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Desaturation of Alkylbenzenes by Cytochrome $P450_{BM3}$ (CYP102A1)

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Cytochrome P450 monooxygenases catalyse the oxidation of a broad array of endogenous and exogenous organic substrates, typically inserting an oxygen atom from atmospheric dioxygen into a carbon–hydrogen bond to give the corresponding alcohol.[1] Other known types of activity include olefin epoxidation, carbon–carbon bond cleavage, heteroatom oxidation, dealkylation and dehydrogenation across C–O, C–N and C–C bonds.[2] Few examples of dehydrogenation by bacterial P450 systems have been reported. CYP102A1 ($P450_{BM3}$), a medium- to long-chain fatty acid hydroxylase from *Bacillus megaterium*, has been shown to aromatise Nifedipine by C–N dehydrogenation,[3] and a triple mutant was able to oxidise acetaminophen by C–N and C–O dehydrogenation.[4] Wild-type CYP102A1 gave 0.9% C–C dehydrogenation (desaturation) with α-thujone (Figure S1), while CYP101A1 ($P450_{cam}$) from *Pseudomonas putida* gave 13.7%.[5] Substrates found to undergo desaturation in microsomal systems include valproic and lauric acids,[6,7] ezlopitant[8] and capsaicinoids[9] (Figure S1). In some cases, aromatisation or the formation of conjugated products, such as styrenes, appeared to provide a driving force for the reaction.[9–11]

Wild-type CYP102A1 (WT) monooxygenates most non-natural substrates at nugatory rates, although directed evolution and site-specific mutagenesis may be employed to enhance activity.[12–14] Introducing mutations can cause substrates to bind in altered orientations, allowing different C–H bonds to be oxidised and transforming product profiles. During an investigation into the oxidation of *p*-cymene by a family of recently reported variants,[15] we were interested to encounter significant quantities of a product that seemed unlikely, on the basis of its short gas chromatography (GC) elution time (Figure S2), to arise from monooxygenase activity. This was in due course identified as the C–C desaturation product, *p*,α-dimethylstyrene, using GC co-elution with an authentic sample. Small amounts of *p*,α-dimethylstyrene oxide, the further oxidation product, were also produced. In the light of this finding, the oxidation of a series of alkylbenzenes structurally related to *p*-cymene was investigated in a search for complementary examples of desaturase activity. In addition to WT CYP102A1, a selection of variants was studied, with F87A/A330P/E377A/D425N (KT5)[15] giving the most striking product distributions (Figure 1, Table 1).

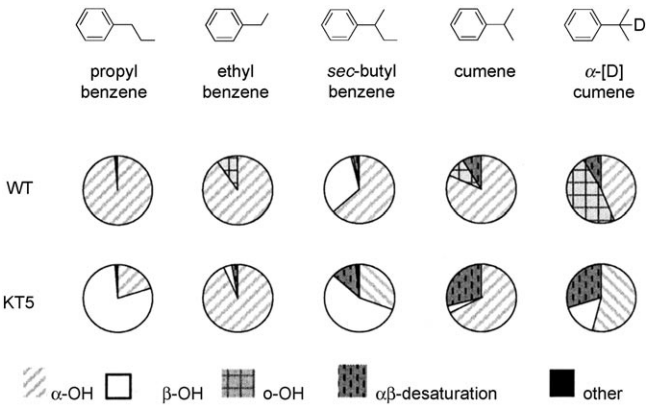

Figure 1. Product distributions in the oxidation of alkylbenzenes by wild-type CYP102A1 (WT) and variant KT5 (F87A7A330P/E377A/D425N).

