Regiospecific Oxidation of Naphthalene and Fluorene by Toluene Monooxygenases and Engineered Toluene 4-Monooxygenases of *Pseudomonas mendocina* KR1

Ying Tao,[1] William E. Bentley,[2] Thomas K. Wood[1]

[1]Departments of Chemical Engineering & Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3222; telephone: (860) 486-2483 fax: (860) 486-2959; e-mail: twood@engr.uconn.edu
[2]Department of Chemical Engineering, University of Maryland, College Park, Maryland 20742

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Abstract: The regiospecific oxidation of the polycyclic aromatic hydrocarbons naphthalene and fluorene was examined with *Escherichia coli* strains expressing wildtype toluene 4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1, toluene *para*-monooxygenase (TpMO) from *Ralstonia pickettii* PKO1, toluene *ortho*-monooxygenase (TOM) from *Burkholderia cepacia* G4, and toluene/*ortho*-xylene monooxygenase (ToMO) from *P. stutzeri* OX1. T4MO oxidized toluene (12.1 $\pm$ 0.8 nmol/min/mg protein at 109 μM), naphthalene (7.7 $\pm$ 1.5 nmol/min/mg protein at 5 mM), and fluorene (0.68 $\pm$ 0.04 nmol/min/mg protein at 0.2 mM) faster than the other wildtype enzymes (2–22-fold) and produced a mixture of 1-naphthol (52%) and 2-naphthol (48%) from naphthalene, which was successively transformed to a mixture of 2,3-, 2,7-, 1,7-, and 2,6-dihydroxynaphthalenes (7%, 10%, 20%, and 63%, respectively). TOM and ToMO made 1,7-dihydroxynaphthalene from 1-naphthol, and ToMO made a mixture of 2,3-, 2,6-, 2,7-, and 1,7-dihydroxynaphthalene (26%, 22%, 1%, and 44%, respectively) from 2-naphthol. TOM had no activity on 2-naphthol, and T4MO had no activity on 1-naphthol. To take advantage of the high activity of wildtype T4MO but to increase its regiospecificity on naphthalene, seven engineered enzymes containing mutations in T4MO alpha hydroxylase TmoA were examined; the selectivity for 2-naphthol by T4MO I100A, I100S, and I100G was enhanced to 88–95%, and the selectivity for 1-naphthol was enhanced to 87% and 99% by T4MO I100L and G103S/A107G, respectively, while high oxidation rates were maintained except for G103S/A107G. Therefore, the regiospecificity for naphthalene oxidation was altered to practically pure 1-naphthol or 2-naphthol. All four wildtype monooxygenases were able to oxidize fluorene to different monohydroxylated products; T4MO oxidized fluorene successively to 3-hydroxyfluorene and 3,6-dihydroxyfluorene, which was confirmed by gas chromatography – mass spectrometry and ^{1}H nuclear magnetic resonance analysis. TOM and its variant TomA3 V106A oxidize fluorene to a mixture of 1-, 2-, 3-, and 4-hydroxyfluorene. This is the first report of using enzymes to synthesize 1-, 3-, and 4-hydroxyfluorene, and 3,6-dihydroxyfluorene from fluorene as well as 2-naphthol and 2,6-dihydroxynaphthalene from naphthalene. © 2005 Wiley Periodicals Inc.

Keywords: naphthalene; fluorene; toluene monooxygenase; regiospecific oxidation

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) consist of two or more fused aromatic rings in linear, angular, or cluster arrangements (Cheung and Kinkle, 2001). They are commonly found in crude oil and coal tar, and are one of the largest groups of priority environmental pollutants because of their toxic, mutagenic, and carcinogenic properties (Finkelstein et al., 2003). Microbial metabolism and biodegradation of PAHs such as naphthalene, phenanthrene, and anthracene have been described previously (Cerniglia, 1984; Evans et al., 1965; Finkelstein et al., 2003; Grifoll et al., 1992; Guerin and Zones, 1988; Janikowski et al., 2002; Nojiri et al., 1999; Schocken and Gibson, 1984; Shindo et al., 2001; Yamazoe et al., 2004). These studies with PAHs may be of importance in developing remediation technologies that address environmental pollution by these chemicals. In addition, these enzymes may provide alternatives for the synthesis of commercially valuable intermediates from PAHs (Bestetti et al., 1995; Resnick and Gibson, 1996; Selifonov et al., 1996; Sello et al., 2004) such as *cis*-diol enantiomers from fluorene, dibenzofuran, and dibenzothiophene (Resnick and Gibson, 1996).

Naphthalene and fluorene (Fig. 1) serve as model bi- and tricyclic aromatic hydrocarbons for understanding the properties of a large class of PAHs (Casellas et al., 1997). Phenanthrene has been often used as a model

