JOURNAL OF BACTERIOLOGY, Feb. 2005, p. 1511–1514 Vol. 187, No. 4
0021-9193/05/$08.00+0 doi:10.1128/JB.187.4.1511–1514.2005

NOTES

Alpha-Subunit Positions Methionine 180 and Glutamate 214 of *Pseudomonas stutzeri* OX1 Toluene-*o*-Xylene Monooxygenase Influence Catalysis

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Received 3 November 2004/Accepted 17 November 2004

Alpha-subunit position M180 of toluene-*o*-xylene monooxygenase influences the regiospecific oxidation of aromatics (e.g., from *o*-cresol, M180H forms 3-methylcatechol, methylhydroquinone, and 4-methylresorcinol, whereas the wild type forms only 3-methylcatechol). Position E214 influences the rate of reaction (e.g., E214G increases *p*-nitrophenol oxidation 15-fold) by controlling substrate entrance and product efflux as a gate residue.

Toluene monooxygenases (1, 5, 7, 15, 21) are multisubunit catalysts that oxidize benzene to phenol, catechol, and trihydroxybenzene (18, 20) and may be engineered to produce a range of methyl-, nitro-, and methoxy-substituted aromatics with industrial and pharmaceutical value (2, 3, 11, 16, 17, 19, 20). Structure-function relationships are beginning to be understood to the extent that it is now possible to hydroxylate the benzene ring of toluene at all possible positions (4). Through DNA shuffling of these oxygenases, alpha-subunit positions I100 (2), A107 (11), E214G/D312N/M399V (19), and M180T/E284G (19, 20) have been identified which influence catalysis. Here, saturation mutagenesis of the alpha-subunit (TouA) of toluene-*o*-xylene monooxygenase (ToMO) showed that TouA M180 influences the regiospecificity of hydroxylation of substituted aromatics and TouA E214 influences the catalysis rate. By substituting glycine, alanine, valine, glutamine, phenylalanine, and tryptophan at TouA position 214, it was found this residue acts as a gate.

Escherichia coli strain TG1 (12) was used to express ToMO from pBS(Kan)ToMO by using exponentially growing cells (20). Chemicals were from Fisher Scientific Co. (Fairlawn, N.J.), Sigma Chemical Co. (St. Louis, Mo.), Acros Organics (Morris Plains, N.J.), Frinton Labs (Vineland, N.J.), Vitas-M (Moscow, Russia), and Apin Chemicals (Abingdon, United Kingdom).

Our approach was to clone *touABCDEF* encoding ToMO into a stable *E. coli* plasmid and express this monooxygenase in a host where the substrates are not oxidized by background oxygenases. Saturation mutagenesis of TouA positions M180 and E214 and site-directed mutagenesis to substitute alanine, valine, and tryptophan at TouA E214 were performed as described previously (19, 20); for saturation mutagenesis, 500 colonies were screened on *o*-cresol, toluene, nitrobenzene, and *p*-nitrophenol to ensure with a 99.96% probability that all 64 codons were screened (10) using a colony-based method that detected altered dihydroxy product formation (19, 20). The interesting variants were examined further with various substrates (Tables 1, 2, and 3) by reverse-phase high-performance liquid chromatography and by gas chromatography as described previously (19, 20).

TouA position M180 (Fig. 1) lies ~8 Å away from the diiron center (6, 8, 14), and most of the TouA M180 mutants gave a shift in the product distribution; for example, from *o*-cresol, variants M180S, M180Q, and M180H formed 3-methylcatechol (59, 63, and 50%, respectively), methylhydroquinone (37, 27, and 43%, respectively), and 4-methylresorcinol (4, 10, and 7%, respectively), whereas wild-type ToMO formed only 3-methylcatechol (100%) (Table 1). This indicates that Tou M180 influences the regiospecificity of oxidation. Most of the enzymes were as active as the wild type on the natural substrate toluene, and some of the M180 variants altered the regiospecificity of its oxidation, too (Table 3). For the related enzyme T4MO, the alpha-subunit (TmoA) variant I180F was studied previously, but no regiospecific changes were observed during toluene, *p*-xylene, and *m*-xylene oxidation (9).

