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Revisiting Cytochrome P450-Mediated Oxyfunctionalization of Linear and Cyclic Alkanes

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Abstract: Cytochrome P450 monooxygenases (CYPs) of the CYP153 family catalyse terminal hydroxylation of *n*-alkanes. Alkane hydroxylating mutants of self-sufficient CYP102A1 have also been described. We evaluated two CYP153s (a three-component system and a fused self-sufficient CYP), wild-type CYP102A1 and nine CYP102A1 mutants, for the conversion of three cycloalkanes (C_6, C_7 and C_8) and three *n*-alkanes (C_6, C_8 and C_{10}) using whole cells (WCs) and crude cell-free extracts (CFEs). The aim was to identify substrate–enzyme combinations that give high product titres and space-time yields (STYs). Comparisons were made using total turnover numbers (TTNs) and turnover frequencies (TOFs) to normalize for CYP expression. Reactions were carried out using high enzyme and substrate concentrations compatible with high STYs. Under these conditions CYP102A1 and the double R47L,Y51F mutant, although not regioselective, performed better on all substrates in terms of product titres over 8 h, and thus STYs and TTNs, than heavily mutated variants that have been reported to give very high TOFs. CYP153A6, with its ferredoxin (Fdx) and ferredoxin reductase (FdR), emerged as the superior catalyst for conversion of *n*-alkanes. In addition to its excellent regioselectivity it also gave the highest final product titres and STYs in WC conversions of hexane and octane. Interaction with FdR and Fdx initially limited performance in CFEs, but with additional FdR and Fdx gave 1-octanol titres of 50 $mmol \cdot L_{BRM}^{-1}$ and TTNs exceeding 12,000 over 18 h, rivalling results reported with self-sufficient CYPs. Selecting biocatalysts for application requires caution, since experimental conditions such as amount of substrate added and solubility as well as cofactor dependence and regeneration can have a profound effect on catalyst performance, while stability and efficiency with regard to cofactor usage (coupling efficiency) are at least as important as TOFs when high product titres and STYs are the target.

Keywords: alkane hydroxylation; C–H activation; CYP102A1 (P450 BM3); CYP153A6; self-sufficient cytochrome P450 monooxygenase

Introduction

Oxyfunctionalization of unactivated carbons in general and in linear and cyclic alkanes specifically is a problem that is currently receiving much attention from both chemists and biochemists as is evident from the number of recent reviews.[1–5] One class of enzymes that is quite successful at these reactions are the cytochrome P450 monooxygenases (CYPs). Cytochrome P450s are heme-dependent enzymes that activate and insert one oxygen atom from molecular oxygen into C–H bonds while the second is reduced to water. Two families of CYPs that can even accomplish terminal hydroxylation of linear alkanes, a difficult and much sought after reaction, are the CYP52 family from alkane utilizing yeasts[6,7] and the CYP153 family from bacteria.[8,9]

Octane hydroxylation reactions using CYP153A6 have reached space-time yields (STYs) of up to 0.8 $g \cdot L^{-1} h^{-1}$,[10] which although not yet feasible for commercial production of a bulk chemical such as 1-octanol is higher than the minimum requirement of 0.1 $g \cdot L^{-1} h^{-1}$ for the application of monooxygenases in the production of fine chemicals.[11] In these studies

Table 1. Published data on alkane conversion by CYP153s, CYP102A1 and some CYP102A1 mutants. Data summarized here are for reactions that were carried out under analytical conditions using low enzyme and substrate concentrations and short reaction times.

Enzyme	Octane TOF [min^{-1}]	Product ratio 1:2:3:4[a]	Cyclohexane TOF [min^{-1}]	Cyclooctane TOF [min^{-1}]	Ref.
CYP153A6 FdRFdx	58	95:5:0:0	NA	NA	[8]
CYP153A13 FdRFdx[b]	1	100:0:0:0	10	NA	[8]
CYP153A13 RhFred	57	100:0:0:0	3	NA	[13]
CYP102A1 WT	4	0:17:40:43	0.75	2	[14,15]
CYP102A1 1-12G	150	5:82:11:3	NA	NA	[16]
CYP102 A1 139-3	480	1:61:20:17	NA	NA	[16]
CYP102A1 R47L,Y51F	NA	NA	14	NA	[15]
CYP102A1 F87V,A328F	22	–:92:–:–[c]	NA	230	[14]

[a] Ratio 1-octanol:2-octanol:3-octanol:4-octanol.
[b] Not analyzed with its own FdR and Fdx, but with Fdx from *Sphingomonas* sp. HXN200 and FdR from *Mycobacterium* sp. HXN1500.
[c] 92% 2-octanol reported, other products were not specified.

CYP153A6 was co-expressed from a single operon together with its ferredoxin (Fdx) and ferredoxin reductase (FdR), required to transfer electrons from NADH to the heme iron. It is generally assumed that electron transfer in such three-component systems slows down catalysis compared to CYPs with an electron transfer partner fused to the heme domain, the so-called self-sufficient CYPs. The best studied example of the latter group is CYP102A1 (P450 BM3), a bacterial subterminal fatty acid hydroxylase.[12] In order to overcome these shortcomings some CYP153s have been fused to the reductase domain from another natural self-sufficient CYP P450RhF.[9] The most extensively studied of these self-sufficient CYP153s is CYP153A13RhFred.[13] The hydroxylation rate, expressed as turnover frequencies (TOFs) towards *n*-octane reported with the CYP153A13RhFred as purified enzyme or in cell-free extracts (CFEs) is much higher than that for CYP153A13 in the three-component system, but similar to the rate of CYP153A6 in the three-component system (Table 1). Although these rates are higher than the octane hydroxylation rates reported with wild-type CYP102A1, octane hydroxylation rates reported for CYP102A1 mutants far exceed these. Nevertheless, no CYP102A1 mutants have been obtained to date that can perform regioselective terminal hydroxylation as is seen with CYP153s. However, two of the mutants listed in Table 1 can produce 2-octanol with >80% selectivity.[14,16]

The rates compared in Table 1 were reported for reactions carried out under analytical conditions with reactions mixtures containing relatively low enzyme and substrate concentrations and with excess NAD(P)H supplied, thus avoiding the need for cofactor regeneration. Such reaction conditions can, however, not be used for preparative scale biotransformations. Maurer and co-workers[15] used equal volumes of buffer containing a purified CYP102A1 mutant (6.6 μM) and cyclohexane in a preparative biphasic biotransformation (10-mL scale) to produce *ca.* 72 $mmol \cdot L_{BRM}^{-1}$ after 100 h with cofactor regeneration by a NADPH-dependent mutant formate dehydrogenase. In the case of CYP153A6, whole cell (WC) biotransformations are currently deemed the most practical for preparative reactions, because it comprises a three-component system and requires cofactor regeneration. In reactions reported thus far *n*-octane was added as a second phase with up to 300 μL octane added to 1 mL biotransformation reaction mixtures. In one such a reaction 67 mmol $octanol \cdot L_{BRM}^{-1}$ were produced within 24 h by a resting WC suspension containing 4.5 μM CYP153A6.[17]