Correspondence to: T.K. Wood

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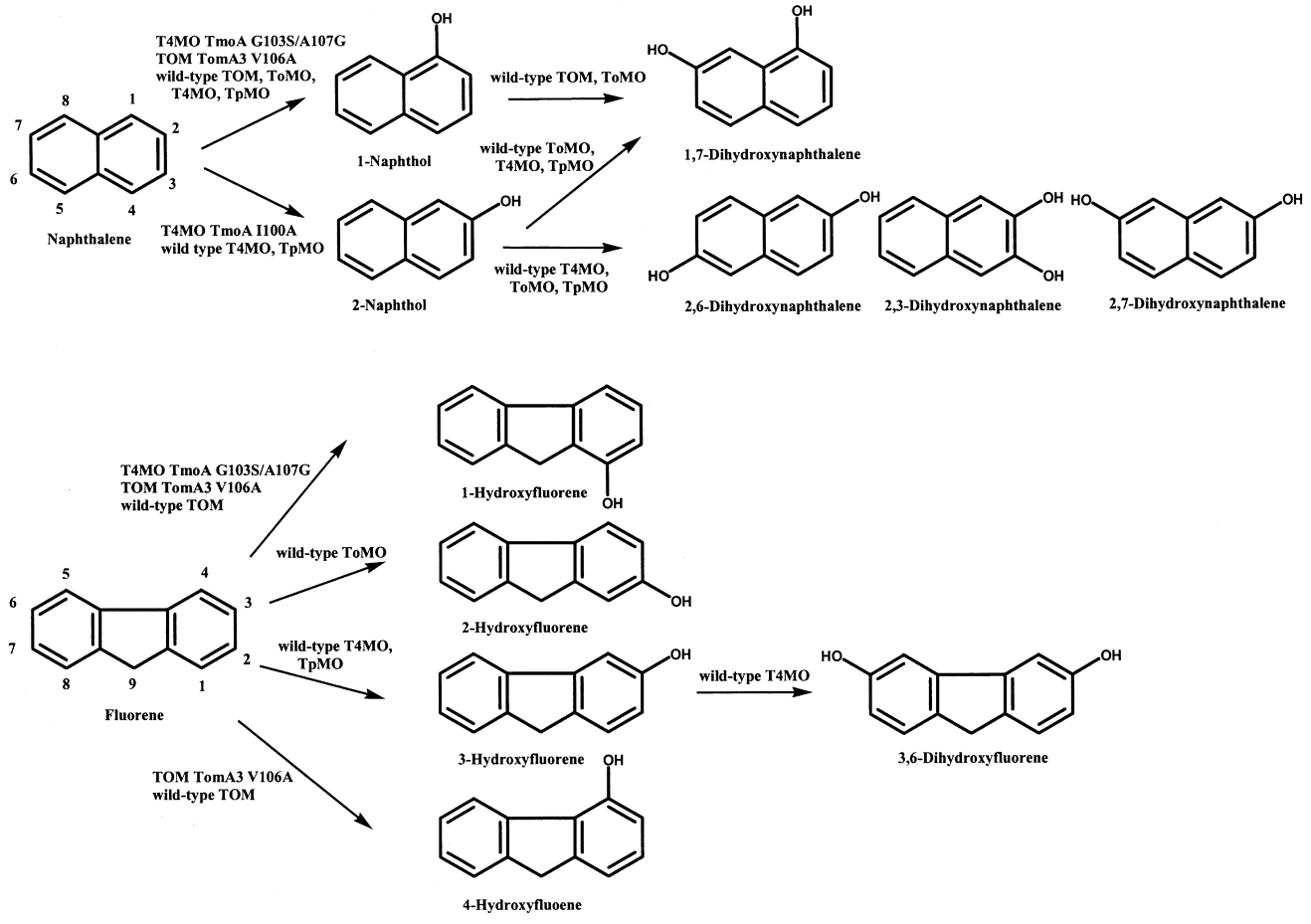

Figure 1. Pathway of naphthalene and fluorene oxidation by wildtype toluene monooxygenases, TOM variant TomA3 V106A, and T4MO TmoA variants.

substance for microbial metabolism of 3-ring PAHs due to its noncarcinogenicity and its abundance in coal tar (Laor et al., 1999; Prabhu and Phale, 2003). In addition, singly and doubly hydroxylated products of naphthalene are widely used in the manufacture of dyes, drugs, perfumes, insecticides, and surfactants such as magnesium methonaphthone (a choleretic) from 1-naphthol (Howe-Grant, 1991). Manufacture of these hydroxylated compounds by chemical routes is hampered by toxic reagents such as naphthalene-1-sulfonic acid, which is combustible, high concentrations of acids and bases such as hydrogen fluoride, and extreme temperature conditions (Howe-Grant, 1991); for example, 98% 2-naphthol is synthesized by treating naphthalene with 90% hydrogen peroxide in fluorosulfuric acid-sulfuryl chloride fluoride at –60 to –78°C (Olah et al., 1991). There are no previous reports for making 2-naphthol with microorganisms, while the Western world demand for 2-naphthol was three times more than that for 1-naphthol (~ 50,000 t/yr vs. 15,000t/yr) (Howe-Grant, 1991). It was previously reported by us that toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4 produces 98% 1-naphthol from naphthalene and its variant TomA3 V106A made 1-naphthol (98%) 6-fold faster than the wildtype TOM (Canada et al., 2002). England et al. (1998) reported that the oxidation of naphthalene to 1-naphthol (97%) was increased 100-fold by a cytochrome $P450_{cam}$ mutant Y96F. Hence, there is great potential for expanding the substrate range of oxygenases. Furthermore, these oxygenases may be used to mimic mammalian metabolism to screen potential pharmaceutical drugs; hence, they may have use in fate and toxicity studies (Smith et al., 1976; Smith and Posazza, 1974).

