Journal of Molecular Catalysis B: Enzymatic 128 (2016) 52–64

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

In vivo and *in vitro* hydroxylation of cineole and camphor by cytochromes P450CYP101A1, CYP101B1 and N242A CYP176A1

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ARTICLE INFO

Article history:
Received 9 February 2016
Received in revised form 4 March 2016
Accepted 16 March 2016
Available online 17 March 2016

Keywords:
Cytochrome P450
Cineole
Hydroxycineole
Camphor
Hydroxycamphor

ABSTRACT

Cytochromes P450 (P450s) are valuable enzymes that can generate a range of useful compounds *via* biocatalytic oxidations that complement traditional synthetic chemistry. In this study three bacterial P450s, $P450_{cam}$ (CYP101A1), CYP101B1 and the mutant N242A-$P450_{cin}$ (N242A-CYP176A1), were used to produce a range of products from the oxidation of the monoterpenes (1*R*)- and (1*S*)-camphor and 1,8-cineole. We demonstrate that both *in vitro* and *in vivo* catalytic turnover with these P450s can produce a complement of up to seven hydroxycamphors and seven hydroxycineoles, in addition to compounds produced from further oxidation. The CYP101B1 whole cell catalytic system was found to produce 300–600 mg/L of culture of oxidation products that could be easily separated chromatographically. The CYP101B1 *in vitro* oxidation of 1,8-cineole primarily produced (1*S*)-5α-hydroxycineole, which was 78% of the total product formed. However, the amount of (1*S*)-5α-hydroxycineole was reduced to 42% of the total products when isolated from the CYP101B1 whole cell system. (1*S*)-6α-Hydroxycineole (96% *ee*) could be isolated from a whole cell catalytic turnover of 1,8-cineole by N242A-$P450_{cin}$ in a yield of 46 mg/L (98% of the total product). However, the amount of product isolated ((1*R*)-5-*endo*-hydroxycamphor, 75% of the total products) from the whole cell catalytic oxidation of (1*R*)-camphor with N242A-$P450_{cin}$ was much lower (6 mg/L) due to the inefficient use of reducing equivalents (3.5 ± 0.5%) for substrate oxidation. These compounds will assist in the identification of specific structures in mechanistic investigations and structure elucidation, but further optimisation is required to generate larger quantities for synthetic applications.

1. Introduction

Cytochromes P450 (P450s)[1] are a family of multipurpose enzymes that are able to carry out numerous oxidative transformations that include, but are not limited to, aliphatic and aromatic hydroxylations, heteroatom oxidation and carbon-carbon bond cleavage [1]. Typically, P450s catalyse the oxidation of their substrate by employing an extremely potent, high-valent iron-oxo species that is able to directly insert an oxygen atom into an unactivated carbon-hydrogen bond [2,3]. The iron-oxo species is formed within the P450 when molecular oxygen is activated following the delivery of two electrons that have been originally sourced from a nicotinamide cofactor (NADH or NADPH). In contrast to traditional methodologies of organic synthesis, P450 catalysed oxidation, and carbon hydroxylation in particular, is a process where the regio- and stereoselectivity are often controlled in a single step [4,5]. Hence, it is the ability of P450s to efficiently catalyse oxidation reactions *via* this exceptionally effective and selective oxidant, together with their catalytic versatility and substrate diversity that make P450s particularly attractive for potential biocatalytic applications [6,7]. This allows P450s to complement traditional chemical synthesis for the production of a wide array of compounds.