CYP153s and the alkane hydroxylating CYP102A1 mutants listed in Table 1, which had been reported to give high TOFs with octane (1-12G and 139-3), cyclooctane (F87V,A328F) and cyclohexane (R47L,Y51F), have never been compared in a single study. In this study we set out to compare the two CYP153s (as a wild-type three-component system and an artificially fused self-sufficient CYP) with wild-type CYP102A1 as well as nine CYP102A1 mutants (five previously described and four new combinatorial mutants; Table 2). These twelve CYPs were evaluated for the biotransformation of three cycloalkanes (cyclohexane, cycloheptane and cyclooctane) and three linear alkanes (*n*-hexane, *n*-octane and *n*-decane) using WCs and crude CFEs. Although regioselectivity is essential for the practical application of *n*-alkane hydroxylations, the focus of this study was to compare these different CYPs in terms of their ability to achieve high product titres and STYs. Comparisons were done using TTNs and TOFs in order to normalize for CYP expression. An important goal was to identify the substrate–enzyme combinations that could give the highest STYs in order to use these in future stud-

Table 2. CYPs investigated in this study.

Enzyme name	Parent enzyme	Mutations		Cofactor dependance	Ref.
		Total number	Amino acid substitutions in binding pocket		
153A6FdxFdR	CYP153A6	1	–	NADH	[18]
153A13RhFred	CYP153A13	–	–	NADPH	[9]
102A1	CYP102A1	–	–	NADPH	[19]
139-3	CYP102 A1	11	V78A	NADPH	[20]
1-12G	CYP102A1	17	V78A, A82L & A328V	NADPH	[16]
47-51	CYP102A1	2	R47L, Y51F	NADPH	[15]
87-328	CYP102A1	2	F87 V, A328F	NADPH	[14]
102A1NADH	CYP102A1	2		NADH	[21]
139-3C1	CYP102A1	13	R47L, Y51F, V78A	NADPH	this study
139-3C1NADH	CYP102A1	15	R47L, Y51F, V78A	NADH	this study
87-328NADH	CYP102A1	4	F87V, A328F	NADH	this study
139C2NADH	CYP102A1	17	R47L, Y51F, V78A, F87V, A328F	NADH	this study

ies to investigate process parameters limiting oxyfunctionalization by CYPs. Biotransformations were therefore carried out under conditions of high enzyme and substrate concentrations compatible with the conditions required for preparative reactions setting a premium on high STYs. We also sought, by comparing a large number of enzymes on a range of substrates, to probe issues relating to substrate solubility and uptake, the type and regeneration of cofactors and the perceived superiority of one component systems.

Results and Discussion

CYP Expression

The CYPs listed in Table 2 were targeted because they had previously been reported to give high TOFs with alkanes while also being relatively regioselective for hydroxylation at C-2 of *n*-alkanes in the case of 139-3, 1-12G and 87-328. New combinatorial mutants were expected to have the same or improved activity and regioselectivity as the parental mutants. NADH-dependent versions of wild-type CYP102A1 and some of the mutants were created to investigate the effect of cofactor dependence on activity and specificity.

All twelve CYPs were cloned into pET28 and expressed in *E. coli* BL21-Gold (DE3) using auto-induction medium. CYP levels in WCs and CFEs varied between CYPs and between experiments. Because the biotransformation reaction mixture (BRM) was always prepared using a constant wet biomass of 50 $g \cdot L^{-1}$ ($\approx$5 $g \cdot L^{-1}$ dry cell weight) final CYP concentrations in the BRM also varied between CYPs (Figure 1) and between experiments. In the case of 1-12G, 87-328 and 139-3C2NADH, CYP concentrations in the WC BRM were always less than 2.5 μM. Müller and co-workers also reported significantly decreased expression levels for a CYP102A1 mutant (19A12) carrying 18 mutations, which included the A328F mutation.[21] WC concentrations for the rest of the CYPs typically varied between 4 and 7 μM, but occasionally dropped below 4 μM. CYP recovery in CFEs was *ca.* 20% in the case of the CYP153s and on average *ca.* 50% for CYP102A1 and its mutants so that CYP concentrations in CFE BRM generally varied between 1 and 5 μM (Figure 1). The relatively low CYP recovery in the CFEs, especially in the case of the CYP153s, might be improved through the use

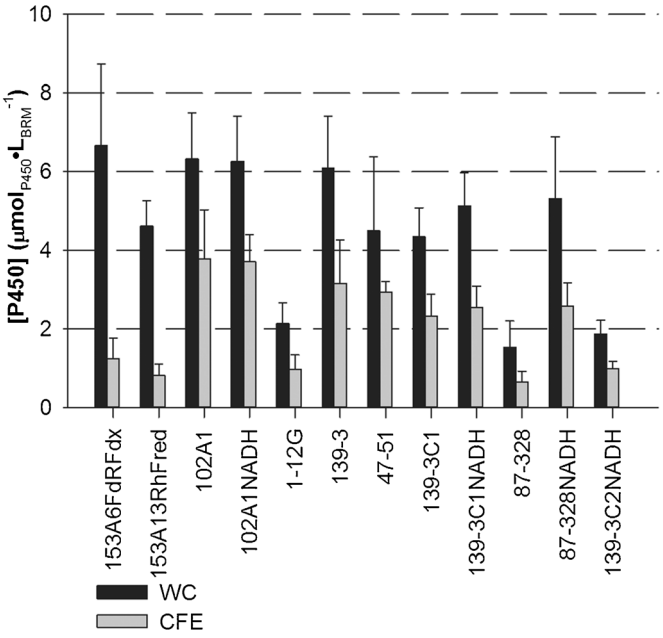

Figure 1. CYP content in WC and CFE containing BRM. Averages and standard deviations are for six and five different experiments, respectively.