In recent years, the toluene monooxygenases including toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1 (Whited and Gibson, 1991; Yen and Karl, 1992; Yen et al., 1991), TOM of *B. cepacia* G4 (Newman and Wackett, 1995; Shields et al., 1989), toluene/*o*-xylene monooxygenase (ToMO) of *P. stutzeri* OX1 (Bertoni et al., 1998; Cafaro et al., 2002), and toluene *para*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1 (Fishman et al., 2004b; Olsen et al., 1994) have been studied extensively due to their potential in degrading environmental pollutants (Chauhan et al., 1998; Folsom and Chapman, 1991; McClay et al., 1996; Ryoo et al., 2000; Shim et al., 2001; Sun and Wood, 1996). They are soluble, nonheme, O_2-dependent monooxygenases with multiple components, including a hydroxylase subunit that contains the catalytically active diiron cofactor (Leahy et al., 2003). These

wildtype toluene monooxygenases and their mutants formed by rational and random mutagenesis have broad substrate ranges, not only oxidizing toluene and xylenes but also nitro-, chloro-, and methoxy-substituted benzenes and phenols to the corresponding monohydroxylated products (Fishman et al., 2004b; Pikus et al., 1997; Rui et al., 2004) or dihydroxylated products (Fishman et al., 2004a; Tao et al., 2004a,b; Vardar et al., 2005; Vardar and Wood, 2004); yet these studies have addressed primarily the oxidation of single-ring aromatics. The substrates naphthalene, anthrone, anthene, anthone, flavonone, flavone, and anthracene were tested for purified wildtype T4MO enzyme but the products were not determined explicitly (Oppenheim et al., 2001). In addition, the analogous substrate fluorene has not been characterized.

Here, we compare the naphthalene and fluorene reaction rates and regiospecificities of whole cells of *Escherichia coli* expressing four wildtype toluene monooxygenases. In addition, eight T4MO and TOM mutant enzymes created in our laboratory were also examined with respect to the synthesis of hydroxylated aromatics including 2-naphthol. Our results indicate that T4MO is the most active enzyme in the toluene monooxygenase family and is capable of oxidizing naphthalene to 1-naphthol, 2-naphthol, and 2,6-dihydroxynaphthalene (2,6-DHN) and catalyzing fluorene to 3-hydroxyfluorene (3-HF), 2-HF, and 3,6-dihydroxyfluorene (3,6-DHF). TOM and its variant TomA3 V106A were able to produce a mixture of 1-, 2-, 3-, and 4-HF from fluorene. A few T4MO mutants were identified with significantly altered regiospecificity for producing 2-naphthol. This is the first report of synthesizing 1-HF, 3-HF, 4-HF, 2-naphthol, 2,6-DHN, and 3,6-DHF using enzymes.

MATERIALS AND METHODS

Chemicals

Naphthalene and fluorene were purchased from Fisher Scientific (Pittsburgh, PA). 1-Naphthol, 2-naphthol, 1,2-DHN, 1,3-DHN, 1,4-DHN, 1,5-DHN, 1,6-DHN, 1,7-DHN, 2,6-DHN, *o*-cresol, *m*-cresol, 2-HF, 3-HF, and 9-HF were purchased from Sigma-Aldrich (Milwaukee, WI). Toluene and *p*-cresol were obtained from Acros Organics (Morris Plains, NJ). 1-HF was purchased from ChemBridge (San Diego, CA). All materials used were of the highest purity available and were used without further purification.

Bacterial Strains and Growth Conditions

Escherichia coli TG1 (Sambrook et al., 1989) with the plasmid constructs was routinely cultivated at 37°C with shaking at 250 rpm on a C25 incubator shaker (New Brunswick Scientific, Edison, NJ) in Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with kanamycin at 100 μg/mL to maintain the plasmids. All under the control of a constitutive *lac* promoter, *E. coli* TG1/pBS(Kan)T4MO contains the T4MO genes *tmoABCDEF* from *P. mendocina* KR1 (Tao et al., 2004b), *E. coli* TG1/pBS(Kan)TpMO contains the TpMO genes *tbuA1UBVA2C* from *R. pickettii* PKO1 (Fishman et al., 2004b), *E. coli* TG1/pBS(Kan)ToMO contains the ToMO genes *touABCDEF* from *P. stutzeri* OX1 (Vardar and Wood, 2004), and *E. coli* TG1/pBS(Kan)TOM contains the TOM genes *tomA012345* from *B. cepacia* G4 (Canada et al., 2002; Rui et al., 2004). *E. coli* TG1/pBS(Kan)TOM V106A contains the mutant enzyme with a single amino acid change in the α subunit TomA3 V106A relative to the wildtype enzyme (Canada et al., 2002; Rui et al., 2004). *E. coli* TG1/pBS(Kan)T4MO I100A, I100S, I100G, I100L, G103A, G103S/A107G, and G103S are mutant strains derived from *E. coli* TG1/pBS(Kan)T4MO (Tao et al., 2004b) which contain single or double amino acid changes in the α subunit TmoA at positions I100, G103, and A107 as described in the strain name (Fishman et al., 2004a; Tao et al., 2004a).

Enzyme Activity and Expression

All experiments were conducted by diluting overnight cells to an optical density at 600 nm (OD) of 0.1–0.2 and growing to an OD of 1.4. The exponentially grown cells were centrifuged at 13,000*g* for 5 min at 25°C in a Beckman J2-HS centrifuge (Palo Alto, CA) and resuspended in Tris-HNO_3 buffer (50 mM, pH 7.4). Two mL of exponentially grown cells (OD 2-10) was sealed with various aromatic substrates (toluene, naphthalene, naphthols, or fluorene) in 15-mL serum vials with a Teflon-coated septum and aluminum crimp seal. The vials were shaken at 37°C (except for toluene, where the reaction was performed at room temperature) and 300 rpm on a IKA-260KS shaker (IKA-Works, Wilmington, NC). Toluene was dissolved in ethyl acetate (0.5 M) and fluorene, the naphthols, and naphthalene were dissolved in dimethyl formamide (0.1, 0.5, and 2.5 M, respectively); 0.25 vol% ethyl acetate or dimethyl formamide was used as the diluents. Ethyl acetate was found to inhibit wildtype T4MO with naphthalene as a substrate; with 0.25 vol% ethyl acetate, there was a 0.4-fold decrease in the oxygenase rates. For dimethyl formamide, there was a 6% decrease in the rates with 0.25 vol% dimethyl formamide; therefore, dimethyl formamide (0.25 vol%) did not inhibit the cells during the oxidation of naphthalene and fluorene (Table I). Since there is substantial inhibition by ethyl acetate, the rates for toluene oxidation should be higher than the values reported in Tables I and III; however, the same amount of ethyl acetate was used for all the enzymes in Tables I and III, so the toluene oxidation rates for the different enzymes should still be comparable.

