Isolation of new P450s and the modification of existing P450s for biocatalysis

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Abstract

Cytochrome P450s are a family of heme-containing monooxygenases that are ubiquitous in nature. Many P450s from bacterial sources, such as from *Frankia* sp. EuI1c which contain genes encoding 68 of these enzymes, have not previously been investigated. These P450s are potentially involved in the metabolism and biosynthesis of novel natural products including steroids, siderophores, fatty acids and antibiotics. Here four of these P450s were successfully expressed and purified. One of these, FraEu2494, was purified at high concentrations suitable for crystallisation. A selection of chemical compounds was screened with these enzymes to determine the substrate range of these P450s. In particular the P450 FraEu1415 exhibited high affinity towards steroid compounds such as testosterone, estrone and progesterone, highlighting the important compounds these P450s may metabolise.

The ferredoxin electron transfer partners of these P450s were also successfully expressed and purified. While the ferredoxin reductases were unable to be produced, a mutant library of the ferredoxin Fdx2495 was created. Non-standard amino acid residues within the iron-sulfur binding motif of the ferredoxins were investigated to ascertain if Fdx2495 could be used as a model for future study.

The cytochrome P450 CYP199A4 from *Rhodopseudomonas palustris* strain HaA2 is highly specific for the regioselective oxidation of *para*-substituted benzoic acids such as 4-methoxybenzoic acid. It has been reported that the activity of the CYP199A4 S244D mutant for the hydroxylation and demethylation of *para*-substituted non-benzoic acid derivatives is greater than with the wild-type enzyme. Here we report the potential scale up of these oxidation reactions by the S244D mutant with a system that contains an excess of NADH. A selection of similar *para*-substituted compounds, including styrenes, methylthio- and dimethyl-substituted benzene derivatives were tested with the enzyme to further investigate the mechanism and productivity of the mutant.

The sulfoxidation and epoxidation reactions by the S244D mutant of *para*-substituted benzene derivatives were investigated. The epoxidation reactions produced small amounts of aldehyde arising from a 1,2-rearrangement reaction, giving evidence of a non-concerted reaction pathway. Chiral analysis for the sulfoxidation and epoxidation reactions revealed a consistent bias for a single enantiomer, suggesting similar binding conformations for these *para*-substituted benzene substrates within the active site.

The crystal structure of 4-methoxybenzoic acid bound to the CYP199A4 S244D mutant (PDB: 5U5J) was solved. This revealed small differences between the mutant and the equivalent wild-type structure (PDB: 4DO1). Aside from the specific 244 amino acid mutation, the substrate binding site was largely unaffected by the S244D variant. A

small shift in the position of the substrate over the active site was observed. A large shift was discovered for the chloride ion which caps the active site from external solvent in the wild-type enzyme. The chloride ion in the S244D mutant coordinates to an asparagine residue, which plays no role in the coordination to this ion in the equivalent wild-type.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations

AcCN	acetonitrile
ADH	alcohol dehydrogenase
AMU	atomic mass units
BA	benzoic acid
CO	carbon monoxide
DCM	dichloromethane
DFT	density functional theory
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ee	enantiomeric excess
EtOH	ethanol
EMM	*E. coli* minimal media
GC content	guanine-cytosine content
GC-MS	gas chromatography-mass spectrometry
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria-Bertani medium
NADH	reduced form of nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
QM/MM	quantum mechanics/molecular mechanics
S244D	CYP199A4 serine-244-aspartate mutant
SOC	super optimal broth with catabolite repression
TFA	trifluoroacetic acid
WT	wild-type

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