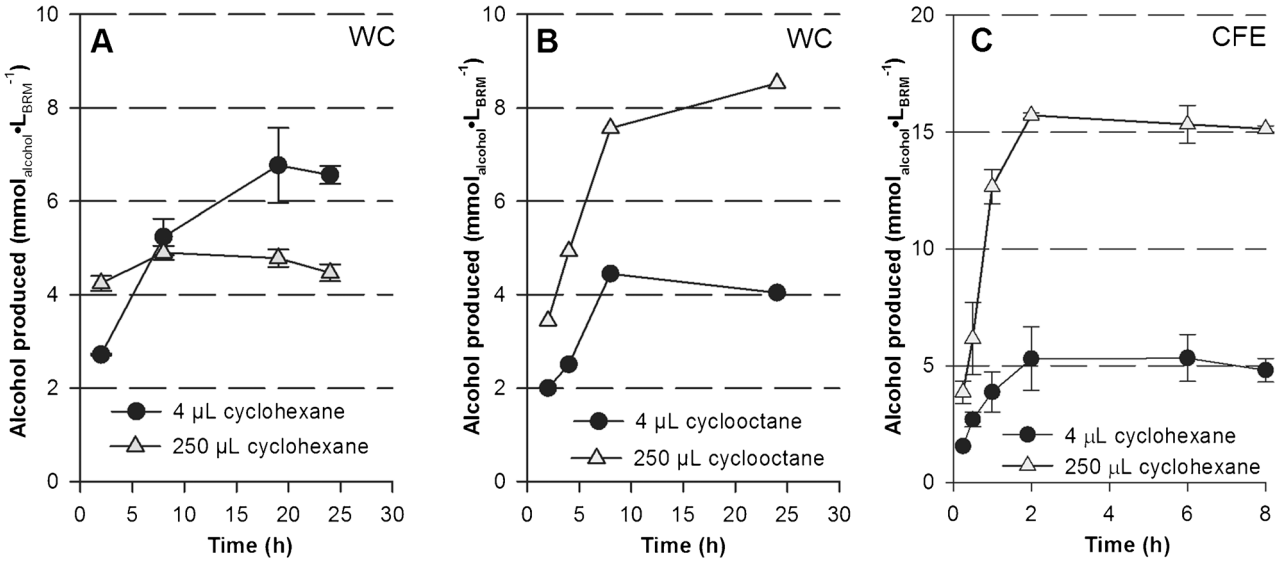

Figure 2. Time course of whole cell (WC) biotransformations of (A) cyclohexane and (B) cyclooctane using 139-3C1NADH to investigate the effect of different amounts of substrate (4 or 250 µL) added to 1 mL BRM. (C) Time course of cyclohexane (4 or 250 µL) conversion by 139-3C1NADH using cell free extracts (CFE) containing BmGDH for cofactor regeneration. Error bars are for duplicate reactions using WCs or CFEs from the same culture.

of a different buffer and requires further investigation.

Reaction Conditions for Comparison

Preliminary WC experiments had indicated that 139-3C1 and 139-3C1NADH were promising catalysts for the conversion of cycloalkanes. Cyclohexane conversion by 139-3C1 and octane conversion by 153A6FdRFdx were therefore used to investigate WC biotransformation conditions by statistically designed experiments (see the Supporting Information, Section A). Conditions which previously had given promising results for WC conversion of octane by 153A6FdRFdx[10,17] were used as starting point for these experiments. Amount of substrate added, the type of buffer and the buffer concentration emerged as significant factors affecting activity in 2 h and 24 h reactions. Buffer (Tris-HCl, pH 8, 200 mM) as well as other parameters were chosen to select a set of conditions that would favour both enzyme–substrate combinations. Although the difference in results obtained with 24-mL and 40-mL vials were not statistically significant, reactions were performed in 40-mL vials to ensure that no oxygen limitation occurred.

The same conditions used for WC reactions were used for CFE reactions. Cellular metabolism of glucose and glycerol was relied on to take care of cofactor regeneration in WC reactions. In the case of CFE reactions, CFEs from *E. coli* BL21-Gold (DE3) expressing the *Bacillus megaterium* glucose dehydrogenase (BmGDH) was added for cofactor regeneration. This enzyme was selected because it accepts both NAD^+ and $NADP^+$ with similar turnover numbers.[22]

Amount of substrate added had opposite effects in the case of cyclohexane and octane. In the case of octane it was evident from the statistically designed experiments that low substrate concentrations not only resulted in lower hydroxylase activity but also in over-oxidation to give significant amounts of the corresponding diol and carboxylic acid. In the case of cyclohexane time-course experiments with all three cycloalkanes indicated that only cyclohexane was inhibitory to WCs when 250 µL were added, while CFEs yielded higher product concentrations when 250 µL cyclohexane were used (Figure 2). It was thus decided to use 4 µL per 1 mL BRM in WC biotransformations of cyclohexane but 250 µL per 1 mL BRM in the case of WC reactions with the other five substrates as well as for all CFE reactions. From the time-course experiments reaction times of 8 h were selected to compare the different enzymes in WCs, while 1 h and 8 h reactions were selected for CFE comparisons. Even with very active enzymes such as 139-3C1NADH on cyclohexane, 1 h was still at the end of the linear range for the calculation of TOFs, while 8 h measurements were used for comparison of WC and CFE conversions using TTNs. Particularly with whole cells there were most likely in many cases, i.e., cyclooctane conversion by 139-2C1NADH (Figure 2B), still residual activity left after 8 h, so that the TTNs calculated after 8 h represent a lower boundary for comparison.

All biotransformations were performed at 20 °C because we had previously observed with WC biotransformations of octane by 153A6FdRFdx that this

lower temperature ensured that activity could be maintained for longer so that higher product titres could be achieved over 24 h.[10] CFE conversions of cyclohexane and octane by 139-3C1NADH and 153A6FdRFdx, respectively, confirmed that 20 °C favoured higher product titres and thus higher TTNs over 8 h.

Cycloalkane Conversion

The cycloalkanes were very poor substrates for the terminal hydroxylases 153A6FdRFdx and 153A13RhFred (Figure 3 and Supporting Information, Figure S3) and thus this section focuses on the results obtained with CYP102A1 and its mutants. Comparison of the TTNs for the different CYPs obtained with WCs and CFEs, indicated that CFEs generally performed better than WCs, despite the fact that activities in CFEs generally levelled off after 1 h (Supporting Information, Figure S3). TTNs calculated over 8 h were thus in some cases up to five times higher with CFEs than with WCs (Figure 3) with TTNs of more than 8000 achieved with 1-12G and 47-51 on cyclohexane and cycloheptane. The higher TTNs observed with CFEs are most likely due to substrate uptake being limited in WCs.

Activities towards cyclohexane and cycloheptane were generally higher than towards cyclooctane with TOFs of *ca.* 100 min^{-1} achieved with cycloheptane during conversions with CFEs using 1-12G, 139-3C1 and 139-3C1NADH. The highest TOFs with cyclohexane were *ca.* 80 min^{-1} obtained with CFEs containing 1-12G, 47-51 and 139-3C1, while the highest TOFs with cyclooctane were only *ca.* 60 min^{-1} obtained with 1-12G, 139-3C1 and 139-3C1NADH. The TOF obtained with 47-51 with cyclohexane was higher than the activity previously described (14 min^{-1}; Table 1[15]). These authors had used 0.4 mM cyclohexane for these assays, but had already pointed out the increase in activity with increase in amount of cyclohexane added. In a biphasic reaction using 6.6 μM purified 47-51, they had obtained over 10 h *ca.* 20 $mmol_{alcohol} \cdot L_{BRM}^{-1}$, while we obtained with 3 μM 47-51 in CFEs over 8 h 26 $mmol_{alcohol} \cdot L_{BRM}^{-1}$. The 1-12G and 139-3 mutants, which had not previously been tested with cyclic substrates, both displayed higher TOFs with cycloheptane and cyclooctane than 102A1. Wild-type 102A1 on the other hand displayed higher TOFs with cyclohexane and cyclooctane than activities previously reported[14,15] (Table 1). This might again be explained by the fact that these authors added less than 0.5 mM substrate instead of a second phase as we used. Surprising though is that the cyclooctane activity we observed with 87-328 was the same as the activity with 102A1 while Weber and co-workers[14] had reported that activity of the