Using a 10 μL syringe, 300 μM toluene in ethyl acetate was added to the 15-mL vials (actual initial liquid concentration was 109 μM based on Henry's Law constant of 0.27) (Dolfing et al., 1993). The reaction was stopped after 0–15 min by adding 2 mL of 500 μM hexadecane (the

Table I. Aromatic oxidation rate and regiospecificity by *E. coli* TGI cells expressing wildtype T4MO, ToMO, TOM, TOM TomA3V106A, and T4MO TmoA variants.

	Toluene oxidation[a]				Naphthalene oxidation[b]			Fluorene oxidation[c]				
		Regiospecificity (%)				Regiospecificity (%)			Regiospecificity (%)			
Enzyme	Rate, nmol/min/mg pro	*o*-cresol	*m*-cresol	*p*-cresol	Rate, nmol/min/mg pro	1-naphthol	2-naphthol	Rate, nmol/min/mg pro	1-HF	2-HF	3-HF	4-HF
Wildtype TOM	2.5 ± 0.1	>98	0	0	0.9 ± 0.2	99	<1	0.10 ± 0.01	12	26	21	41
TOM TmoA3 V106A	4.8 ± 1.1	50	33	17	3.4 ± 0.5	99	<1	0.17 ± 0.02	33	7	8	51
Wildtype ToMO	6.1 ± 0.1	32	21	47	4.2 ± 2.3	90	10	0.03 ± 0.01	4	29[d]	67[d]	0
Wildtype TpMO	4.1 ± 0.2	0	10	90	0.9 ± 0.2	63	37	0.21 ± 0.01	0	14[d]	86[d]	0
Wildtype T4MO	12.1 ± 0.8	<1	3	96	7.7 ± 1.5	52	48	0.68 ± 0.04	0	14[d]	86[d]	0
TmoA I100A	19.1 ± 0.8	0	20	80	7.0 ± 1.5	8	92	0.57 ± 0.03	0	16[d]	84[d]	0
TmoA I100S	22.7 ± 1.6	0	20	80	5.5 ± 1.6	5	95	0.49 ± 0.13	0	17[d]	83[d]	0
TmoA I100G	15.1 ± 2.3	0	13	87	7.6 ± 0.5	12	88	0.57 ± 0.11	0	17[d]	83[d]	0
TmoA I100L	17.7 ± 0.2	7	3	90	3.2 ± 1.5	83	17	0.38 ± 0.18	0	13[d]	87[d]	0
TmoA G103S/A107G	1.5 ± 0.3	82	7	11	0.5 ± 0.1	99	<1	0.15 ± 0.04	28	18	53	0
TmoA G103A	20.1 ± 0.4	12	13	75	5.6 ± 1.5	81	19	0.38 ± 0.04	0	22[d]	78[d]	0
TmoA G103S	18.1 ± 1.7	9	15	76	5.9 ± 0.1	83	17	0.23 ± 0.02	0	14[d]	86[d]	0

[a]Toluene oxidation rate and regiospecificity determined via GC with 109 μM toluene calculated based on Henry's law.
[b]Total naphthol formation rate and regiospecificity determined via HPLC with 5 mM naphthalene (solubility is 0.23 mM in water).
[c]Fluorene oxidation rate determined via HPLC and regiospecificity determined via GC except for TOM and TOM TomA3V106A where regiospecificity was determined via GC-MS. Activity determined at the saturation concentration of 0.2 mM so the initial rate values represents V_{max}.
[d]Regiospecificity confirmed by HPLC with a supelcosil-ABZ alky-amide column.

internal GC standard) in ethyl acetate to the vial with a syringe, and the vial was vortexed thoroughly to ensure full extraction of the toluene (96% efficiency). The extract was centrifuged and analyzed by gas chromatography (GC). For the naphthalene and naphthol substrates, 5,000 μM naphthalene, 500 μM 1-naphthol, or 500 μM 2-naphthol was added to the reaction vials. The reaction was stopped after 0–30 min by centrifugation. The supernatant was analyzed by high-pressure liquid chromatography (HPLC). For fluorene, a concentration of 200 μM was added to the reaction vials and the reaction was stopped and extracted after 0–150 min by adding 2 mL ethyl acetate to the vial with a syringe. The vial was vortexed thoroughly to ensure full extraction of the fluorene (99% efficiency). The extract was analyzed by HPLC for oxidation rate and by GC for the monohydroxylation regiospecificity except for wildtype TOM and TomA3 V106A, where GC-mass spectrometry (MS) analysis was used to determine the regiospecificity. All rates were determined from linear regions of concentration vs. time graphs and are reported in the form of the mean ± 1 SD (based on at least two independent results). For regiospecificities, the values were verified by at least two independent experiments except for wildtype TOM.

For determining the apparent V_{max} and K_m for toluene oxidation by the wildtype TOM and TOM TomA3 V106A, exponentially grown cells were centrifuged at 13,000*g* for 5 min at 25°C, washed once with phosphate buffer (50 mM, pH 7.4), resuspended in the same buffer, and contacted at 25°C with 2 mL of cells (OD 6-10) in sealed vials with 17–232 μM toluene.

