Investigations and Applications of Self-Sufficient Cytochrome P450 Monooxygenases

Samuel David Munday

Supervisors:
Dr Stephen Bell
Prof. Simon Pyke
Assoc. Prof. Christopher Ford

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THE UNIVERSITY of ADELAIDE

Contents

Abstract

The cytochrome P450 superfamily catalyses the oxidation of a vast array of organic molecules. Most commonly, this oxidation process ensues by the insertion of a single oxygen atom from dioxygen into an unreactive C–H bond. There is a high degree of interest for this reaction type in conventional synthesis, but it is difficult to achieve high levels of selectivity and is often performed under harsh conditions. CYP102A1 or P450Bm3 from *Bacillus megaterium* however, can perform this oxidative process under physiological conditions and so researchers have a strong interest in exploiting the potential benefits of this enzyme. The natural substrates of P450Bm3 are fatty acids but this thesis will address both modern and classical techniques to improve catalytic performance with a variety of non-natural substrates. The first two results chapters of this thesis (Chapters 3 and 4) describe the effect of decoy molecules on non-natural substrate oxidation with the aim of improving rates of product formation while maintaining the selectivity of the enzyme. Analysis of the oxidation of these substrates by wild-type P450Bm3 and the variant KT2 showed substantial increases in product formation rate while maintaining the regioselectivity. As a rigorous test of regioselectivity, a selection of xylenes were used that have previously been shown to generate multiple products upon P450Bm3 oxidation. Retention of enantioselectivity was also assessed by using prochiral substrates that have stereocentres introduced upon P450Bm3 oxidation. Chiral chromatography analysis of these turnovers showed that in most cases, the enantioselectivity of the enzyme was either maintained or marginally improved. Knowing that xylenes give a range of oxidation products upon P450Bm3 activity, a wider range of disubsituted benzene compounds were also analysed (Chapter 5). These substrates were chosen to resemble potential xenobiotic compounds in order to assess what metabolites may be produced by P450Bm3 and therefore other P450 systems. These substrates were analysed with several P450Bm3 variants and significantly improved rates of product formation were observed, enabling identification of the likely metabolites. Chapter 6 describes an investigation into two potential CYP102 family members from the bacterium *Ktedonobacter racemifer* DSM44963 (Krac0936 and Krac9955). Their sequenced genes show similarities to P450Bm3, which encouraged the investigation of a range of fatty acid substrates with these two enzymes. Although their product distributions differed, both Krac0936 and Krac9955 were active with straight-chain saturated and unsaturated fatty acids.

Declaration

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Samuel David Munday

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Abbreviations

AMU: atomic mass units
BA: benzyl alcohol
BID: barrier discharge ionisation detector
BSTFA: *N,O*-bis(trimethylsilyl)trifluoroacetamide
CPR: cytochrome P450 reductase
CYP: cytochrome P450
DMP: dimethylphenol
DTT: dithiothreitol
ee: enantiomeric excess
EMM: *E. coli* minimal media
FAD: flavine adenine dinucleotide
FMN: flavine mononucleotide
GC: gas chromatography
HPLC: high performance liquid chromatography
HS: high spin
IPTG: isopropyl-β-D-thiogalactopyranoside
kan: kanamycin
LB: Luria-Bertani broth
LS: low spin
MBA: methylbenzyl alcohol
MP: methylphenol
NAD(P)(H): nicotinamide adenine dinucleotide (phosphate)(H = reduced form)
NIH: National Institutes of Health
NPG: *N*-palmitoyl glycine
PAH: polyaromatic hydrocarbon
PFR: product formation rate
SB: substrate bound
SF: substrate free
TE: trace elements solution
TIC: total ion count
TMCS: trimethylchlorosilane
Tris: tris(hydroxylmethyl)aminomethane
WT: wild-type
2×YT: yeast extract tryptone medium

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