Desaturation took place between the α- and β-positions of the alkyl substituents of all the substrates studied, though to varying extents. WT gave small quantities, while higher percentages were obtained from F87A-containing variants such as KT5. Isopropylbenzene (cumene) gave 29% α,β-desaturation (including α-methylstyrene oxide formation) with variant KT5, while *sec*-butylbenzene gave 11%, ethylbenzene 3% and propylbenzene just 0.7%. *p*-Cymene and *p*-isopropylanisole gave 22 and 23%, respectively (Tables S5 and S6). Desaturation was therefore most pronounced when the α-carbon of the alkyl substituent was branched and the

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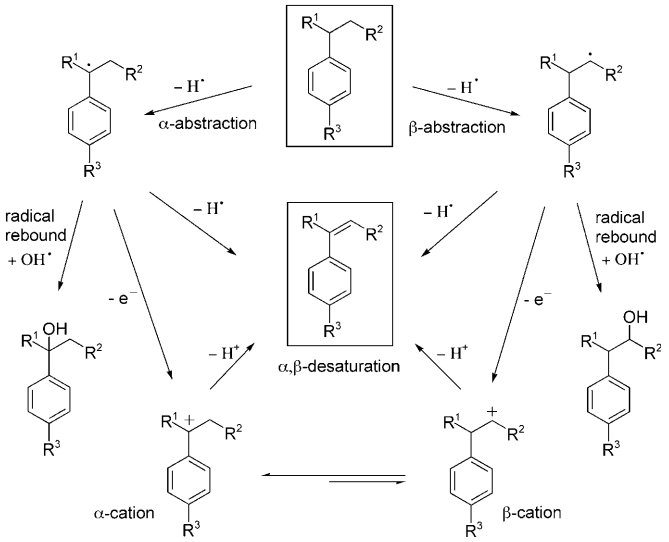
Table 1. Product distributions in the oxidation of alkylbenzenes by wild-type CYP102A1 (WT) and KT5 (F87A/A330P/E377A/D425N).

		% Hydroxylation				% Desaturation		
		α-	β-	γ-	*o*-	α,β-	β,γ-	oxides
propylbenzene								
	WT	98.7	0.3	–	0.6	0.2	0.2	–
	KT5	20.3	78.3	0.3	–	0.6	0.4	0.1
ethylbenzene								
	WT	90.2	–	n/a	9.8	–	n/a	–
	KT5	93.2	3.9	n/a	–	2.9	n/a	–
sec-butylbenzene								
	WT	63.2	32.8	–	1.8[a]	1.5	–	0.7
	KT5[b]	30.5	56.3	1.1	–	7.9	–	3.2
cumene								
	WT	81.8	–	n/a	9.3	8.9	n/a	–
	KT5	67.9	3.2	n/a	–	27.5	n/a	1.4
α-[D]cumene								
	WT	44.0	–	n/a	47.5	8.5	n/a	–
	KT5	54.1	16.3	n/a	–	28.8	n/a	0.8
β-[D_6]cumene								
	WT	84.3	–	n/a	8.8	6.9	n/a	–
	KT5	72.3	–	n/a	–	25.5	n/a	2.2
p-cymene								
	WT	83.7	–	n/a	10.4	5.9	n/a	–
	KT5	77.9	–	n/a	–	18.9	n/a	3.2

[a] Includes 0.7% *p-sec*-butylphenol. [b] percentages do not sum to 100 due to a minority product. n/a = not applicable. See Tables S1–S6 for detailed product profiles.

β-carbon(s) terminal. NADPH utilisation efficiencies (coupling) and product formation rates (PFRs) did not appear to be adversely affected by high desaturation levels. *p*-Cymene, for example, gave a PFR of 702 nmol min^{-1} (nmol P450)$^{-1}$ with KT5 at a coupling efficiency of 50% (Table S7). The principal oxidation pathway was generally α-hydroxylation, but significant levels of β-hydroxylation occurred in substrates possessing a sub-terminal β-carbon (e.g., 33% from *sec*-butylbenzene with WT). Substrates with terminal β-carbon atoms gave smaller quantities of β-alcohols, consistent with C–H bond reactivity trends, while terminal γ-alcohols were produced by substrates possessing a γ-carbon atom. With certain substrates, *o*-hydroxylation or *O*-demethylation (Table S6) were encountered. Interestingly, trace amounts of β,γ-desaturation were observed with propylbenzene (though not with *sec*-butylbenzene). In addition to giving higher desaturation percentages than WT across all substrates, KT5 also gave more β-hydroxylation (e.g., 78 vs 0.3% with propylbenzene), while WT gave more *o*-hydroxylation than KT5 (e.g., 11 vs 0% with cumene).