The fluorene samples for GC-MS and ^{1}H nuclear magnetic resonance spectroscopy (NMR) were prepared by incubating 20 mL cells (OD 10) with 300 μM fluorene or 300 μM 3-HF for 20–60 h. Cells were removed by centrifugation and compounds in the supernatant were extracted by 20 mL ethyl acetate once. The extracts were dried in a nitrogen evaporator (Organomation Associates, South Berlin, MA). The dried samples were dissolved in 0.5 mL ethyl acetate or deuterated dimethyl sulfoxide (for ^{1}H NMR).

The relative protein expression levels of the different toluene monooxygenases and their variants were evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8, 12, and 15% polyacrylamide) (Sambrook et al., 1989). Cellular protein samples were prepared by growing cells under the same conditions used for determining enzyme activity.

Analytical Methods

Reverse-phase HPLC was conducted to determine the product formation rates and the regiospecificity from various substrates. A sample of the supernatant from the oxidation of naphthalene, 1-naphthol, and 2-naphthol or ethyl acetate extract from fluorene oxidation was injected by an autosampler (Waters, Milford, MA, 717 plus) and analyzed using a Zorbax SB-C8 column (Agilent Technologies, Palo Alto, CA; 5 μm, 4.6 × 250 mm) with a Waters 515 solvent delivery system coupled to a photodiode array detector (Waters 996). The injection volume was 20 μL. The elution gradient for detecting naphthalene and naphthol oxidation products was performed with H_2O (0.1% formic acid) and acetonitrile (65:35 for 0–5 min, gradient to 35:65 at 12 min, and gradient to 65:35 at 20 min) as a mobile phase at a flow

Table II. Physical properties of oxidation products from fluorene and 3HF by *E. coli* TG1 cells expressing wildtype T4MO, ToMO, and TOM variant enzyme TomA3 V106A.

Enzyme	Substrate	GC[a] R_t (min)	HPLC[b] R_t (min)	UV-visible spectrum (nm)	Major peaks in mass spectra, m/z (%)	^{1}H NMR chemical shifts (δ)[c]	Product identified
T4MO	Fluorene	31.3	37.5	257/309	182 (M^+, 100), 181 (78), 165 (11), 153 (19), 152 (44), 151 (16), 127 (6), 126 (3), 91 (4), 76 (10)	9.37 (s, [1OH]), 7.77 (d, *J* = 7.4 Hz, H-5), 7.54 (d, *J* = 7.2 Hz, H-8), 7.36 (t, *J* = 7.2, H-7), 7.35 (d, *J* = 8.0 Hz, H-1), 7.30 (td, *J* = 7.4 and 1.1 Hz, H-6), 7.23 (sd, *J* = 2.2 Hz, H-4), 6.73 (dd, *J* = 8.0 and 2.2 Hz, H-2), 3.77 (s, H-9 [2H])	3-HF
		31.5	40.5	269	ND[d]	9.47 (s, [1OH]), 7.71 (d, *J* = 7.5 Hz, H-5), 7.65 (d, *J* = 8.2 Hz, H-4), 7.48 (d, *J* = 7.4 Hz, H-8), 7.35 (t, *J* = 7.4 Hz, H-6), 7.19 (t, *J* = 7.3 Hz, H-7), 6.97 (s, H-1), 6.78 (dd, *J* = 8.2 and 2.0 Hz, H-8), 3.80 (s, H-9 [2H])	2-HF
	Fluorene, 3-HF	ND	14.2	258/317	198 (M^+, 100), 197 (62), 181 (25), 152 (8), 139 (8)	9.33 (s, [2OH]), 7.30 (d, *J* = 8.2 Hz, [2H]), 7.10 (*J* = 2.3 Hz, [2H]), 6.70 (*J* = 8.2 Hz and 2.3 Hz [2H]), 3.63 (s, H-9 [2H])	3,6-DHF
TOM TomA3 V106A	Fluorene	30.8	41.6	263	182 (M^+, 100), 181 (69), 165 (6), 153 (16), 152 (40)	ND	1-HF
		31.1	ND	ND[d]	182 (M^+, 100), 181 (52), 165 (16), 164 (17), 154 (16), 153 (27), 152 (41), 126 (6), 76 (12)	ND	4-HF
		31.3	37.6	257/309	182 (M^+, 100), 181 (67), 165 (11), 151 (18), 152 (40), 151 (16), 127 (3), 76 (10)	ND	3-HF
		31.5	40.2	269	182 (M^+, 100), 181 (64), 165 (13), 153 (22), 152 (36), 151 (13), 76 (8)	ND	2-HF
ToMO	Fluorene	31.3	38.5	257/309	ND	ND	3-HF
		31.5	41.7	269	ND	ND	2-HF
TpMO	Fluorene	31.0	37.5	257/309	ND	ND	3-HF
		31.2	40.5	269	ND	ND	2-HF

[a]GC analysis with a HP-5 column.
[b]HPLC analysis with a supelcosil-ABZ alky-amide column.
[c]Chemical shift multiplicities are abbreviated as follows: s, singlet; d, doublet; t, triplet; sd, singlet of doublets; dd, doublet of doublets; td, triplet of doublets. Coupling constants (*J* values) are given in hertz. Proton signal assignments for 2-HF were determined with nuclear overhauser effect.
[d]ND, not determined.

rate of 1 mL/min. The isocratic elution method for detecting fluorene for substrate oxidation rate determination was H_2O (0.1% formic acid) and acetonitrile (15:85). The separation of mono- and dihydroxylation products from fluorene was achieved with a supelcosil-ABZ alky amide column (Supelco, Bellefonte, PA; 5 μm, 4.6 × 150 mm). The elution gradient was performed with H_2O (0.1% formic acid) and acetonitrile (65:35 for 0–45 min, gradient to 15:85 at 52 min, and gradient to 65:35 at 60 min). Compounds were identified by comparison of retention times and UV-Vis spectra to those of authentic standards as well as by coelution with standards.

