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Efficient hydroxylation of cycloalkanes by co-addition of decoy molecules to variants of the cytochrome P450 CYP102A1

Shaghayegh Dezvarei[a], Hiroki Onoda[b], Osami Shoji[b], Yoshihito Watanabe[c], Stephen G. Bell[a,*]

[a] Department of Chemistry, University of Adelaide, Adelaide 5005, Australia
[b] Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan
[c] Research Center for Materials Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan

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ABSTRACT

The wild-type cytochrome P450 (CYP) monooxygenase enzyme CYP102A1 (P450Bm3) has low activity for cycloalkane oxidation. The oxidation of these substrates by variants of this enzyme in combination with perfluorinated decoy molecules (PFCs) was investigated to improve productivity. The use of rate accelerating variants, which have mutations located outside of the substrate binding pocket as well as an active site variant of CYP102A1 (A74G/F87V/L188Q) all enhanced cycloalkane oxidation (C5 to C10). The addition of the decoy molecules to the wild-type and the rate accelerating mutants of CYP102A1 boosted the substrate oxidation rates even further. However, the levels of cycloalkanol product decreased with the larger alkanes when the decoy molecules were used with the variant A74G/F87V/L188Q, which contained mutations within the substrate binding pocket. For the majority of the enzymes and PFC decoy molecule combinations the highest levels of oxidation were obtained with cyclooctane. When larger second generation decoy molecules, based on modified amino acids were utilised there was a significant improvement in the oxidation of the smaller cycloalkanes by the wild-type enzyme and one other variant. This resulted in significant improvements in biocatalytic oxidation of cyclopentane and cyclohexane. However, the use of these optimised decoy molecules did not significantly improve cycloalkane oxidation over the fluorinated fatty acid derivatives when combined with the best rate accelerating variant, R47L/Y51F/I401P. Overall our approach enabled the cycloalkanes to be oxidised 300- to 8000-fold more efficiently than the wild-type enzyme at product formation rates in excess of 500 and up to 1700 nmol·nmol-CYP^{-1}·min^{-1}.

1. Introduction

Cyclic alcohols are important building blocks for the production of valuable industrial chemicals such as cyclohexanol which is used in the production of Nylon-6,6 [1,2]. The direct oxidation of cycloalkanes as a primary step for preparing the corresponding alcohols is therefore of significant interest [1]. The high stability of saturated C—H bonds in alkanes makes these compounds unreactive and they require forcing conditions such as high temperature and pressure, or a reactive oxidant in order to proceed [3,4]. Various organic and inorganic methods have been used to facilitate the oxidation of cycloalkanes [5–7]; nevertheless these reactions remain challenging. The catalysts used in these chemical methods can be expensive, cannot be recovered or reused or proceed with low selectivity and the reactions often undergo further undesired oxidation. In addition these catalysts are often toxic and have an adverse impact on the environment [8].

Enzymatic approaches for C—H hydroxylation have emerged as an alternative method for alkane and cycloalkane oxidation [8–13]. Efficient enzyme biocatalysis for these processes would benefit industry and the environment as they occur under mild conditions in aqueous media. Taking advantage of enzymes as biocatalysts could overcome many of the hurdles of chemocatalysts, namely the expense, poor selectivity and environmental toxicity [13,14]. The cytochrome P450 heme monooxygenases (CYPs) are often Nature's enzyme of choice when selective oxidation of a complex substrate is required. They catalyse the hydroxylation of unreactive C—H bonds at ambient temperature and pressure using a reactive compound I iron-oxo species [15–24].

The CYP102A1 enzyme (P450Bm3) has often been employed as a biocatalyst to catalyse hydroxylation reactions [25–29]. This enzyme was discovered in the 1970s by Fulco, it was the third enzyme isolated from *Bacillus megaterium* and as such was named Bm3 [30,31]. CYP102A1 is one of the most utilised P450 enzyme systems due to its high activity, solubility and self-sufficient nature. The electron

* Corresponding author.
E-mail address: stephen.bell@adelaide.edu.au (S.G. Bell).

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transferring reductase domain is fused to the heme domain and it only needs the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) and dioxygen to function [29]. Fatty acids of carbon chain length 12–15 are good substrates for this enzyme and hydroxylation of the acids occurred at subterminal position (ω-1 to ω-3) [29,31]. Cycloalkanes are not the natural substrates of CYP102A1 therefore the enzyme must be modified to find conditions under which their hydroxylation will occur. Enzyme engineering by altering the amino acid sequences has been used to exploit this enzyme for alkane oxidation [25–27]. Two broad approaches have been utilised to adapt CYP102A1. The first makes changes in the substrate binding pocket (rational mutagenesis) [29,32,33] and the second involves directed evolution or random mutagenesis with a suitable screening method [28,34–36].

