxyphenol	*m*-methoxyphenol	*p*-methoxyphenol
Wild-type	2.6	1.30 ± 0.01	1.6 ± 0.1	7.4 ± 3	0.43
A101V	2.9	2.0 ± 0.3	1.0 ± 0.5	8.9	2.0
A110C	2.1	1.0 ± 0.1	1.2 ± 0.1	5 ± 2	1.0
A101V/A110C	nm[1]	0.83 ± 0.1	0.8 ± 0.2	4.1	nm

nm[1], not measured.

A110C formed methylhydroquinone (7% and 3%, respectively) as a novel product (Table II). Hence, the regiospecific products from these two variants were analyzed with eight additional substrates (Table III). From *m*-cresol, variants A101V and A110C formed twofold more 3-methylcatechol compared to wild-type ToMO, and methylhydroquinone (2%) was formed as a novel product by variant A110C. Similarly, the regiospecificity of variant A101V was altered to synthesize threefold more 3-methoxycatechol from *m*-methoxyphenol (Table III, Fig. 3). Hence, Tou A101 and A110 influence the regiospecific oxidation of methyl- and methoxy-substituted aromatics. Variants A101V and A110C were as active as the wild-type on the natural substrate toluene (initial toluene degradation rates of 2.9 and 2.1, respectively, versus 2.6 nmol/min/mg protein) and altered the regiospecificity of its oxidation, too (Tables II and IV). Table IV also shows that variants A101V and A110C were as active as wild-type on other substrates such as *o*-cresol, *o*-methoxyphenol, *m*-methoxyphenol, and *p*-methoxyphenol.

The double TouA mutant A101V/A110C was active as the wild-type enzyme and the single variants (Table IV); however, the two beneficial mutations did not alter the regiospecfic oxidation of *o*-methoxyphenol, *m*-methoxyphenol, and naphthalene oxidation very much (Table III) although there was a slight increase in methoxyhydroquinone production from *o*-methoxyphenol. Thus, this double mutant A101V/A110C behaves more like the single mutant A101V.

There is no previous report about the hydroxylation of methoxy aromatics by ToMO. Here, it was also discovered that ToMO oxidizes *o*-methoxyphenol to methoxyhydroquinone (60%) and 4-methoxyresorcinol (40%); *m*-methoxyphenol to 4-methoxycatechol (96%) and 3-methoxycatechol (4%); and *p*-methoxyphenol to 4-methoxycatechol (100%) (Table III). These results differ from that of T4MO, which produces 4-methoxyresorcinol (87%) and 3-methoxycatechol (11%) from *o*-methoxyphenol (Tao et al., 2004a).

After screening 500 colonies from each A101 and A110 library on nitrobenzene agar plates, none of the variants generated by saturation mutagenesis had significantly-enhanced rates of nitroaromatic oxidation. The two best variants that formed darker red halos, TouA A110G and TouA A110P, had only a 20% and 30% increase in nitrobenzene oxidation (e.g., 0.17 vs. 0.13 nmol/min/mg protein for 200 μM nitrobenzene for TouA A110P and wild-type ToMO, respectively). However, the regiospecific oxidation of nitrobenzene by these variants was altered to synthesize twofold less *p*-nitrophenol compared to the wild-type enzyme with more *m*-nitrophenol produced (86% to 88% vs. 72% for the wild-type enzyme).

It is clearly shown in this study that residues A101 and A110 in the alpha subunit of ToMO affect catalytic activity. Hence, all of the known beneficial residues that influence regiospecificity for toluene monooxygenases are nearby the active site, and most of them are located on the alpha subunit helix-B (Fig. 2). In contrast, TouA position E214G, which is 23 Å from the active site, influences the rate of oxidation of nitroaromatics, but does not influence the regiospecificity of the reaction (Vardar and Wood, 2005a). By discovering two more residues that influence catalysis, those who wish to tailor this family of monooxygenases (which includes soluble methane monooxygenase of *Methylosinus trichosporium* OB3b (Jahng and Wood, 1994)) now have more tools to allow them, for example, to produce pharmaceuticals from indigoid compounds (Rui et al., 2005) and to control the regiospecific hydroxylation of toluene (Fishman et al., 2005).

References

Bertoni G, Bolognese F, Galli E, Barbieri P. 1996. Cloning of the genes for and characterization of the early stages of toluene and *o*-xylene catabolism in *Pseudomonas stutzeri* OX1. Appl Environ Microbiol 62:3704–3711.

Byrne AM, Kukor JJ, Olsen RH. 1995. Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii*PKO1. Gene 154:65–70.

Cafaro V, Scognamiglio R, Viggiani A, Izzo V, Passaro I, Notomista E, Piaz FD, Amoresano A, Casbarra A, Pucci P et al. 2002. Expression and purification of the recombinant subunits of toluene/*o*-xylene monooxygenase and reconstitution of the active complex. Eur J Biochem 269:5689–5699.

Canada KA, Iwashita S, Shim H, Wood TK. 2002. Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. J Bacteriol 184:344–349.

Fishman A, Tao Y, Bentley WE, Wood TK. 2004a. Protein engineering of toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 for synthesizing 4-nitrocatechol from nitrobenzene. Biotechnol Bioeng 87:779–790.