None of the E214 (Fig. 1) variants generated by saturation mutagenesis had altered regiospecificities for the substrates *o*-cresol, *m*-cresol, *p*-cresol, phenol, naphthalene, *o*-nitrophenol, *m*-nitrophenol, *p*-nitrophenol, and nitrobenzene; hence, position E214 does not influence the regiospecific hydroxylation of substituted aromatics. However, variant E214G oxidizes *p*-nitrophenol 15 times better than wild-type ToMO (Table 2). Since the rates of oxidation by variant E214G for *o*-nitrophenol, *p*-nitrophenol (Table 2), *m*-nitrophenol (0.56 versus 0.53 nmol of 4-nitrocatechol formed/min/mg of protein), and nitrobenzene (0.83 versus 0.78 nmol of nitrophenols formed/min/mg of protein) are similar to those of the variant

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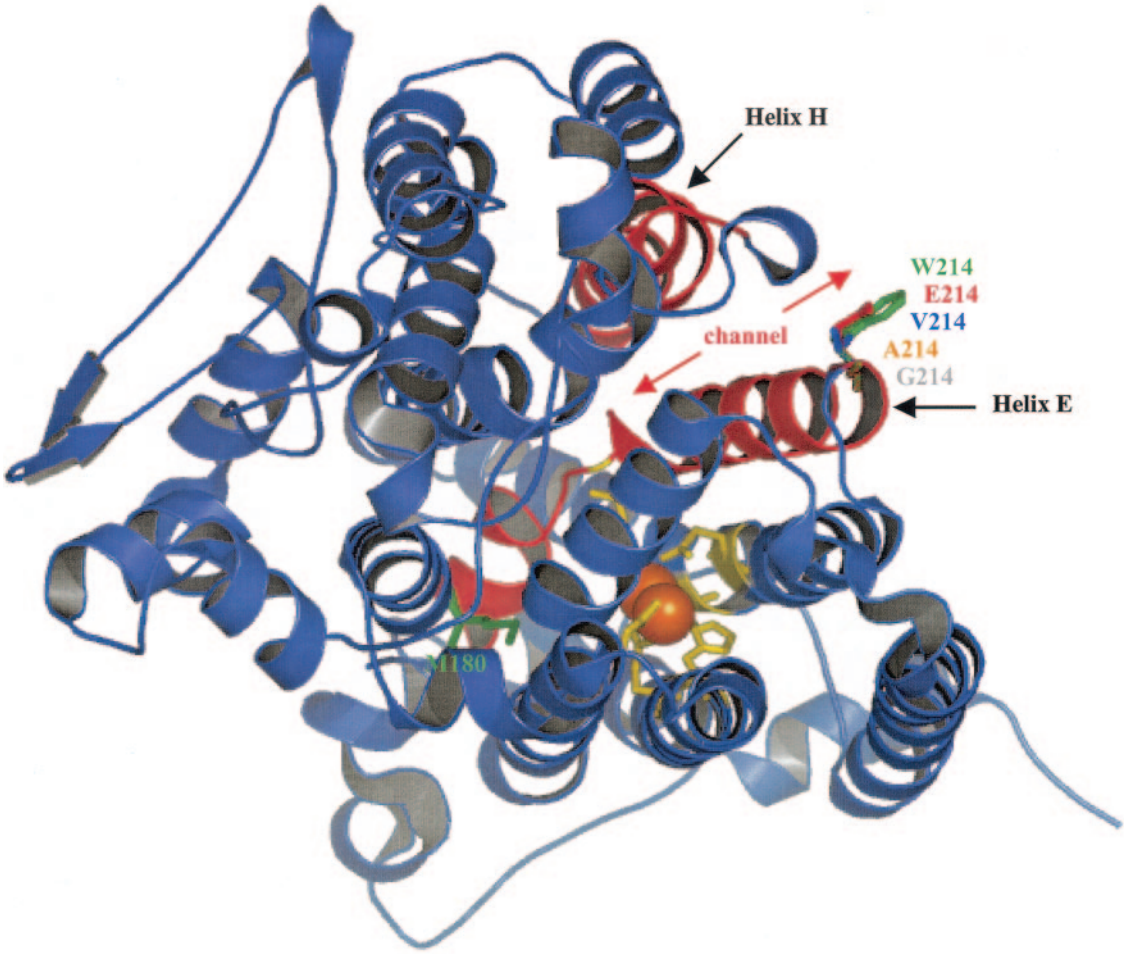

FIG. 1. Catalytic residues M180 and E214 of the hydroxylase alpha-subunit (TouA) of ToMO. The C-terminal loop of helix E (red) and the N-terminal loop of helix H (red) form a channel opening at the surface of TouA. Side chains shown in yellow are the metal-binding residues forming the diiron center (TouA E104, E134, H137, E197, E231, and H234). TouA M180 is green, and TouA E214 is red. The wild-type ToMO hydroxylase (Protein Data Bank accession code 1t0q [13]) was visualized using the Swiss-Pdb Viewer program (DeepView) (6, 8, 14), and the final figure is from Pymol. TouA E214 forms the entrance of the channel and is ~23 Å away from the active site, whereas TouA M180 is much closer to the active site (~8 Å). Mutations at E214 that show this residue controls the channel are indicated (E214A, E214G, E214V, and E214W). Also, variants E214F, E214Q, and E214P were created but are not shown, for clarity.