$P450_{cam}$ (CYP101A1) is a bacterial P450 that catalyses the stereospecific hydroxylation of the monoterpene (1*R*)-camphor **1** to 5-*exo*-hydroxycamphor **2a** (Fig. 1). Originally described in 1968 [8], $P450_{cam}$ has become one of the most well-known and extensively studied P450s. Much of the mechanistic understanding of P450s in general has been established *via* studies employing $P450_{cam}$ as a model system [9]. However, these mechanistic investigations have not only allowed a more detailed understanding of P450 catalysed

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[1] *Abbreviations:* P450, cytochrome P450; $P450_{cam}$, CYP101A1; $P450_{cin}$ or WT-$P450_{cin}$, wild type CYP176A1; Cdx, cindoxin; Arx/ArR, [2Fe-2S] ferredoxin/flavin dependant ferredoxin reductase from *Novosphingobium aromaticivorans*.

http://dx.doi.org/10.1016/j.molcatb.2016.03.004
1381-1177/

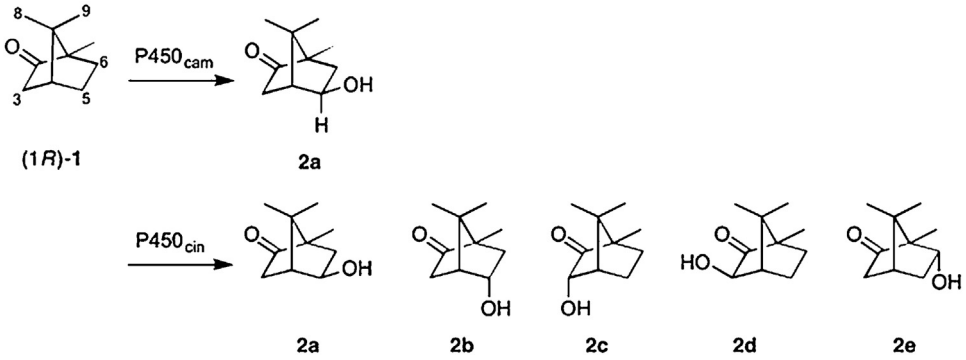

Fig. 1. Products identified from the catalytic turnover of (1*R*)-camphor **1** by $P450_{cam}$ and $P450_{cin}$ [8,43].

oxidation but they have also revealed the biocatalytic potential of $P450_{cam}$. Structural investigations including both the crystallographic [10–16] and *in silico* studies [17–19] have established that a variety of different molecules can either bind or are predicted to bind within the active site of $P450_{cam}$. Further investigations have demonstrated that $P450_{cam}$ is also able to catalyse the oxidation of some of these molecules to chemically useful compounds, which has consequently led to development of mutants that are optimised to catalyse the oxidation of alternative substrates [20–33]. For example, a number of active-site mutations introduced into $P450_{cam}$ were found to improve its selectivity toward two monoterpenes: *S*-limonene and (+)-α-pinene [22,24,34]. This direct manipulation of $P450_{cam}$ can therefore provide access to a range of compounds for use as standards in structure elucidation and mechanistic studies and also for the potential production of compounds for the fragrance, flavour and pharmaceutical industries [6,35].

Mechanistic investigations of another bacterial P450, $P450_{cin}$ (CYP176A1) have also highlighted the biocatalytic potential of P450s. $P450_{cin}$ was originally isolated from *Citrobacter braakii* and catalyses the enantiospecific hydroxylation of a monoterpene 1,8-cineole **3** to produce (1*R*)-6β-hydroxycineole **4a** (Fig. 2), which is the first step in the biodegradation of cineole in *C. braakii* [36–38]. During the initial analysis of $P450_{cin}$ it was observed that it did not possess the almost universally conserved threonine found in other P450s [39]. Instead, in WT-$P450_{cin}$ (Wild type $P450_{cin}$) this conserved threonine residue was replaced with an asparagine. To probe this anomaly, an N242A-$P450_{cin}$ mutant was prepared to examine the role of this asparagine in WT-$P450_{cin}$ [40,41]. It was determined that the Asn242 was not a functional replacement for the mechanistically important threonine found in other P450s, but rather it was important for determining the regio- and enantioselectivity of the oxidation of cineole [40,41]. Interestingly, this single amino acid change (N242A) was also shown to result in formation of only 5% of the product observed with WT-$P450_{cin}$ ((1*R*)-6β-hydroxycineole **4a**) and instead resulted in conversion of cineole **3** to predominately (1*S*)-6α-hydroxycineole **4c** (90%; Fig. 2) [40]. Further mutagenesis investigations have demonstrated that a range of hydroxycineole isomer mixtures can also be obtained from a number of single amino acid mutations [41,42], again underlining the potential of $P450_{cin}$ mutants in the generation of chemically useful compounds.