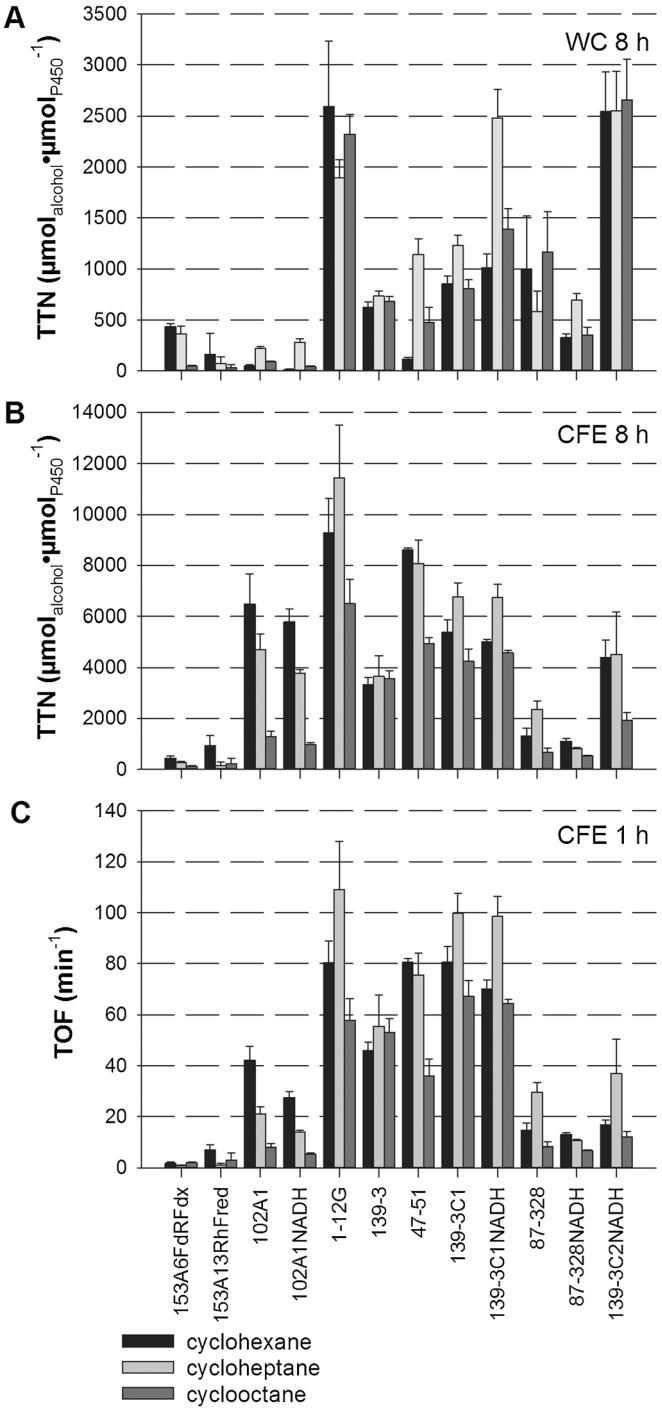

Figure 3. Comparison of activities for the hydroxylation of cyclohexane, cycloheptane and cyclooctane by twelve different CYPs. (A) Total turnover numbers (TTNs) achieved over 8 h in whole-cell (WC) biotransformtions. (B) TTNs achieved over 8 h in cell-free extracts (CFE) biotransformations. (C) Turnover frequencies (TOFs) for CFE reactions calculated for 1 h reactions. Initial CYP concentrations in the BRM were used to calculate TOFs. Results are the averages for triplicate reactions using WCs or CFEs from the same culture. The same trends were observed in two independent experiments.

F87V,A328F mutant towards cyclooctane was 115 times improved (Table 1). From our results it appears as if the F87V,A328F mutations destroyed activity towards cyclic substrates, since TOFs of 139-3C2NADH towards all three cycloalkanes are less than 40% of those of 139-3C1NADH

Although some enzymes such as 102A1 and 47-51 had lower TOFs, they were able to produce over 8 h more cyclohexanol and cycloheptanol (up to 25 mmol·L_{BRM}^{-1}) than 1-12G, 139-3C1 and 139-3C1NADH (Supporting Informatrion, Figure S3), because their concentrations in CFEs were higher and they also maintained activity over longer reaction times. It is not clear why activity of the highly active enzymes almost ceased after 1 h. This levelling off in activity after 1 h is, however, not strictly activity related, since alcohol production from cyclooctane also levelled off even though cyclooctanol concentrations after 1 and 8 h were between 5 and 10 mmol·L_{BRM}^{-1} less than from cyclohexanol and cycloheptanol. Müller and co-workers also observed that heptane hydroxylation activity of two highly active CYP102A1 mutants levelled off after 1 h.[21] They had used purified enzymes and lower enzyme and substrate concentrations so that activity levelled off when only 0.6 mmol·L_{BRM}^{-1} heptanols (mixture of regioisomers) had been produced.

Linear Alkane Conversion

All the enzymes were also tested for the conversion of *n*-hexane, *n*-octane and *n*-decane. Enzymes 153A6FdRFdx, 153A13RhFred, 1-12G, 139-3 and 87-328 were included in this study because of their previously reported activities towards linear alkanes (Table 1), but activity data have not been reported for all of them on all these substrates. For linear alkanes regiospecific hydroxylation is an important requirement. Regiospecificities of the previously described enzymes were as expected and changing the cofactor specificity of 102A1 and its mutants did not affect the regioselectivity (Figure 4). The new combinatorial constructs 139-3C1 and 139C1NADH displayed for all three *n*-alkanes the same regioselectivity as 139-3, while 139-3C2NADH displayed the same strong preference for hydroxylation at the 2-position as the previously described 87-328 and 1-12G. These results again confirm that mutation of Ala at position 328 to a larger residue forces hydroxylation to the 2-position.[14] It is unfortunate that all these 2-specific enzymes, except 87-328NADH were expressed at relatively low levels compared with 102A1 and its other mutants.