Oxidation of toluene to cresol was measured by GC with a Hewlett-Packard 6890N GC equipped with an EC-WAX capillary column (30 m × 0.25 mm, 0.25 μm thickness; Alltech Associates, Deerfield, IL) and a flame ionization detector as reported previously (Tao et al., 2004b). Oxidation of fluorene to monohydroxylation products was detected by GC with a HP-5 capillary column (30 m × 0.32 mm, 0.25 μm thickness; Agilent Technologies). The column temperature was held isothermally at 50°C for 1 min and then increased to 290°C at a rate of 5°C/min. A split ratio of 3:1 and helium as carrier gas at a constant flow rate of 0.9 mL/min were used.

The identities of hydroxylated fluorenes were confirmed by GC-MS and ^{1}H NMR analysis. GC-MS samples were analyzed with a Hewlett-Packard 5970B GC/MS instrument equipped with an HP-1 column (12 m × 0.2 mm,

0.33 μM thickness) and the ionization voltage was 70 eV. The temperature was 50°C for 1 min followed by 50–270°C at a rate of 5°C/min. ^{1}H NMR analysis was performed on a DRX-400 (400.144 MHz) instrument (Bruker BioSpin, Billerica, MA) with a standard 30° pulse and 1.5-sec relaxation delay.

RESULTS

Oxidation of Toluene, Naphthalene, and Naphthols by Wildtype Toluene Monooxygenases

A whole-cell system was used to oxidize substrates due to the multiple subunits of T4MO, TpMO, ToMO, and TOM (3-component hydroxylase, reductase, mediating protein, and ferredoxin for T4MO, TpMO, and ToMO and a relatively unknown subunit for TOM) and their dependence on the cofactor NADH. *E. coli* cells expressing four different toluene monooxygenases were characterized for their specific rates and their regiospecificity on the single ring substrate toluene and bicyclic substrate naphthalene (Table I, Fig. 1). Wildtype T4MO was the best enzyme tested in terms of rate, and the rates of toluene and naphthalene oxidation were 5–9-fold higher than those of wildtype TOM (Table I). However, it produced a mixture of 1-naphthol (52%) and 2-naphthol (42%) from naphthalene.

Previously, we reported that TOM variant TomA3 V106A showed 2–6-fold increased activity toward toluene and naphthalene relative to wildtype TOM (Canada et al., 2002; Rui et al., 2004), as was found here with a different assay for naphthalene, although absolute values were changed slightly for toluene oxidation (Table I). The apparent V_{max} and K_m for toluene oxidation were 1.7 ± 0.2 nmol/min/mg protein and 53 ± 16 μM for wildtype TOM and 3.9 ± 0.3 nmol/min/mg protein and 82 ± 14 μM for TomA3 V106A using washed cells. The washing step decreases the oxidation rates to about one-half that of unwashed cells; hence, since the initial rate data reported in Table I were obtained without washing cells, the initial toluene oxidation rates for TOM and TomA3 V106A in Table I are slightly higher than those rates used to find the apparent V_{max} and those reported previously (Canada et al., 2002; Rui et al., 2004).

In addition to naphthalene oxidation to naphthol products, these monooxygenases were able to further oxidize naphthol substrates to DHN. Wildtype TOM oxidized 1-napthhol to 1,7-DHN (Fig. 1) while no product was detected for 2-naphthol. ToMO utilized both 1-naphthol and 2-naphthol as substrates, catalyzing 1-naphthol specifically to 1,7-DHN and 2-naphthol to a mixture of 2,7-, 2,3-, 2,6-, and 1,7-DHN (1%, 26%, 22%, and 44%, respectively). Wildtype T4MO did not oxidize 1-naphthol, but 2-naphthol was converted to a mixture of 2,3-, 2,7-, 1,7-, and 2,6-DHN (7%, 10%, 20%, and 63%, respectively). Wildtype TpMO oxidized 2-naphthol to a mixture of 2,3-, 1,7-, and 2,6-DHN (29%, 34%, and 36%, respectively). No dihydroxynaphthalene products were detected from TpMO after incubating with 1-naphthol.

Oxidation of Fluorene by Wildtype Toluene Monooxygenases

Fluorene oxidation was determined at a concentration of 0.2 mM which was found to be in the saturation range for wildtype T4MO. GC analysis of ethyl acetate extracts after fluorene oxidation by *E. coli* TG1 strains expressing wildtype toluene monooxygenases revealed various monohydroxylated products (Table I, Fig. 1), which were identified by comparing the GC retention times (R_t), by coeluting with authentic standards, by GC-MS, and by ^{1}H NMR analysis (Table II).

In addition, the ^{1}H NMR spectra of the T4MO fluorene reaction mixture indicated an additional fluorene derivative was formed, DHF. HPLC analysis of the same sample indicated a more polar peak than that of monohydroxyfluorenes, and GC-MS analysis identified a peak with a molecular ion at *m/z* 198 (Table II), which is consistent with the MS spectra of dihydroxyfluorene in the literature (Finkelstein et al., 2003). GC-MS and ^{1}H NMR analysis of the reaction mixture following 3-HF (the major product of fluorene oxidation) oxidation by T4MO revealed the same MS and NMR spectra as that of fluorene oxidation by T4MO (Table II). The singlet for the two hydroxyl groups on the benzene rings (δ = 9.32) and 4 of 7 proton signals identified indicated the structure of the DHF is symmetrical. Considering 3-HF as the starting substrate, this allowed the position of one hydroxyl group of the DHF compound to be identified at the C3 atom and the other hydroxyl group to be identified at the symmetric C6 atom; hence, the additional product was 3,6-DHF.