The formation of β- and γ-alcohols indicates that the first hydrogen abstraction by the P450 oxyferryl compound I species can take place from the β- or γ-carbon of an alkyl substituent as well as from the more activated benzylic α-carbon. Desaturation between C_α and C_β could therefore be initiated either by α- or β-abstraction (Scheme 1). Intramolecular deuterium isotope effects show that the first abstraction in the desaturation of valproic acid and ezlopitant (see Figure S1) by microsomal P450s takes place from one of the more activated carbon atoms.[6,8] By contrast, the desaturation of capsaicinoids is thought to be initiated by abstraction from the less activated ω-position.[9] Carbocation formation via electron transfer from the incipient ω-radical to the oxidising species then occurs, followed by rearrangement to the more stable ω-1 carbocation. In principle, an analogous mechanism could operate in CYP102A1, with successive abstractions taking place from the less activated β-carbon. β-Abstraction followed by electron transfer would yield a β-cation with an intrinsic tendency to rearrange to the more highly substituted benzylic α-cation (Scheme 1). Rearrangement should be facile in cumene, where the α-carbon is tertiary and the β-carbon terminal, potentially allowing α,β-desaturation to compete effectively against β-hydroxylation as well as α-hydroxylation. In propylbenzene, however, there would be a limited probability of rearrangement, which could explain the low desaturation levels observed (0.7% with KT5 vs 29% for cumene).



Scheme 1. Possible reaction pathways in the oxidation of alkylbenzenes by CYP102A1 (R^1, R^2, R^3 = H or Me).

In a search for evidence of a carbocationic rearrangement, we investigated the oxidation of α-[D]cumene to establish whether desaturation entailed the migration of the single α-deuterium to the β-carbon. GC-MS analysis gave the mass of the α-methylstyrene formed as 118.176 versus a theoretical mass for the non-deuterated product of 118.176, while NMR spectroscopy showed the non-aromatic protons to be in a 1:1:3 ratio, with no loss of fine structure at any of the three resonances when compared to an authentic sample. The absence of deuterium in the product demonstrated that desaturation did not proceed via carbocationic rearrangement with α-to-β proton transfer. The NADPH consumption rates measured in these experiments were similar to those observed with non-deuterated cumene, but coupling and PFRs were substantially lower (Table 2). Peroxide uncoupling levels were unchanged, indicating that the oxidase pathway was primarily responsible for the decrease in cou-

pling. α-Deuteration resulted in significant levels of metabolic switching. KT5 gave higher β-hydroxylation levels at a higher absolute PFR while WT gave higher *o*-hydroxylation percentages, again at a higher PFR. The isotope effects observed for desaturation were lower than those for α-hydroxylation. These metabolic switching levels and isotopic sensitivities were higher than those observed in the microsomal P450 oxidation of monodeuterated ezlopitant[8] (Figure S1), which has the same desaturation site structure as α-[D]cumene. β-Deuteration of cumene[16] had contrastingly little impact on oxidation profiles, the only significant isotope effect being that associated with the abolition of β-hydroxylation in KT5.

Table 2. Product formation rates (PFRs) and isotopic sensitivities for the oxidation of cumene (H), α-[D]cumene (α-[D]) and β-[D_6]cumene (β-[D]) by wild-type CYP102A1 (WT) and variant KT5.