In WC reactions 153A6FdRFdx performed very well over 8 h with both *n*-hexane and *n*-octane producing up to 36 and 23 mmol·L_{BRM}^{-1} 1-hexanol and 1-octanol, respectively, with TTNs of up to 4000 (Supporting Information, Figure S4 and Figure 5). The 102A1s in general, and specifically mutants 47-51 and 139-3C1, also performed very well in WC biotransformations of *n*-hexane achieving TTNs of more than 4000. However, 102A1 and its mutants gave compared to 153A6FdRFdx relatively poor conversions in WC biotransformations of *n*-octane, while WC biotransformation of *n*-decane yielded very poor results with all the enzymes. This probably points to substrate uptake limitation that is more pronounced at lower water solubilities.

In reactions with CFEs, TOFs were generally lower than observed for cycloalkanes, with the exception of 47-51 which displayed very high activity with *n*-

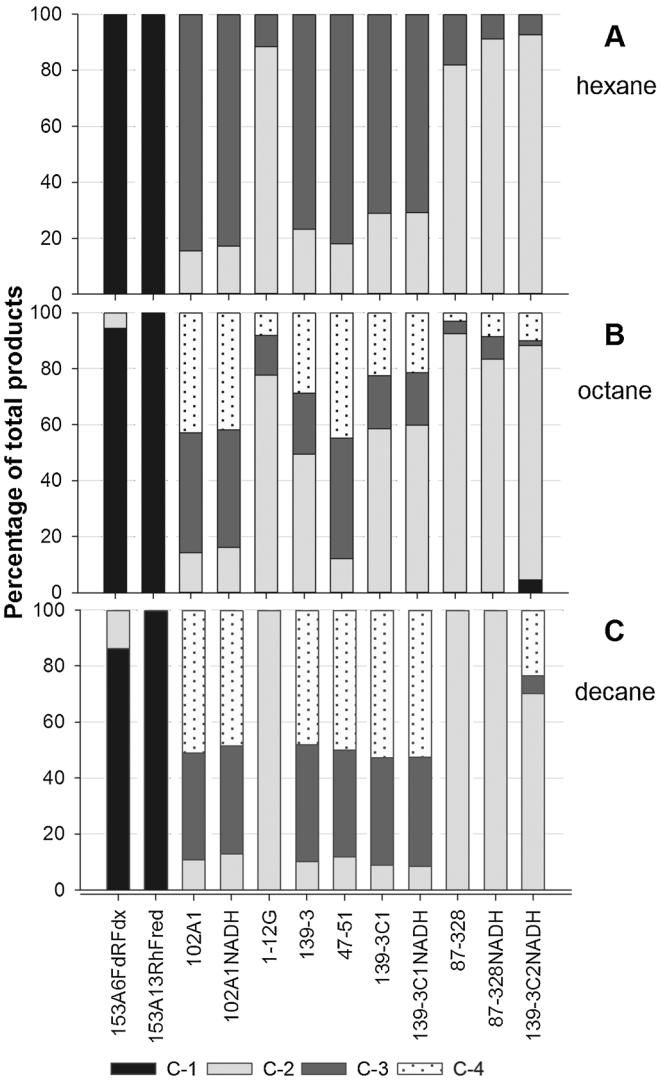

Figure 4. Regioselectivity of (A) hexane, (B) octane and (C) decane hydroxylation by twelve different CYPs. Data from 8 h reactions with cell free extracts were used to calculate regioselectivities. Results are the averages for triplicate reactions using WCs or CFEs from the same culture. The same trends were observed in two independent experiments.

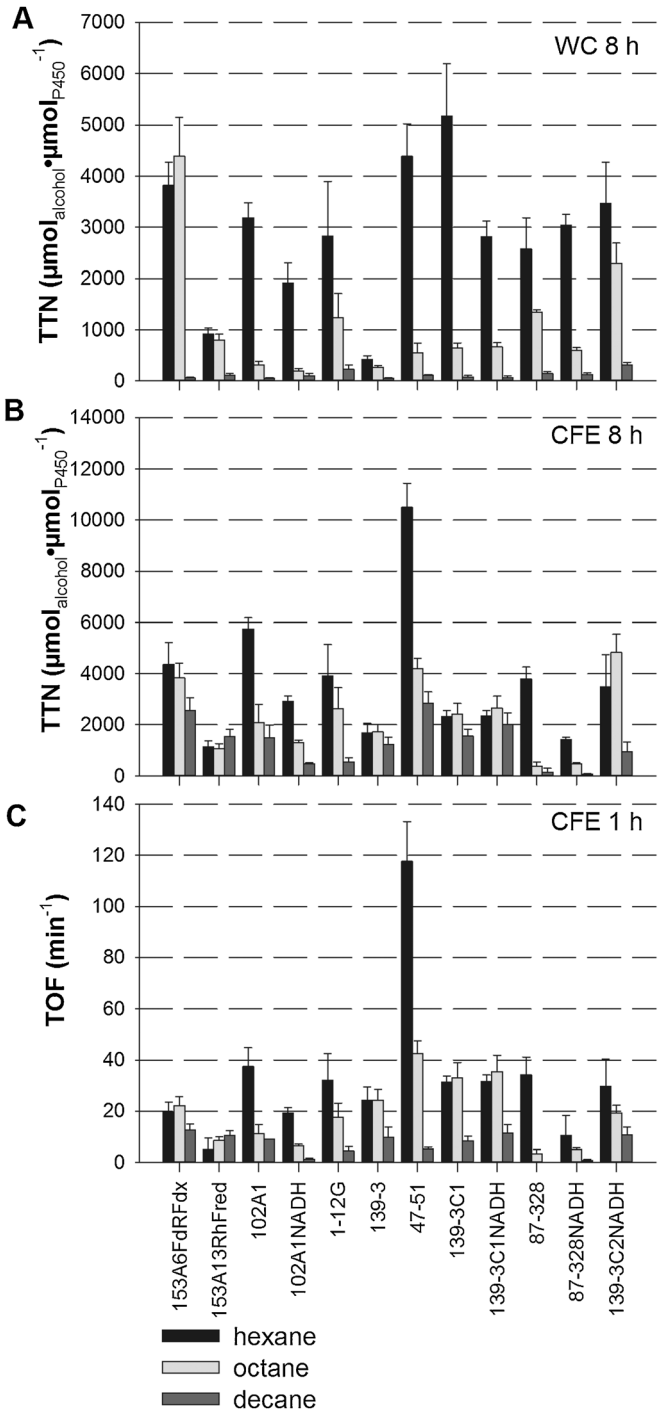

Figure 5. Comparison of activities for the hydroxylation of hexane, heptane and decane by twelve different CYPs. (A) TTNs achieved over 8 h in WC biotransformtions. (B) TTNs achieved over 8 h in CFE biotransformations. (C) TOFs for CFE reactions calculated for 1 h reactions. Initial CYP concentrations in the BRM were used to calculate TTNs and TOFs. Results are the averages for triplicate reactions using WCs or CFEs from the same culture. The same trends were observed in two independent experiments.

hexane. In the case of *n*-hexane and *n*-octane, 139-3 and its two derivatives 139-3C1 and 139-3C1NADH produced little additional products between 1 and 8 h (Supporting Information, Figure S4). As in the case of the cycloalkanes this is probably an indication that these enzymes have very high activities, but activities could not be maintained. Interestingly, although TOFs with *n*-decane for 47-51, 139-3C1 and 139-3C1NADH were approximately 30% of that for *n*-octane, activity lasted longer, resulting in decane TTNs over 8 h being 60 to 70% of octane TTNs. This is an indication that, different from what we argued in the case of the cyclooctane, deactivation of these 102A1 mutants is, after all, activity related.

