Protein engineering of the archetypal nitroarene dioxygenase of Ralstonia sp strain U2 for activity on aminonitrotoluenes and dinitrotoluenes through alpha-subunit residues leucine 225, phenylalanine 350, and glycine 407

Naphthalene dioxygenase (NDO) from Ralstonia sp. strain U2 has not been reported to oxidize nitroaromatic compounds. Here, saturation mutagenesis of NDO at position F350 of the alpha-subunit (NagAc) created variant F350T that produced 3-methyl-4-nitrocatechol from 2,6-dinitrotoluene (26DNT), that released nitrite from 23DNT sixfold faster than wild-type NDO, and that produced 3-amino-4-methyl-5-nitrocatechol and 2-amino-4,6-dinitrobenzyl alcohol from 2-amino-4,6-dinitrotoluene (2A46DNT) (wild-type NDO has no detectable activity on 26DNT and 2A46DNT). DNA shuffling identified the beneficial NagAc mutation G407S, which when combined with the F350T substitution, increased the rate of NDO oxidation of 26DNT, 23DNT, and 2A46DNT threefold relative to variant F350T. DNA shuffling of NDO nagAcAd also generated the NagAc variant G50S/L225R/A269T with an increased rate of 4-amino-2-nitrotoluene (4A2NT; reduction product of 2,4-dinitrotoluene) oxidation; from 4A2NT, this variant produced both the previously uncharacterized oxidation product 4-amino-2-nitrocresol (enhanced 11-fold relative to wild-type NDO) as well as 4-amino-2-nitrobenzyl alcohol (4A2NBA; wild-type NDO does not generate this product). G50S/L225R/A269T also had increased nitrite release from 23DNT (14-fold relative to wild-type NDO) and generated 2,3-dinitrobenzyl alcohol (23DNBA) fourfold relative to wild-type NDO. The importance of position L225 for catalysis was confirmed through saturation mutagenesis; relative to wild-type NDO, NDO variant L225R had 12-fold faster generation of 4-amino-2-nitrocresol and production of 4A2NBA from 4A2NT as well as 24-fold faster generation of nitrite and 15-fold faster generation of 23DNBA from 23DNT. Hence, random mutagenesis discovered two new residues, G407 and L225, that influence the regiospecificity of Rieske non-heme-iron dioxygenases.