TOM and its variant TOM TomA3 V106A were found to make four different products based on GC analysis. GC-MS analysis of the TomA3 V106A samples revealed that they are four monohydroxylation isomers with a molecular ion at *m/z* 182 (Table II). 1-HF, 2-HF, and 3-HF were identified as three products by comparing GC R_t and by coeluting with authentic standards. Since the GC R_t did not match with authentic 9-HF, most likely the other monohydroxylation isomer was 4-HF. However, this assignment conflicts with another report where a compound tentatively identified as 4-HF (based on MS data) eluted earlier than 1-HF using similar GC conditions (Selifonov et al., 1996). Here, 4-HF produced by TomA3 V106A eluted after 1-HF (Table II).

Oxidation of Naphthalene, Fluorene, and 2-Naphthol by *E. coli* Strains Expressing T4MO Mutant Enzymes

The best mutants (T4MO I100A, I100S, I100G, I100L, G103A, G103S, and G103S/A107G) initially identified by

Table III. Apparent V_{max} and K_m values of wildtype T4MO and T4MO TmoA variant I100A.[a]

Enzyme	Toluene oxidation[b]			Naphthalene oxidation[c]			2-Naphthol oxidation[c]		
	V_{max}, nmol/min/mg pro	K_m, μM	V_{max}/K_m	V_{max}, nmol/min/mg pro	K_m, μM	V_{max}/K_m	V_{max}, nmol/min/mg pro	K_m, μM	V_{max}/K_m
Wildtype T4MO	26 ± 0.9	30 ± 2.5	0.88	15 ± 5	49 ± 8	0.30	9.6 ± 2	31 ± 9	0.31
I100A	37 ± 8	63 ± 17	0.59	9 ± 1	26 ± 8	0.35	15 ± 2	58 ± 5	0.27

[a]Cell OD was 2, and kinetic constants were calculated from a double reciprocal Lineweaver-Burk plot.

[b]Initial specific rates were determined by monitoring toluene oxidation rates using GC; toluene concentrations in the liquid phase are 20, 56, 96, 148, 360, and 725 μM based on Henry's law constant of 0.27 (Dolfing et al., 1993) (53, 154, 263, 408, 993, and 2,000 μM added if all the toluene is in the liquid phase). Initial rates were determined at every 2 min over 15 min, thus the initial rates were higher than those in the earlier study (Fishman et al., 2004b) leading to a higher V_{max} and smaller K_m for wildtype T4MO.

[c]Initial specific rates were determined by monitoring all product formation rates using HPLC; naphthalene concentrations in the reaction mixtures are 10, 20, 50, 100, 250, 500, and 5,000 μM, and 2-naphthol concentrations are 20, 50, 100, 150, 250, 500, and 1,000 μM.

screening a saturation mutagenesis library for substituted catechol synthesis (Fishman et al., 2004a; Tao et al., 2004a) were further examined for 1-naphthol and 2-naphthol production with 5 mM naphthalene (naphthalene solubility is 0.23 mM in water [Perry and Chilton, 1973]). From 5 mM naphthalene, four T4MO TmoA mutants (TmoA I100L, G103A, G103S, and G103S/A107G) had elevated 1-naphthol synthesis compared to wildtype T4MO (81–99% vs. 52%) as shown in Table I. Interestingly, T4MO variant G103S/A107G produced 1-naphthol as pure as wildtype TOM, but at half the wildtype TOM rate. Notably, three T4MO TmoA mutants (TmoA I100G, I100A, and I100S) were found to produce primarily 2-naphthol (88–95%). All three variants had the same naphthalene oxidation rates as wildtype T4MO.

For fluorene oxidation, most of the T4MO mutants demonstrated conserved regiospecificity (Table I). However, TmoA G103S/A107G produced 28% 1-HF, which was not made by wildtype T4MO and the other T4MO variants (Fig. 1). Using the other toluene monooxygenases, only TOM and TOM variant TomA3 V106A were found to make significant amounts of 1-HF (Table I). Therefore, T4MO variant TmoA G103S/A107G behaved more like TOM for its regiospecificity reaction with toluene, naphthalene, and fluorene oxidation.

The regiospecificity of 2-naphthol oxidation by these T4MO mutant enzymes was also examined (wildtype T4MO produces 7% 2,3-DHN, 10% 2,7-DHN, 20% 1,7-DHN, and 63% 2,6-DHN). T4MO TmoA variant I100A made a mixture 8% 2,3-DHN, 18% 2,7-DHN, 5% 1,7-DHN, and 71% of 2,6-DHN, and T4MO TmoA variants I100S, G103S/A107G, G103A, and G103S made 45–49% 2,6-DHN. Since TmoA variant I100A was more specific for both 2,6-DHN and 2-naphthol, its maximum reaction rate for I100A for substrate toluene, naphthalene, and 2-naphthol was measured (Table III). Variant I100A followed saturation kinetics with all the substrates tested as did wildtype T4MO. I100A showed a 42–59% increase in the apparent V_{max} towards toluene and 2-naphthol, while it showed 60% of the wildtype apparent V_{max} towards naphthalene. This mutant also demonstrated 88–114% increased K_m towards toluene and 2-naphthol and 49% decreased K_m towards naphthalene. The kinetic data indicate the clone I100A could be very useful for synthesizing 2-naphthol from naphthalene and 2,6-DHN from 2-naphthol.