This discrepancy between the cyclooctane and *n*-decane results can be explained if these enzymes are more uncoupled with cyclooctane as substrate than with *n*-decane. In the case of 153A6FdRFdx, 153A13RhFred as well as 102A1 and its NADH mutant there was still significant product formation by CFEs between 1 h and 8 h on all three linear alkanes (Supporting Information, Figure S4). Activity of these enzymes could most likely be maintained for longer, because their initial activities were slower and they are less uncoupled than some of the 102A1 mutants (uncoupling of these mutants is discussed later).

It is common practice to add catalase to the BRM when purified CYPs are used, in order to scavenge hydrogen peroxide produced by uncoupling.[15,21,23] However, addition of catalase to CFE reactions carried out with 153A6FdRFdx and 139-3C1NADH did not have a significant effect. Hydrogen peroxide scavenging activity in CFEs of *E. coli* is probably sufficient to eliminate hydrogen peroxide produced by the CYPs.[24] However, it is still possible that hydrogen peroxide or other reactive oxygen species cause immediate damage to the enzyme thereby contributing to the instability of the 102A1 mutants.

From the TTNs for CFE reactions it can be concluded that the enzymes with the highest sustained activity towards *n*-hexane are 47-51 and 102A1 followed by 153A6FdRFdx, 1-12G and 87-328. When regioselectivity is also considered 153A6FdRFdx is of interest for 1-hexanol production, while 1-12G and 87-328 might be of interest for 2-hexanol production.

In the case of *n*-octane the TOFs for CFE reactions indicate that 47-51 as well as the combinatorial mutants 139-3C1 and 139-3C1NADH have the highest activities with *n*-octane (33–36 min^{-1}). However, their regiospecificities are poor, making them uninteresting for practical application. On the other hand 153A6FdRFdx and the newly created combinatorial mutant 139-3C2NADH gave somewhat lower TOFs (19–22 min^{-1}), but both displayed comparable or better TTNs (*ca.* 4000) and excellent regioselectivity to produce 1- and 2-octanol, respectively.

The superior performance of 153A6FdRFdx in WCs towards *n*-hexane and *n*-octane, when compared to all the other enzymes and when compared to its relative performance in CFE, should be noted. Performance of 153A6FdRFdx is probably relatively better in WCs, because it functions as a three-component system with separate FdR and Fdx and interaction with the redox partners is improved in the confined space within the cell.

Factors Limiting Activity: Substrate Solubility

The fact that TTNs over 8 h for WC reactions were generally much lower than for CFE reactions, although many CFE reactions levelled off after 1 h, gives a very strong indication that substrate uptake was limiting. This has previously been reported by several authors using such hydrophobic substrates.[25–27] When polymyxin B, which is known to permeabilize *E. coli* cells through disruption of the cell wall and membranes,[28] was added to the BRM prior to biotransformations with linear alkanes using 153A6FdRFdx and 153A13RhFred, activity was enhanced in the case of *n*-octane and *n*-decane, but not for *n*-hexane (Supporting Information, Figure S5). Also the improvement in activity was more with *n*-decane than with *n*-octane, again supporting the notion that substrate uptake becomes more limiting as solubility decreases. In the case of *n*-hexane the addition of polymyxin B caused reduced product formation, probably because the stress caused by cell permeabilization outweighed the advantage of improved substrate uptake in this case where the substrate is more water soluble.

Even with CFEs substrate solubility apparently limits activity. When maximum activities of the different 102A1 mutants towards the different cyclic and linear alkanes were plotted against solubility data (experimentally observed and predicted by two different algorithms[29,30]), a clear correlation emerged (Figure 6). The correlation with the predicted solubility data is better, although the predicted solubility values from the two models are quite different. Two opposing solubility related factors probably influence activity, so that activity initially increases as solubility increases, but a maximum is reached at around the solubility of cycloheptane and *n*-hexane. Thus when solubility further increases the substrate becomes inhibitory (toxic) to the enzyme. This observation is particularly intriguing when it is further considered that activity increases as the ratio of substrate to aqueous phase increases even when the smallest amount of substrate added already exceeds the solubility limit, i.e., the 37 mmol·L_{BRM}^{-1} cyclohexane added to CFE of 139-3C1NADH was more than the solubility of cyclohexane in water, but activity was still improved when more cyclohexane was added (Figure 2C).

