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Carbon–carbon bond cleavage by cytochrome $P450_{BioI}$ (CYP107H1)†

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Cytochrome $P450_{BioI}$ (CYP107H1) is believed to supply pimelic acid equivalents for biotin biosynthesis in *Bacillus subtilis*: we report here that the mechanistic pathway adopted by this multifunctional P450 for the in-chain cleavage of fatty acids is via consecutive formation of alcohol and *threo*-diol intermediates, with the likely absolute configuration of the intermediates also reported.

The cytochromes P450 (P450s) comprise a superfamily of oxidative hemoproteins that catalyse an impressive array of oxidative transformations. Typically, this involves oxidation at an unactivated carbon centre to yield the corresponding alcohol.[1] However, the oxidative potential of these enzymes is perhaps best illustrated by their ability to catalyse the cleavage of C–C bonds.[2] We report here that the mechanistic pathway adopted by the multifunctional $P450_{BioI}$ for the in-chain cleavage of fatty acids is *via* alcohol and *threo*-diol intermediates. This represents the first prokaryotic P450 capable of catalysing C–C bond cleavage in an unfunctionalised substrate.

A number of mechanistically different reaction types can be distinguished for P450 catalysed C–C bond scission, but each type is represented by only a few examples.[2] Additionally, almost all of the described reactions are restricted to eukaryotes; CYP51, which occurs in both eukaryotes and prokaryotes, is the only prokaryotic example apart from $P450_{BioI}$.[3] Most P450 C–C bond scissions occur α to an existing functional group,[2,4–8] but perhaps more impressive are those catalysed by multifunctional P450s which oxidatively cleave an unactivated alkyl moiety. One such reaction that has been fully described is the transformation of cholesterol into pregnenolone and 4-methylpentanal catalysed by the eukaryotic $P450_{scc}$.[2]‡ In this, two hydroxylation reactions lead specifically to 20(*R*),22(*R*)-dihydroxycholesterol and the C–C bond between the two oxygenated carbons is then oxidatively cleaved. A similar process occurs in the metabolism of the antimicrobial agent olanexidine, but it is unclear whether a single P450 is responsible for all of the oxidative steps.[9]

We have recently reported the over-expression and characterisation of a *Bacillus subtilus* enzyme, $P450_{BioI}$, that is involved in biotin biosynthesis.[10] Analysis of mutant phenotypes had suggested that it played a role in the formation of pimelic acid (heptanedioic acid) which provides the majority of the carbon backbone of biotin.[11] Expression of $P450_{BioI}$ in *E. coli* resulted in the isolation of the enzyme alone and in complex with an acyl carrier protein acylated with C14 to C18 fatty acids (acyl ACP).[10] It was demonstrated that turnover of this complex resulted in the formation of a pimeloyl ACP, *via* cleavage of the alkyl chain of the acyl moiety. Free fatty acids also serve as substrates for a catalytically active $P450_{BioI}$ system.[10,12,13] Initially, it was postulated that ω-functionalisation of the fatty acid may occur, providing an alternative route for the production of pimelic acid *via* subsequent chain shortening reactions.[13] However, we have recently demonstrated that the major positions of free fatty acid oxidation are exclusively non-terminal.[12] For example, tetradecanoic acid (**1**) is oxidised to mainly 11-, 12- and 13-hydroxytetradecanoic acids along with a small amount of pimelic acid. The lack of specificity observed in the oxidation of the free fatty acids is unsurprising, given that it appears likely the natural substrate is an ACP thioester of a fatty acid.[10] However, we believed that as pimelic acid was indeed produced from free fatty acids, the route by which this was formed would likely be the same as that by which the pimeloyl ACP was produced. We thus set out to determine the pathway by which $P450_{BioI}$ catalysed the C–C bond cleavage of fatty acids to produce pimelic acid using tetradecanoic acid **1** as the illustrative substrate.

$P450_{scc}$ provides the only delineated example of oxidative bond fission of an unactivated alkyl chain.‡ However, it was clear that other pathways might be followed as direct P450 catalysed C–C bond cleavage has also been observed in simple alcohols[7] and ketones.[2] Thus, we planned to synthesise a range of possible intermediates and determine the relative level of pimelic acid production, reasoning that more advanced intermediates would lead to greater pimelic acid production.