Protein Expression

To verify that the changes in activity and regiospecificity of the TomA3 V106A and the TmoA mutants derive from amino acid substitutions rather than from nonuniform protein expression, SDS-PAGE was used to visualize three of the six subunits: α-hydroxylase (54.4 kDa), β-hydroxylase (37.7 kDa), and reductase (40 kDa) for TOM and TOM variant TomA3 V106A; and α-hydroxylase (55–57.6 kDa) along with a combined band from β-hydroxylase (35–37.5 kDa) and reductase (36–38 kDa) for TpMO, ToMO, T4MO, and the T4MO variants. The bands of the wildtype enzymes and mutants in Table I had comparable intensities except for T4MO variant G103S/A107G, which had one-third the expression of wildtype T4MO. Taking into account the lower expression level of the G103S/A107G variant, this mutant showed significant activity with toluene and the other substrates (Table I).

DISCUSSION

Oppenheim et al. (2001) reported purified, wildtype T4MO enzyme did not oxidize a number of 3-ring aromatics (e.g., anthrone, anthene) in aqueous buffer. However, monohydroxylation was identified using a perfluorohexane/buffer system and LC-MS while the positions of monohydroxylation were not determined. Regiospecific oxidation of the 3-ring substrate fluorene has not been characterized previously for T4MO, TpMO, and ToMO. TOM and its variant TomA3 V106A (TOM-Green) expressed in *E. coli* were reported by us to oxidize phenanthraene, fluorene, and anthracene based on a colorimetric assay but the products were not identified (Canada et al., 2002). Here we sought to

elucidate the reactivity of 3-ring aromatics in water and to characterize the regiospecificity of toluene monooxygenase family of enzymes.

By using identical conditions (promoter, vector, monooxygenase expression, and reaction conditions) in the absence of interfering host reactions, the rates and regioselective oxidation of these substrates were compared. All four wildtype toluene monooxygenases (T4MO, TpMO, ToMO, and TOM) were able to oxidize toluene, naphthalene, and fluorene in aqueous buffer to a number of hydroxylated products. It is noted that there is no absolute pattern of regiospecificity for these four wildtype toluene monooxygenases. Wildtype T4MO was regiospecific for toluene and fluorene, while it was nonspecific for naphthalene attacking both 1- and 2-position of naphthalene. Wildtype ToMO is known as a relaxed enzyme for toluene, while it showed high regiospecific for naphthalene and remained relaxed for fluorene, and wildtype TOM was a regioselective enzyme for both toluene and naphthalene, while it produced as many as four monohydroxylation isomers from fluorene. However, the relative rates were consistent: T4MO was the most active enzyme for the 1, 2, and 3-ring aromatic substrates. Also, as the number of rings increased, the oxidation rate (apparent V_{max}) decreased markedly; for example, the naphthalene oxidation rate for wildtype T4MO was 57% that of the toluene oxidation rate (Table III) and the fluorene oxidation was only 3% of the toluene oxidation rate (Table I). The solubility difference and bioavailability partially explain the lower oxidation rates.

Bacterial oxygenation of fluorene has been classified into three different types of reactions (Yamazoe et al., 2004): lateral dioxygenation by oxidizing one of the aromatic rings to form dihydrodiol, angular dioxygenation, and five-membered ring monooxygenation by oxidizing the C9 atom to form 9-hydroxyfluorene. 1,2- and 3,4-Dihydroxyfluorene were proposed to be the intermediates of fluorene degradation by *Arthrobacter* sp. strain F101; however, they were not isolated and have not been identified (Casellas et al., 1997; Grifoll et al., 1992). Monohydroxylation at the C9 position of fluorene and lateral dihydroxylation were also reported for naphthalene dioxygenase using strain *P. aeruginosa* PAO1(pRE695) (Selifonov et al., 1996) and for carbazole 1,9a-dioxygenase from *Pseudomonas* sp. strain CA10 (Nojiri et al., 1999) in which the putative *cis*-dihydrodiols were dehydrated to 1-HF, 2-HF, 3-HF, and 4-HF (phenolic compounds) due to a nonenzymatic reaction (Nojiri et al., 1999; Selifonov et al., 1996; Shindo et al., 2001). There is only one report of monohydroxylation of fluorene at the C2 position (2-HF) (Finkelstein et al., 2003), whereas there are many at the C9 position (Casellas et al., 1997; Grifoll et al., 1992; Nojiri et al., 1999; Selifonov et al., 1996; Yamazoe et al., 2004). Here we found that T4MO oxidizes fluorene to a major product identified as 3-HF, which can be further oxidized to 3,6-DHF. In addition, we found TOM and its variant TomA3 V106A make 1- and 4-HF as the main products. The 1-HF, 3-HF, 4-HF, and 3,6-DHF products from fluorene by these toluene monooxygenases were not previously seen in the literature using enzymes, which expands the product spectrum and the pathway of fluorene oxidation. In addition, Finkelstein et al. (2003) identified 2-HF and 2,7-DHF as hydroxylation products from fluorene with *Rhodococcus* strains. Hence, all reported hydroxylations of fluorene have been found to produce a symmetrical molecule (3,6-DHF and 2,7-DHF).

Considering the high oxidation rate of wildtype T4MO and its high regiospecificity for monocyclic substrates, one of the goals of this study was to increase T4MO regiospecificity for 1- or 2-naphthol synthesis while maintaining the high rates using mutagenesis. Three T4MO mutants containing the mutations I100A, I100S, or I100G were determined to be 2-naphthol producing enzymes. The reason that this single mutation at position I100 tuned this enzyme beneficially for 2-naphthol synthesis (as much as 95%) is not understood. However, this is the first report of 2-naphthol formation during the microbial transformation of naphthalene. The results of the present study clearly show the capacity of toluene monooxygenases for regiospecific PAH oxidation and the potential of these enzymes for industrially significant singly or doubly hydroxylated products (like 2-naphthol, 2,6-DHN, 3-HF, and 3,6-DHF). For example, 2,6-DHN, which is made by enzymes for the first time here, is used for the manufacture of aromatic polyesters with liquid crystal characteristics (Howe-Grant, 1991).

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