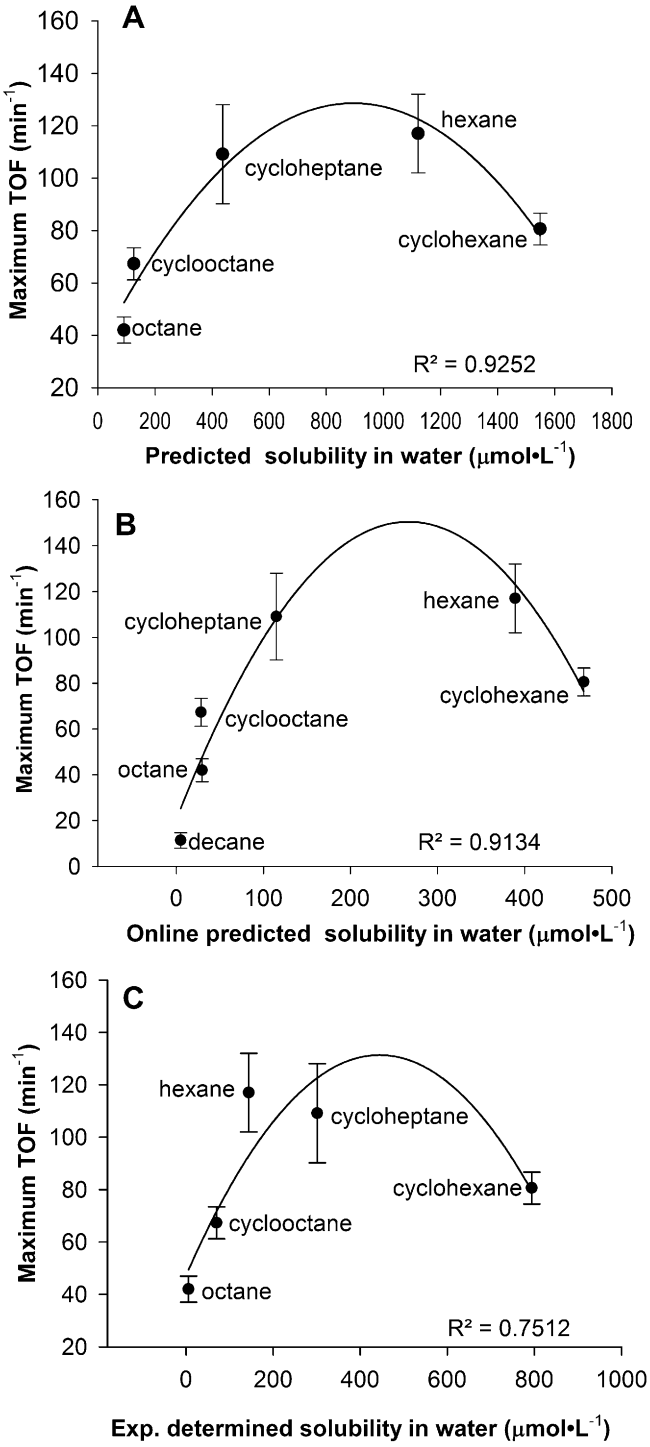

Figure 6. Relationship between water solubility and maximum TOFs observed with 102A1 mutants towards the different substrates fitted to polynomic functions. Predicted solubility data (A),[30] (B)[29] vcclab.org/web/alogps/and (C)[30] experimental data. Large discrepancies exist between experimentally determined and predicted solubility of hexane.

Factors Limiting Activity: Cofactor Dependence and Regeneration

153A6FdRFdx is NADH-dependent, while 153A13RhFred as well as 102A1 and all its original mutants are NADPH-dependent. Since NAD(H) levels in *E. coli* WCs are higher than NADP(H) levels[31] and attractive cofactor regenerating enzymes such as formate dehydrogenase and phosphite dehydrogenase are as WT enzymes NAD^+-dependent,[32,33] it would have been convenient if all the enzymes used in this study could have been NADH-dependent. In order to determine whether this would have been feasible and whether changing cofactor dependence will change other properties of the enzyme, cofactor dependence of 102 A1 and two of its mutants 139-3C1 and 87-328 was changed to NADH. Changing cofactor dependence did not significantly change substrate specificity or regioselectivity. However, despite the fact that an excess of the relevant added cofactors as well as GDH were present in CFE reactions, 102A1NADH as well as 87-328NADH in most instances displayed lower TOFs than their original NADPH-dependent counterparts, while performance of 139-3C1NADH was mostly the same as its parent. Strangely, expression of 87-328NADH was consistently much higher than expression of 87-328 (Figure 1). Several *E. coli* transformants carrying 87-328 were tested and all displayed low expression levels. It is not possible to explain without further experiments and creating more mutants why the 87-328 mutants had lower expression levels and how the mutations in the CPR domain could alleviate the problem. One might speculate that it is due to altered codons that disrupt (in the case of the 87-328 mutations) and restore (in the case of the CPR mutations) synchronization between transcription and translation.

In WCs the glucose and glycerol metabolisms of *E. coli* should be adequate for cofactor regeneration.[5] Efforts to co-express various dehydrogenases using Duet vectors repeatedly resulted in significantly lower expression levels of the CYPs and this route was not pursued. In order to ensure that cofactor regeneration in CFEs will be sufficient, CYP containing CFEs were mixed with an equal volume of CFE from a strain expressing the BmGDH. Comparing results for 153A6FdRFdx, 139-3C1 and 139-3C1NADH from reactions carried out with and without GDH showed that in the case of the 102A1 mutants the added GDH significantly improved activity, but with 153A6FdRFdx this was not the case (Supporting Information, Figure S6). It thus appears that the 102A1 mutants with higher initial reaction rates become limited by cofactor regeneration if the GDH is not present, while the wild-type *E. coli* enzymes are sufficient for the slower 153A6FdRFdx. Additionally the 102A1 mutants are probably much more uncoupled and therefore wasting reducing equivalents; 139-3 and 1-12G reportedly display respectively only 22 and 37% coupling to NADPH.[16] It has also been reported that the gluconolactone produced by the GDH causes acidification.[34] The pH in the reactions of the 102A1 mutants thus probably drops much faster causing denaturation of the CYPs. This problem was not anticipated, because the BRM contains 200 mM Tris buffer and product formation in CFE never reached concentrations of more than 27 $mmol \cdot L_{BRM}^{-1}$. Additionally 153A6FdRFdx reactions carried out with CFEs from more concentrated cell suspensions demonstrated that cofactor regeneration by GDH caused inhibition of the CYP reaction after 1 h (Supporting Information, Figure S7). It thus appears that wild-type enzymes in *E. coli* are also wasting cofactors regenerated by the GDH, thus contributing to the acidification.

Factors Limiting Activity: Redox Partners – Three Component System *vs.* a Single Protein

TTNs over 8 h for 153A6FdRFdx were similar with WC and CFE reactions of *n*-hexane and *n*-octane, while 102A1 and its mutants generally performed significantly better in CFE reactions (Figure 5). One possible explanation for this might be that the separate FdR and Fdx might become too dilute when they are no longer confined to the cell.[18] It was thus decided to test reactions with more concentrated cell suspensions. Initially in the presence of GDH the reactions stopped after 1 h as described above, and there was no improvement in the TOF over 1 h (Supporting Information, Figure S8). When the GDH was omitted the TOF remained the same but the reaction continued for 8 h. When in a new experiment additional CFE from a strain expressing only FdR and Fdx was added, the TOF increased significantly when additional FdR and Fdx were added (Figure 7). The TOF of the reaction without additional FdR and Fdx was lower than in the previous experiment (Supporting Information, Figure S7), because reactions in this case were carried out without the addition of exogenous NADH, thus relying solely on the NADH available from the cytosol. By using concentrated cell suspensions, excluding GDH and adding additional FdR and Fdx it was thus possible to nearly double the TTN of 153A6FdRFdx over 8 h. The TTN over 18 h exceeded 12,000 (volumetric yield of 50 $mmol \, L_{BRM}^{-1}$·1-octanol and STY 0.4 $g \cdot L_{BRM}^{-1} \, h^{-1}$) while the reaction was still not levelling off. Similar TTN have been obtained over 100 h (STY 0.02 $g \cdot L_{BRM}^{-1} \, h^{-1}$) in a bioreactor conversion of cyclohexane with 47-51 using purified CYP and a commercially available formate dehydrogenase.[15] Bordeaux and co-workers[23] have recently reported a TTN of 3247 (STY 0.003 $g \cdot L_{BRM}^{-1} \, h^{-1}$) for a 78 h reaction with purified