Therefore, compounds **2–6** were synthesised and incubated with a catalytically active $P450_{BioI}$ system consisting of purified *E. coli* flavodoxin reductase, cindoxin and $P450_{BioI}$[10] and the level of pimelic acid production quantified by GC analysis.

The results (Fig. 1) show clear differences in the levels of pimelic acid production and delineate the pathway by which $P450_{BioI}$ cleaves the fatty acid chain. The C7 alcohol **2** is a better substrate than **1** but the C7 oxo fatty acid **4** does not improve pimelic acid production. Neither the C8 alcohol **3** nor the C8 oxo fatty acid **5** is processed at all. The *erythro* 7,8-diol (*rac* **6a**) is a poorer substrate than the *threo* diastereomer (*rac* **6b**) which leads to an approximately 15-fold increase in C–C bond cleavage relative to **1**. These results are consistent with an oxidation pathway that is initiated by C7 hydroxylation (Scheme 1). Subsequent C8 oxidation forms the *threo* diol that then undergoes oxidative cleavage. The *threo* diol would be predicted to arise from the enzyme acting upon one face of the extended fatty acid chain.

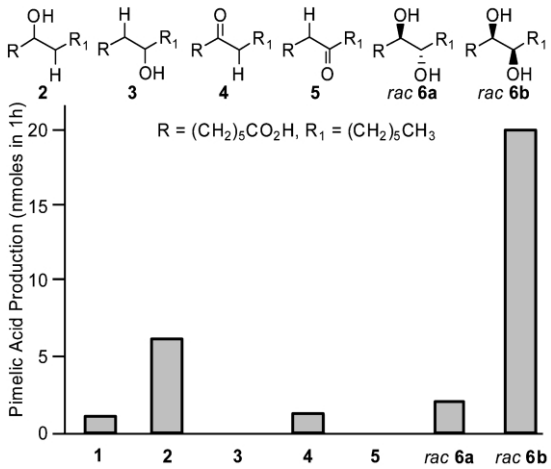

Fig. 1 Pimelic acid production by $P450_{BioI}$ from racemic substrates (substrate concentration = 1 mM).

† Electronic Supplementary Information (ESI) available: **2**, *R* **2**, *S* **2**, **4**, *SS* **6b**, *RR* **6b** characterisation and synthesis. Enzymatic oxidation and analysis protocols. See http://www.rsc.org/suppdata/cc/b3/b311652b/

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Unambiguous identification of the products of C–C bond cleavage was necessary, as a mechanism involving diol cleavage would predict formation of two aldehyde fragments. The increased turnover of *threo*-7,8-dihydroxytetradecanoic acid (*rac* **6b**) revealed the presence of heptanal and 7-oxoheptanoic acid in comparable amounts at short reaction times. Both compounds, either as synthetic standards or produced *via* P450 catalysed bond scission, were converted to the corresponding acids over time (> 70% after 24 h) under enzyme turnover conditions *via* aerial oxidation, independent of NADPH. All of these results are consistent with C–C bond cleavage *via* a route corresponding to that seen for $P450_{scc}$.[2]

The absolute configurations of the preferred enzymic substrates were investigated by the synthesis and evaluation of scalemic samples of the 7-hydroxy (**2**) and *threo* 7,8-dihydroxytetradecanoic acids (**6b**). The former was available in ~70% ee as determined by enantioselective HPLC (Chiracel OD), with the key step in the syntheses being a CBS borane reduction of the appropriate acetylenic ketone.[14] The enantiomerically pure *threo* diols (*R,R* and *S,S* **6b**) were synthesised from chiral, non-racemic tartaric acid, utilizing methodology developed by Seebach for differentiating either end of the starting diacid.[15] Incubation of these substrates with a catalytically active system indicated that the *S* **2** and the corresponding *R,R* diol were preferentially processed by $P450_{BioI}$ (Fig. 2). The enantioselectivity of the enzyme was low, with less favored *R* **2** and *S,S* **6b** still being good substrates for the enzyme. A plausible explanation for this lack of selectivity stems from the fact that the enantiomers of **6b** differ only in the location of a carboxyl or a methyl group five methylene groups removed from the diol moiety. In the putative natural substrate, an acyl ACP, the carrier protein would presumably dictate the binding orientation. However, with the free fatty acids, two binding orientations are possible that present the same apparent stereochemistry of the diol at the active site differing only in the location of a distal methyl or carboxylate.

