Utilising CYP199A4 from Rhodopseudomonas palustris HaA2 for Biocatalysis and Mechanistic Studies

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Abstract

The cytochrome P450 enzyme CYP199A4 from *Rhodopseudomonas palustris* strain HaA2 is highly specific for the regioselective oxidation of *para*-substituted benzoic acids. A selection of these compounds was tested with the enzyme with the aim of investigating the mechanism of different P450-catalysed reactions. These studies revealed that the binding affinity and oxidative activity of CYP199A4 is influenced by the substituent at the *para*-position, and that to the enzyme's known oxidative activities (demethylation, hydroxylation, heteroatom oxidation and desaturation) can be added alkene epoxidation, alkyne oxidation and aldehyde oxidation.

The active oxidants involved in these CYP199A4-catalysed oxidations were investigated using two active site mutants at the conserved acid-alcohol pair, $T252A_{CYP199A4}$ and $D251N_{CYP199A4}$, which should disrupt different steps of the catalytic cycle. There was a general increase in hydrogen peroxide uncoupling in the $T252A_{CYP199A4}$ mutant but significant levels of product formation were observed with each substrate. The D251N mutation reduced the activity of the enzyme dramatically in all but one case, suggesting that this mutation interferes with proton delivery as expected. The elevated rate of 4-ethynylbenzoic acid oxidation by $T252A_{CYP199A4}$ when compared to the wild-type enzyme suggested the involvement of Cpd 0 in alkyne oxidation, while a reduction in activity with 4-methoxybenzoic acid implicated Cpd I in demethylation. Additionally, the notable increase in product formation and coupling efficiency of $D251N_{CYP199A4}$ with 4-formylbenzoic acid suggested the involvement of the peroxo-anion in aldehyde oxidation.

Larger cinnamic acids and closely related substrates were also investigated with CYP199A4. The binding affinity and oxidative activity of the enzyme decreased in the order 4-methoxybenzoic acid > 4-methoxycinnamic acid > 3-(4-methoxyphenyl)propionic acid > 4-methoxyphenylacetic acid, highlighting its selectivity for a planar, benzoic acid- or cinnamic acid-like framework. The exclusive oxidation of cinnamic acids and related derivatives at the *para*-position further demonstrated the high regioselectivity of CYP199A4.

While CYP199A4 exhibited low oxidation activity towards *para*-methoxy substituted benzene derivatives, considerably higher levels of activity reminiscent of the demethylation of 4-methoxybenzoic acid were observed for the Ser244 → Asp244 (S244D) mutant of CYP199A4. The exclusive demethylation of the *para*-methoxy substituted benzenes by S244D revealed that the regioselectivity of CYP199A4 oxidation is maintained in this mutant.

The regioselectivity of the S244D mutant was further investigated using a selection of methyl- and ethyl-substituted derivatives. The methyl analogues were exclusively oxidised at the *para*-position to a single α-hydroxylation product. α-Hydroxylation and C_α-C_β desaturation products were generated in the turnovers of the ethyl derivatives. The alcohol was formed with high stereoselectivity. The electronic properties of the ethyl substrates were found to influence the ratio of hydroxylation/desaturation product, with the more electron donating substrates giving rise to a greater proportion of the latter. This suggested the involvement of a cationic intermediate in CYP199A4-catalysed desaturation.

Declaration

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Abbreviations

AcCN	acetonitrile
BA	benzoic acid
BSTFA-TMCS	N,O-*bis*(Trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS)
CA	cinnamic acid
DCM	dichloromethane
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EMM	*E. coli* minimal media
GC-MS	gas chromatography-mass spectrometry
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
K_2CO_3	potassium carbonate
LB	Luria-Bertani medium
NADH	Reduced form of nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NCS	*N*-chlorosuccinimide
NMR	Nuclear magnetic resonance
SOC	Super Optimal broth with Catabolite repression
TBACl	tetrabutylammonium chloride
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TFA	trifluoroacetic acid
WT	wild-type

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