Fishman A, Tao Y, Wood TK. 2004b. Toluene 3-monooxygenase of *Ralstonia pickettii* PKO1 is a *para*-hydroxylating enzyme. J Bacteriol 186:3117–3123.

Fishman A, Tao Y, Rui L, Wood TK. 2005. Controlling the regiospecific oxidation of aromatics via active site engineering of toluene *para*-monooxygenase of *Ralstonia pickettii* PKO1. J Biol Chem 280:506–514.

Guex N, Peitsch MC. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling. Electrophoresis 18:2714–2723.

Jahng D, Wood TK. 1994. Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 60:2473–2482.

Kolanczyk R, Schmieder P, Bradbury S, Spizzo T. 1999. Biotransformation of 4-methoxyphenol in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes. Aquatic Toxicol 45:47–61.

Mitchell KH. 2002. Mutagenesis studies revealing the complementary roles of effector protein binding and the hydroxylase active site catalysis by toluene 4-monooxygenase. Madison: University of Wisconsin.

Mitchell KH, Studts JM, Fox BG. 2002. Combined participation of hydroxylase active site residues and effector protein binding in a *para* to *ortho* modulation of toluene 4-monooxygenase regiospecificity. Biochemistry 41:3176–3188.

Newman LM, Wackett LP. 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry 34:14066–14076.

Peitsch MC. 1995. Protein modeling by e-mail. Bio/Technology 13:658–660.

Pikus JD, Studts JM, McClay K, Steffan RJ, Fox BG. 1997. Changes in the regiospecificity of aromatic hydroxylation produced by active site engineering in the diiron enzyme toluene 4-monooxygenase. Biochemistry 36:9283–9289.

Pikus JD, Mitchell KH, Studts JM, McClay K, Steffan RJ, Fox BG. 2000. Threonine 201 in the diiron enzyme toluene 4-monooxygenase is not required for catalysis. Biochemistry 39:791–799.

Rui L, Kwon YM, Fishman A, Reardon KF, Wood TK. 2004. Saturation mutagenesis of toluene *ortho*-monooxygenase from *Burkholderia cepacia* G4 for enhanced 1-naphthol synthesis and chloroform degradation. Appl Environ Microbiol 70:3246–3252.

Rui L, Reardon KF, Wood TK. 2005. Protein engineering of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for regiospecific hydroxylation of indole to form indigoid compounds. Appl Microbiol Biotechnol 66:422–429.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sazinsky MH, Lippard SJ. 2005. Product bound structures of the soluble methane monooxygenase hydroxylase from *Methylococus capsulatus* (Bath): Protein motion in the alpha-subunit. J Am Chem Soc 127:5814–5825.

Sazinsky MH, Bard J, Di Donato A, Lippard SJ. 2004. Crystal structure of the toluene/*o*-xylene monooxygenase hydroxylase from *Pseudomonas stutzeri* OX1: Insight into the substrate specificity, substrate channeling and active site tuning of multicomponent monooxygenases. J Biol Chem 279:30600–30610.

Schwede T, Kopp J, Guex N, Peitsch MC. 2003. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385.

Shields MS, Reagin MJ, Gerger RR, Campbell R, Somerville C. 1995. TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl Environ Microbiol 61:1352–1356.

Shim H, Ryoo D, Barbieri P, Wood TK. 2001. Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylenes, and vinyl chloride by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1. Appl Microbiol Biotechnol 56:265–269.

Steffan RJ, McClay KR. 2000. Preparation of enantio-specific epoxides. USA patent WO 00/73425 A1.

Studts JM, Mitchell KH, Pikus JD, McClay K, Steffan RJ, Fox BG. 2000. Optimized expression and purification of toluene 4-monooxygenase hydroxylase. Protein Expr Purif 20:58–65.

Tao Y, Fishman A, Bentley WE, Wood TK. 2004a. Altering toluene 4-monooxygenase by active-site engineering for the synthesis of 3-methoxycatechol, methoxyhydroquinone, and methylhydroquinone. J Bacteriol 186:4705–4713.

Tao Y, Fishman A, Bentley WE, Wood TK. 2004b. Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1. Appl Environ Microbiol 70:3814–3820.

Vardar G, Wood TK. 2004. Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for synthesizing 4-methylresorcinol, methylhydroquinone, and pyrogallol. Appl Environ Microbiol 70:3253–3262.

Vardar G, Wood TK. 2005a. Alpha subunit positions methionine 180 and glutamate 214 of *Pseudomonas stutzeri* OX1 toluene-*o*-xylene monooxygenase influence catalysis. J Bacteriol 187:1511–1514.

Vardar G, Wood TK. 2005b. Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for enhanced chlorinated ethene degradation and *o*-xylene oxidation. Appl Microbiol Biotechnol Vol. 68.

Vardar G, Ryu K, Wood TK. 2005. Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for oxidizing nitrobenzene to 3-nitrocatechol, 4-nitrocatechol, and nitrohydroquinone. J Biotechnol 115:145–156.

Yen K-M, Karl MR, Blatt LM, Simon MJ, Winter RB, Fausset PR, Lu HS, Harcourt AA, Chen KK. 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J Bacteriol 173:5315–5327.