	PFR			PFR(H)/PFR(D)	
	H	α-[D]	β-[D]	α-[D]	β-[D]
WT					
α-hydroxylation	106	26	116	4.0	0.9
β-hydroxylation	–	–	–	–	–
o-hydroxylation	12	29	12	0.4	1.0
α,β-desaturation	12	5.1	10	2.3	1.2
α,β-oxide formation	–	–	–	–	–
TOTAL	130	60	138	2.2	0.9
coupling (%)	31	13	34		
peroxide (%)	33	33	34		
KT5					
α-hydroxylation	189	45	217	4.2	0.9
β-hydroxylation	8.9	13	–	0.7	Inf.
o-hydroxylation	–	–	–	–	–
α,β-desaturation	77	24	76	3.2	1.0
α,β-oxide formation	3.9	0.7	6.6	5.9	0.6
α,β-desaturation + α,β-oxide formation	81	25	83	3.3	1.0
total	279	83	300	3.4	0.9
coupling [%]	37	10	41		
peroxide [%]	14	14	14		

Coupling = total product formation as % of NADPH consumption. Inf. = infinite. PFRs expressed in nmol min^{-1} (nmol P450)$^{-1}$. All data are means of at least three experiments with standard deviations < 5% of the mean.

The fact that the desaturation of cumene by CYP102A1 is sensitive to α-deuteration but relatively insensitive to β-deuteration implies that the first of the two hydrogen abstractions takes place predominantly from the α-carbon.[6] Given that α-hydroxylation is more sensitive to α-deuteration than α,β-desaturation, there could, however, be a competing desaturation pathway initiated by β-abstraction. The increased β-hydroxylation percentages given by KT5 with most substrates suggest that the β-carbon lies closer to the ferryl oxygen in KT5 than in WT, but the concomitantly high desaturation yields given by KT5 do not in fact substantiate the existence of a desaturation pathway initiated by β-abstraction, since a partition shift in favour of desaturation would also be expected if the first abstraction took place from the α-carbon. Nor is the relatively short oxyferryl–C_β distance in KT5 sufficient in itself to tilt the hydroxylation/desaturation partition in favour of desaturation. Whereas desaturation competes effectively against hydroxylation in the oxidation of cumene by KT5, hydroxylation dominates when propylbenzene is the substrate even though C_α and C_β are both accessible to the ferryl oxygen (1% desaturation vs 20% α-hydroxylation and 78% β-hydroxylation). Evidently, the substitution pattern at C_α and C_β is critical; in this context, it is worth noting that the isopropyl group has featured prominently among the examples of desaturation reported in other P450 enzymes.[5,8,9] It remains to be established whether substitution levels influence desaturation yields by retarding radical rebound, accelerating the second hydrogen abstraction, causing substrates to orientate differently in the active site, or by some other means. It is also unclear at this point whether electronic effects are involved. While we have demonstrated that deuterium does not migrate to C_β during the oxidation of α-[D]cumene, which argues against the involvement of β-cation rearrangement, this mechanism cannot yet be ruled out as the Fe^{III}(OH) intermediate could capture the α-proton while in transit to the β-carbon in preference to abstracting a second β-proton.

In conclusion, the oxidation of alkylbenzenes, a relatively simple class of compounds, by CYP102A1 involves a gamut of competing P450 activity types: terminal, sub-terminal, benzylic and aromatic hydroxylation, terminal and sub-terminal desaturation, epoxidation of the resulting olefins and, in one case, *O*-dealkylation (*p*-isopropylanisole, Table S6). Research into alkylbenzenes with different branching structures and other compound types will be required to support and develop the mechanistic framework outlined above. It will be interesting, for example, to see whether natural substrates, such as unsaturated fatty acids, particularly branched fatty acids,[17] are as prone to desaturation as non-natural substrates.[7,18] CYP102A1 is a convenient vehicle to explore desaturation as it is self-sufficient and has variants that can rapidly oxidise a wide array of non-natural substrates in vitro. KT5 is also well suited for the study of the partition between desaturation and ω-hydroxylation,[19,20] as it is an effective desaturase and also possesses some ω-hydroxylase activity. However, even the wild-type enzyme shows slight desaturase activity with most of the substrates investigated, suggesting that this rarely reported P450 activity type may occur more widely than hitherto supposed.