In addition, $P450_{cin}$ has also been observed to catalyse the oxidation of other monoterpenes, such as (1*R*)- and (1*S*)-camphor [43,44]. Camphor **1** is structurally similar to $P450_{cin}$'s natural substrate cineole **3** and was initially investigated to analyse the interaction between the active site and the oxygen of camphor [43]. An *in vivo* system was constructed where *Escherichia coli* had been transformed with a bicistronic plasmid that expressed $P450_{cin}$ and cindoxin (Cdx) with endogenous *E. coli* flavodoxin thought to be facilitating the terminal electron transfer. Both (1*R*)- and (1*S*)-camphor **1** oxidation by $P450_{cin}$ was observed. However, surprisingly no selectivity was observed between (1*R*)- and (1*S*)-camphor **1**, as both were found to be converted to a similar mixture of five hydroxycamphor isomers (Fig. 1) [43].

A group of P450s from *Novosphingobium aromaticivorans* were isolated to explore their potential as biocatalysts and to investigate the mechanism of electron transfer [45]. It was observed that several of these P450s, which are also from the CYP101 family, were able to catalyse the oxidation of a number of mono- and norisoprenoids. Subsequent research led to the development of *in vivo* systems for the expression of each of the P450s in *E. coli* that included both redox partners required to generate a catalytically active system, the [2Fe-2S] ferredoxin Arx and the flavin dependant ferredoxin reductase ArR [46]. One of these P450s, CYP101B1, was able to catalyse the turnover of (1*R*)-camphor **1** [45] to four different hydroxycamphors including 5-*exo*-hydroxycamphor (**2a**; 46%), the natural product of $P450_{cam}$ catalysed oxidation of camphor. The three other metabolites were assigned by coelution experiments with the Y96F mutant of $P450_{cam}$ which in turn had been reported based on the GC retention times and relative polarity of the metabolites [47].

The aim of this present study was to continue to explore monoterpene oxidation by $P450_{cam}$, WT-$P450_{cin}$, CYP101B1 and N242A-$P450_{cin}$. In particular, we wanted to confirm the identity of the hydroxycamphors produced by the CYP101B1 and compare them to those previously reported from $P450_{cam}$ and $P450_{cin}$. Additionally, as N242A-$P450_{cin}$ produced a different hydroxycineole isomer to that produced by WT-$P450_{cin}$ during cineole **3** oxidation, it was of interest to determine the selectivity of this mutant with another monoterpene, camphor **1**. Due to the structural similarity between cineole **3** and camphor **1**, a detailed investigation into the products obtained from cineole **3** oxidation by both $P450_{cam}$ and CYP101B1 was also undertaken to compare with those seen from N242A-$P450_{cin}$ and WT-$P450_{cin}$. *In vivo* systems for each P450 were also utilised in order to obtain structural information for each isomer and assess the efficiency of the system. Collectively, it was anticipated that by assessing the activity of these four P450s with cineole **3** and camphor **1**, a collection of hydroxycamphor **2** and hydrocineole **4** isomers could be produced that may be useful for structure elucidation purposes and potential biocatalytic applications.

2. Experimental

2.1. Chemicals

The two entantiomers of both 5-ketocineole (1*S*)- and (1*R*)-**5** and (1*S*)-5-α-hydroxycineole **4d** and its (1*R*) enantiomer **4e** were prepared as racemic mixtures according to a previously published synthesis [48]. The two enantiomers of 6-ketocineole (1*S*) and (1*R*)-**8** and 6-α-hydroxycineole **4b** and **4c** were also synthesised