Accurate dissociation constants for the enzyme–substrate complexes were difficult to determine spectrophotometrically, probably due to the instability of the uncomplexed enzyme.[10] However, it was clear from the estimated association constants[10] that the hydroxylated fatty acids bound significantly (at least 4 fold) more tightly to the enzyme than the starting fatty acid, but both alcohol and diol appeared to bind equally well. This is analogous to the situation seen with $P450_{scc}$ in which the hydroxylated intermediates were bound equally, but more tightly than cholesterol.[2] This is presumed to increase the efficiency of the reaction: the intermediates are not released or displaced by the initial substrate but continue on to product. The fact that the C7 alcohol and the 7,8-diol both facilitate and direct subsequent oxidation reactions is clearly reflected in the analysis of the enzymic turnovers. While the 11- and 12-hydroxytetradecanoic acids are the major products isolated from $P450_{BioI}$ catalyzed hydroxylation of **1**, they are not substrates for further oxidation.[12] **2** does not accumulate in enzymic oxidations of **1**, nor does **6** appear in turnover of **1** or **2**. Finally, the only alternative oxidation pathway apart from C–C bond cleavage seen for **2** is conversion into the corresponding ketone **4**. Interestingly, this pathway was much more significant for *R* **2** than *S* **2** (> 10 fold, quantified by GC), which produces less pimelic acid. This suggests that the orientation of the substrate in the active site is determined by the stereochemistry at C7 and this in turn directs subsequent oxidation reactions.

In conclusion, we have identified the pathway by which the multifunctional, prokaryotic $P450_{BioI}$ catalyses the cleavage of a C–C bond in a fatty acid (Scheme 1). By analogy we predict cleavage of an acyl ACP, its natural substrate, will occur in the same way. The mechanism involves the consecutive formation of an alcohol and a vicinal diol and subsequent C–C bond cleavage. This is analogous to that seen with $P450_{scc}$ and $P450_{BioI}$ is thus only the second P450 identified as being capable of such bond fission, and the first from a prokaryote.

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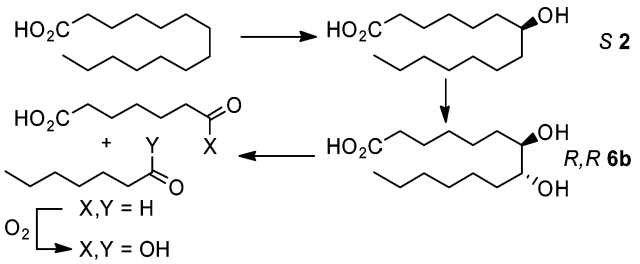

Scheme 1

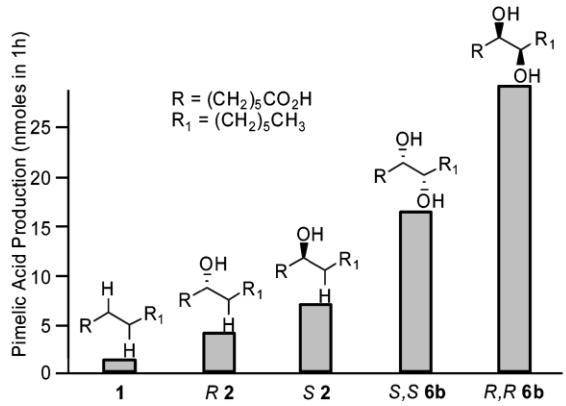

Fig. 2 Pimelic acid production by $P450_{BioI}$ from scalemic substrates (substrate concentration = 1 mM).

Notes and references

‡ Whilst C–C bond cleavage is observed with CYP19 and CYP51 in steroid biosynthesis, both proceed *via* aldehyde intermediates which are inaccessible to $P450_{BioI}$.

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