E214G/D312N/M399V, positions D312 and M399 do not appear to play an important role in catalysis and TouA E214G is responsible for the enhanced rate of oxidation of the nitroaromatics.

TouA E214 is the last residue of helix E (~23 Å away from the active site as calculated via Swiss-PDB viewer) (6, 8, 14) and forms an opening of a substrate channel at the northern end of the hydroxylase (13) (Fig. 1). Our hypothesis was that DNA shuffling identified a residue that functions as a gate and controls substrate and product flow; hence, smaller residues should speed the reaction compared to larger ones. As shown in Table 2, the rate of oxidation of *p*-nitrophenol to 4-nitrocatechol was inversely related to the size of the residue at position E214: TouA variants E214G, E214A, and E214V formed 4-nitrocatechol 15-, 4.3-, and 1.3-fold faster, respectively, than wild-type ToMO, whereas variant E214W with the bulky side chain formed 4-nitrocatechol 6.3-fold slower. Similar behavior was also observed with *o*-nitrophenol: TouA variants E214G, E214A, E214V oxidized *o*-nitrophenol and formed 3-nitrocatechol 2.1-, 1.6-, and 1.2-fold faster, respectively, than wild-type ToMO, whereas variant E214W formed 3-nitrocatechol 3-fold slower (Table 2). The activity of variants with R groups roughly equivalent in size to glutamate, E214Q and E214F, oxidized *o*-nitrophenol at the same rate (Table 2). These results confirm that position E214 is a gate amino acid that controls the rate of nitroaromatic oxidation (Fig. 1). Similarly, DNA shuffling was used to discover the hydroxylase gate residue V106A of the alpha-subunit (TomA3) of toluene *o*-monooxygenase of *Burkholderia cepacia* G4 (equivalent to ToMO position I100), which allowed greater access to the catalytic center for large substrates, such as naphthalene, phenanthrene, fluorene, and anthracene (2).

The TouA, TouE, and TouF subunit expression levels for variants M180T and E214G remained approximately the same as that of wild-type ToMO as evidenced by sodium dodecyl

TABLE 2. Enhanced rates of oxidation of nitrophenols by the TouA E214 variants[a]

Enzyme	Substrate							
	o-Nitrophenol				*p*-Nitrophenol			
	Product	Rate[b]	Relative	Mol%	Product	Rate[b]	Relative	Mol%
Wild-type ToMO	3-NC NHQ	0.12 ± 0.10 0.95 ± 0.15	1.0 1.0	12 88	4-NC	0.0082 ± 0.0002	1.0	100
E214G/D312N/M399V	3-NC NHQ	0.28 1.8	2.4 1.9	14 86	4-NC	0.164 ± 0.002	20	100
E214G	3-NC NHQ	0.25 ± 0.01 1.9 ± 0.2	2.1 2.0	12 88	4-NC	0.124 ± 0.004	15	100
E214A	3-NC NHQ	0.19 1.2	1.6 1.3	14 86	4-NC	0.034 ± 0.001	4.3	100
E214V	3-NC NHQ	0.14 0.76	1.2 0.8	16 84	4-NC	0.011 ± 0.001	1.3	100
E214W	3-NC NHQ	0.038 ± 0.003 0.21 ± 0.01	0.32 0.22	15 85	4-NC	0.0013 ± 0.0001	0.16	100
E214Q	3-NC NHQ	0.12 0.86	1.0 0.9	12 88	NM	NM	NM	NM
E214F	3-NC NHQ	0.09 0.74	0.8 0.8	12 88	NM	NM	NM	NM

[a] *o*-Nitrophenol and *p*-nitrophenol initial concentrations were 500 μM. NM, not measured; 3-NC, 3-nitrocatechol; NHQ, nitrohydroquinone; 4-NC, 4-nitrocatechol. Products were identified via HPLC.
[b] Initial rate, in nanomoles per minute per milligram of protein.

sulfate-polyacrylamide gel electrophoresis, which was conducted with and without 1 mM isopropyl-β-D-thiogalactopyranoside (Fisher Scientific Co.) (20); hence, the increase in the activity and changes in the regiospecific hydroxylation from variants M180T and E214G derive from the amino acid substitutions, not expression differences. The ribosome binding site and 80% of the alpha-subunit were sequenced for most of the variants, and there were no differences found other than at codons M180 and E214.