Another approach which has been applied to enhance CYP102A1 biocatalysis is the use of decoy molecules [37]. These are inert dummy substrates which promote the catalytic activity of the enzyme [38]. Perfluorocarboxylic acids (PFCs) have been used with CYP102A1 with promising results for small alkanes, including cyclohexane, and benzene [39]. The binding of the PFCs, which resemble the natural fatty acid substrates, initiates conformational changes in the heme domain enhancing substrate hydroxylation. The PFCs leave enough space in the substrate binding pocket for an additional molecule to bind. The decoys also appear to compel the substrates to bind more closely to the heme centre and facilitate the exclusion of water molecules. This is reflected in a lower proportion of the reducing equivalents being channelled into uncoupling pathways and resulted in higher levels of hydroxylation [39]. The use of decoy molecules can be combined with mutated variants to oxidise unnatural substrates with high catalytic productivity [39–41]. Second generation decoy molecules based on PFC modified L-amino acids (e.g. PFC9-L-Ala; *N*-perfluorononanoyl-L-alanine) have recently been synthesised to further enhance the activity of small molecules, such as propane. These bind to CYP102A1 with higher affinity than the PFCs. A crystal structure of the *N*-perfluorononanoyl-L-tryptophan (PFC9-L-Trp) bound CYP102A1 heme domain has been obtained which revealed the conformational changes that are induced within the enzyme (Fig. 1) [42].

Here we use decoy molecules in combination with the wild-type (WT) enzyme and four engineered variants of CYP102A1 for the hydroxylation of differently sized cycloalkanes. Three of the variants employed KT2 (A191T/N239H/I259V/A276T/L353I), R19 (R47L/Y51F/H171L/Q307H/N319Y) and RP (R47L/Y51F/I401P) are rate accelerating forms of the enzyme, generated by random or rational mutagenesis or a combination of both [43,44]. These have higher activity with non-natural substrates, including alkanes, but maintain the selectivity of the WT enzyme [39–41]. The substrate free forms of the KT2 and I401P mutants have structural conformations which more closely resemble the fatty acid bound form of the enzyme. This, in combination with a longer heme-iron axial water length, results in substrate induced conformational changes playing a less significant role in promoting the catalytic cycle and enables the enhanced oxidation of non-natural substrates [43,44]. The arginine 47 and tyrosine 51 residues, which have been altered in the R19 and RP variants, form hydrophilic interactions with fatty acid substrates and the changes render the active site more hydrophobic [28]. The combination of this pair of mutations with the rate accelerating mutation found in the R19 and RP variants have been shown to favour the hydroxylation of hydrophobic substrates [39–41]. This has been shown to enhance the oxidation of hydrophobic substrates but could reduce the effectiveness of the acid derived decoy molecules [28,43]. The fourth variant GVQ (A74G/F87V/L188Q) was obtained from site saturation mutagenesis of three residues located in the active site or substrate access channel of CYP102A1 [25,45,46]. This variant increased the activity of the enzyme for hydrophobic substrates but can change the selectivity of oxidation as the modifications are in the active site of the enzyme [25,47]. By using both first and second generation decoy molecules with smaller and larger cycloalkanes than cyclohexane we set out to gain a better understanding of the relationship between the size and shape of the decoy molecule and substrate to the performance of the enzyme catalysed oxidations.

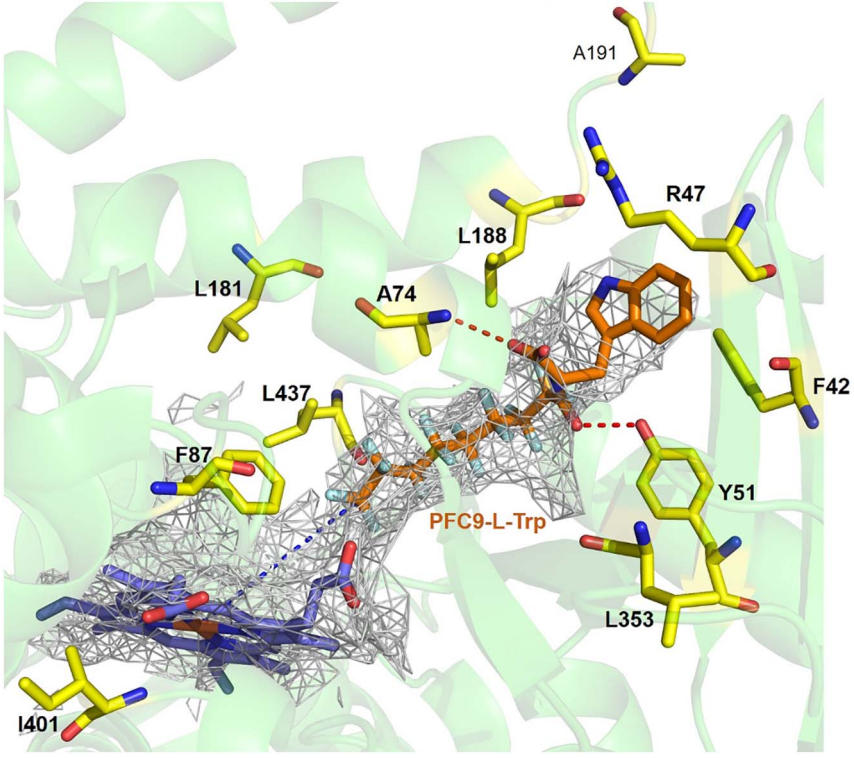

Fig. 1. The crystal structure of CYP102A1 with PFC9-L-Trp decoy molecule (PDB: 3WSP), the access channel (grey wireframe) and hydrophobic or mutated residues (yellow) are shown. The hydrogen bond between PFC9-L-Trp/Y51 and PFC9-L-Trp/A74 (dashed line) and the distance between PFC9-L-Trp and heme domain (dashed line) have been indicated.