Tailoring Toluene *para*-Monooxygenase of *Ralstonia pickettii* PKO1 for Regiospecific Oxidation of Aromatics Using Active Site Engineering

A. Fishman[1], Y. Tao[1], W. E. Bentley[2], and **T. K. Wood[1]**
[1]University of Connecticut, Storrs, CT [2]University of Maryland, College Park, MD

Oxygenases are promising biocatalysts for performing selective hydroxylations not accessible by chemical methods. Toluene *para*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1, formerly known as toluene 3-monooxygenase, has been shown recently by our group to perform primarily *para* hydroxylation of monosubstituted benzenes (rather than *meta*) producing 90% *p*-cresol and 10% *m*-cresol from toluene oxidation and successively transforming them into 4-methylcatechol (*J. Bacteriol.* 186:3117, 2004). Here, using protein engineering at the α– subunit of the hydroxylase, TbuA1, a double mutant I100S/G103S was constructed, capable of producing 75% *m*-cresol from toluene and 100% *m*-nitrophenol from nitrobenzene, thus exhibiting for the first time true *meta*-hydroxylation capabilities for a toluene monooxygenase. One *ortho* TbuA1 variant was created, A107G, which oxidized toluene to mainly *o*-cresol (80%), methoxybenzene to *o*-methoxyphenol (88%), and naphthalene to 97% 1-naphthol, all of which are comparable regiospecificities with toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4. A *para* TbuA1 variant, A107T, produced only *p*-cresol (>98%), *p*-methoxyphenol (>99%), and *p*-nitrophenol (>99%) from toluene, methoxybenzene, and nitrobenzene respectively, exceeding T4MO of *P. mendocina* KR1 in its complete regiospecificity. Thus, using saturation and site-specific mutagenesis at positions I100, G103, and A107 we have constructed variants of TpMO with all possible regiospecificities for ring-hydroxylation of toluene (*ortho*, *meta*, and *para* positions) and naphthalene; this is the first report of the transformation of a single enzyme into all possible regiospecific variants. Furthermore, we have found that these positions influence regiospecific changes in hydroxylation of substituted phenols. Five previously-uncharacterized wild-type TpMO substrates *o*-cresol, *m*-cresol, *p*-cresol, guaiacol, and *m*-nitrophenol were identified, and TpMO TbuA1 variants I100S, G103S, I100S/G103S, A107G, and A107T were identified which produce four novel, industrially-significant products (methylhydroquinone, methoxyhydroquinone, 4-methylcatechol, and 4-nitrocatechol).

Key words: regiospecificity, toluene *para*-monooxygenase, protein engineering.