It is clearly shown in this paper that residues M180 and E214

TABLE 3. Toluene oxidation rate and regiospecificity by the TouA M180 and E214 variants[a]

Enzyme	Oxidation rate (nmol/min/mg of protein)	Regiospecificity (%)		
		o-Cresol	*m*-Cresol	*p*-Cresol
Wild-type ToMO	2.6	32	21	47
M180S	2.7	52	19	29
M180Q	2.3	59	15	26
M180Y	2.6	52	20	28
M180H	2.0	27	24	49
M180F	0.67	21	30	49
M180D	0.56	29	20	51
M180N	0.18	19	19	62
M180P	0.09	28	24	48
M180T	3.4	30	28	42
M180T/E284G	3.1	32	26	42
E214F	NM	39	22	39
E214Q	2.2	34	20	45
E214G	2.5	32	20	48
E214G/D312N/M399V	2.3	35	22	43

[a] Products were identified via GC. Initial toluene concentration was 91 μM based on Henry's law (250 μM if all the volatile organic was in the liquid phase). Note that E214P has negligible activity. NM, not measured.

TABLE 1. Regiospecific oxidation of various substrates by TouA M180 variants[a]

Enzyme	Product (regiospecificity, %) from substrate						
	o-Cresol	*m*-Cresol	*p*-Cresol	Phenol	Naphthalene	Catechol	Resorcinol	*o*-Xylene
Wild-type ToMO	3-MC (100)	4-MC (96), 3-MC (4)	4-MC (100)	C (100)	1-N (88), 2-N (12)	1,2,3-THB (100)	1,2,4-THB (100)	3,4-DMP (82), 2,3-DMP (18)
M180F	3-MC (88), MHQ (12)	4-MC (98), 3-MC (2)	4-MC (100)	NM	1-N (83), 2-N (17)	1,2,3-THB (100)	1,2,4-THB (100)	NM
M180Y	3-MC (89), MHQ (11)	4-MC (97), 3-MC (2), MHQ (1)	4-MC (96), 4-MR (4)	C (98), HQ (2)	1-N (87), 2-N (13)	1,2,3-THB (94), 1,2,4-THB (6)	1,2,4-THB (60), 1,2,3-THB (40)	NM
M180S	3-MC (59), MHQ (37), 4-MR (4)	4-MC (81), MHQ (13), 3-MC (6)	4-MC (100)	C (86), HQ (14)	1-N (87), 2-N (13)	1,2,3-THB (100)	1,2,4-THB (74), 1,2,3-THB (26)	3,4-DMP (85), 2,3-DMP (15)
M180D	3-MC (95), MHQ (5)	4-MC (100)	4-MC (100)	NM	1-N (88), 2-N (12)	1,2,3-THB (100)	1,2,4-THB (100)	NM
M180N	3-MC (97), MHQ (3)	4-MC (90), 3-MC (10)	4-MC (100)	C (100)	1-N (89), 2-N (11)	1,2,3-THB (100)	1,2,4-THB (100)	3,4-DMP (92), 2,3-DMP (8)
M180Q	3-MC (63), MHQ (27), 4-MR (10)	4-MC (75), MHQ (16), 3-MC (8)	4-MC (100)	C (74), HQ (24), R (2)	1-N (92), 2-N (8)	NM	1,2,4-THB (85), 1,2,3-THB (15)	3,4-DMP (69), 2,3-DMP (31)
M180T	3-MC (87), MHQ (13)	4-MC (95), 3-MC (3), MHQ (2)	4-MC (88), 4-MR (12)	C (98), HQ (2)	1-N (87), 2-N (13)	1,2,3-THB (91), 1,2,4-THB (9)	1,2,4-THB (77), 1,2,3-THB (23)	3,4-DMP (89), 2,3-DMP (11)
M180H	3-MC (50), MHQ (43), 4-MR (7)	4-MC (86), MHQ (14)	4-MC (100)	C (85), HQ (15)	1-N (86), 2-N (14)	NM	1,2,4-THB (87), 1,2,3-THB (13)	3,4-DMP (90), 2,3-DMP (10)
M180T/E284G	3-MC (85), MHQ (15)	4-MC (92), 3-MC (4), MHQ (4)	4-MC (88), 4-MR (12)	C (98), HQ (2)	1-N (88), 2-N (12)	1,2,3-THB (86), 1,2,4-THB (14)	1,2,4-THB (77), 1,2,3-THB (23)	3,4-DMP (88), 2,3-DMP (12)

[a] Products were identified by HPLC. NM, not measured; MC, methylcatechol; MHQ, methylhydroquinone; MR, methylresorcinol; C, catechol; HQ, hydroquinone; R, resorcinol; N, naphthol; THB, trihydroxybenzene; DMP, dimethylphenol.

in the alpha-subunit of ToMO affect catalytic activity. Hence, all of the known beneficial residues that influence regiospecificity for toluene monooxygenases are near the active site, and another gate residue, TouA E214, has been discovered.

This study was supported by U.S. Army Research grant DAAD19-00-1-0568.

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