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Keywords: C–H activation · cytochromes · dehydrogenation · isotope effects · oxidase

[1] J. T. Groves in *Cytochrome P450: Structure, Mechanism and Biochemistry*, 3rd ed. (Ed.: P. R. Ortiz de Montellano), Kluwer Academic/Plenum, New York, **2005**, pp. 1–85.
[2] P. R. Ortiz de Montellano, J. J. De Voss in *Cytochrome P450: Structure, Mechanism and Biochemistry*, 3rd ed. (Ed.: P. R. Ortiz de Montellano), Kluwer Academic/Plenum, New York, **2005**, pp. 183–245.
[3] G. Di Nardo, A. Fantuzzi, A. Sideri, P. Panicco, C. Sassone, C. Giunta, G. Gilardi, *J. Biol. Inorg. Chem.* **2007**, *12*, 313–323.
[4] B. M. van Vugt-Lussenburg, M. C. Damsten, D. M. Maasdijk, N. P. Vermeulen, J. N. Commandeur, *Biochem. Biophys. Res. Commun.* **2006**, *346*, 810–818.
[5] Y. Jiang, X. He, P. R. Ortiz de Montellano, *Biochemistry* **2006**, *45*, 533–542.
[6] A. E. Rettie, M. Boberg, A. W. Rettenmeier, T. A. Baillie, *J. Biol. Chem.* **1988**, *263*, 13733–13738.
[7] X. Guan, M. B. Fisher, D. H. Lang, Y. M. Zheng, D. R. Koop, A. E. Rettie, *Chem.-Biol. Interact.* **1998**, *110*, 103–121.
[8] R. S. Obach, *Drug Metab. Dispos.* **2001**, *29*, 1599–1607.
[9] C. A. Reilly, G. S. Yost, *Drug Metab. Dispos.* **2005**, *33*, 530–536.
[10] H. Sun, W. J. Ehlhardt, P. Kulanthaivel, D. L. Lanza, C. A. Reilly, G. S. Yost, *J. Pharmacol. Exp. Ther.* **2007**, *322*, 843–851.
[11] K. Kassahun, K. Skordos, I. McIntosh, D. Slaughter, G. Doss, T. Baillie, G. Yost, *Chem. Res. Toxicol.* **2005**, *18*, 1427–1437.
[12] S. G. Bell, N. Hoskins, C. J. C. Whitehouse, L. L. Wong, *Met. Ions Life Sci.***2007**, *3*, 437–476.
[13] V. B. Urlacher, S. G. Bell, L. L. Wong in *Modern Biooxidation* (Eds.: R. D. Schmid, V. Urlacher), Wiley-VCH, Weinheim, **2007**, pp. 99–122.
[14] J. D. Bloom, M. M. Meyer, P. Meinhold, C. R. Otey, D. MacMillan, F. H. Arnold, *Curr. Opin Struct. Biol.* **2005**, *15*, 447–452.
[15] C. J. Whitehouse, S. G. Bell, H. G. Tufton, R. J. Kenny, L. C. Ogilvie, L. L. Wong, *Chem. Commun.* **2008**, 966–968.
[16] K. Sugiyama, W. F. Trager, *Biochemistry* **1986**, *25*, 7336–7343.
[17] M. J. Cryle, R. D. Espinoza, S. J. Smith, N. J. Matovic, J. J. De Voss, *Chem. Commun.* **2006**, 2353–2355.
[18] P. H. Buist, *Nat. Prod. Rep.* **2004**, *21*, 249–262.
[19] A. E. Rettie, P. R. Sheffels, K. R. Korzekwa, F. J. Gonzalez, R. M. Philpot, T. A. Baillie, *Biochemistry* **1995**, *34*, 7889–7895.
[20] D. Kumar, S. P. De Visser, S. Shaik, *J. Am. Chem. Soc.* **2004**, *126*, 5072–5073.

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