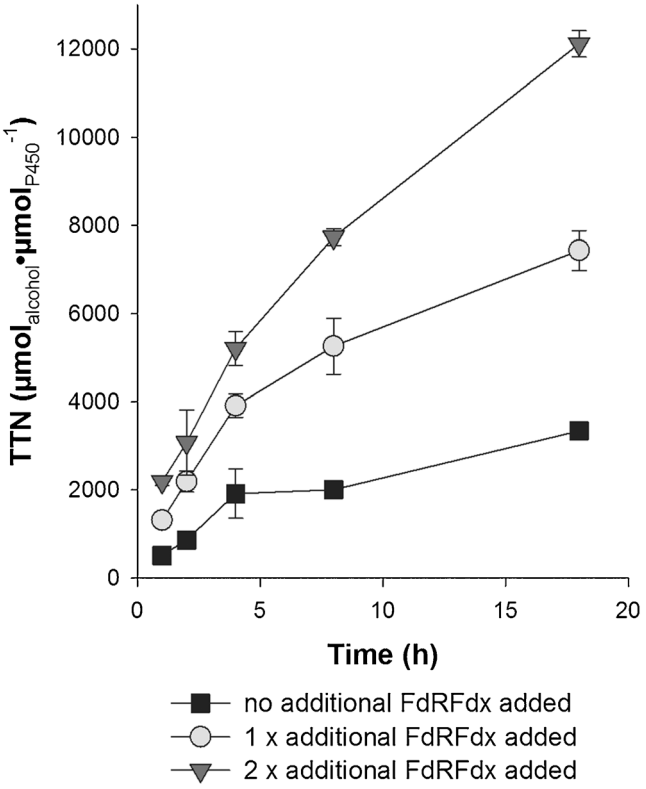

Figure 7. Effect of additional FdR and Fdx on octane conversion by CYP153A6FdRFdx when using CFEs from concentrated cell suspensions (500 g_{WCW}·L^{-1}). CYP containing CFE (250 μL) and CFEs from FdRFdx expressing cells or cells carrying empty vector (125 or 250 μL) were used to prepare BRM (final volume 1.2 mL). No NADH was added. TTNs were calculated using the initial CYP concentration (4.1 μM).

153A13RhFred, using a commercial isocitrate dehydrogenase for cofactor regeneration. These results demonstrate that it is possible to achieve with a three-component system TTNs and STYs rivalling those obtained with self-sufficient CYPs, even in CFE reactions.

Conclusions

CYPs have been extensively studied for hydroxylation of hydrocarbons such as linear and cyclic alkanes. Although very high TOFs have been reported for some CYP102A1 mutants, the STYs achieved with these reactions were too low to be of interest for synthetic application, because relatively low CYP and substrate concentrations were used. We confirmed that activities of both CYP153s and CYP102A1s towards cyclic and linear alkanes benefit when relatively large volumes of substrate, equivalent to what would be used for a co-solvent, are added. Under these conditions substrate specificity apparently changes so that 102A1 and the 47-51 mutant generally performed better in terms of product titres, and thus STYs over 8 h, than 1-12G, 139-3 and combinatorial mutants created from 139-3. This is remarkable since 1-12G and 139-3 were reported as promising alkane hydroxylases obtained from several rounds of directed evolution, while WT 102A1 is often proclaimed as having negligible activity towards alkanes. Although substrate uptake generally limited WC reactions, regioselectivity, final product titres and STYs obtained in WC conversions of *n*-hexane and *n*-octane using 153A6FdRFdx revealed this natural alkane hydroxylase as the superior catalyst for conversion of linear alkanes, when compared with 102A1 and its mutants. Although improved expression might still make 1-12G and 139-3C2NADH attractive as 2-hydroxylases, poor coupling efficiencies will always hamper the heavily mutated 102A1s, even if they are to be used with an alternative cofactor regeneration system that will not cause acidification. However, even for 2-hydroxylation a CYP153 might become the preferred catalyst, since Yang and coworkers have recently created mutants of another CYP153 (CYP153A7; P450pyr), which are >99% regioselective and >95% enantioselective for C-2 hydroxylation of octane while maintaining >90% activity when compared with the wild-type CYP.[35]

The STY of 0.37 g·L_{BRM}^{-1} h^{-1} and final product titre of 23 mmol·L_{BRM}^{-1} obtained with WC octane biotransformations using 153A6FdRFdx were higher than those obtained in bioreactor studies with WCs of *E. coli* expressing the diiron monooxygenase from *Pseudomonas putida* GPo1 (STY 0.23 g·L_{BRM}^{-1} h^{-1} and final total product titre 11 mmol·L_{BRM}^{-1}),[25] but not as high as the STY of 1 g·L_{BRM}^{-1} h^{-1} obtained in deep well plates with an *E. coli* strain co-expressing the outer membrane protein AlkL, which facilitates the uptake of hydrophobic substrates by *E. coli*.[36] Co-expression of AlkL also allowed WC conversion of dodecane by the diiron monooxygenase which accepts longer chain alkanes than CYP153A6. AlkL however did not affect WC activity towards hexane, confirming our own results that permeabilization did not improve CYP activity towards hexane. A drawback of WC biotransformations with the diiron monooxygenase is that alcohols are over oxidised to the corresponding acids.

Co-expression of AlkL with the diiron monooxygenase has demonstrated the potential of *E. coli* whole cells to hydroxylate hydrophobic substrates. By combining reaction engineering with catalyst engineering Schrewe et al. has managed to obtain within 17 h total product titres of more than 200 mM in WC conversions of dodecanoic acid methyl ester.[37] It is therefore quite likely that such productivities can also be achieved with the three-component 153A6FdRFdx through further catalyst and reaction engineering.

153A6FdRFdx did not initially perform as well in CFE reactions as it did in WC reactions, while the self-sufficient CYPs benefitted significantly when the substrate uptake barrier was removed. This was probably because interactions with FdR and Fdx were not optimal in CFEs. This problem was alleviated by the addition of CFEs containing only FdR and Fdx to the BRM, giving a STY of 0.4 $g \cdot L_{BRM}^{-1} h^{-1}$ over 18 h and a final 1-octanol titre of 50 $mmol \cdot L_{BRM}^{-1}$. These results are in terms of final product titre and STY better than any reported thus far for *n*-alkane conversion by a self-sufficient CYP and point the way for further improvement of the 153A6FdRFdx system in both CFEs and WCs.

This study demonstrated that given the importance of high product titres and STYs, efficient heterologous expression, stability (TTN) and efficiency with regard to cofactor usage (coupling efficiency) outweigh TOFs derived from short reaction times, especially from kinetic assays, when comparing recombinant CYPs for preparative biocatalysis. It also indicates that one should tread with caution when comparing biocatalysts either based on published data or in your own experiments, since experimental conditions such as amount of substrate added and solubility as well as cofactor dependence and regeneration can have a profound effect on catalyst performance.

Experimental Section

Cloning of Enzymes

Cloning of the CYP153A6 operon into pET28b(+) was as previously described.[17] The complete open reading frame of CYP153A13 fusion was synthesized (GenScript) with added 5′ *Nde*I and 3′ *Hind*III restriction sites (supplied in pMK). CYP153A13 fusion was sub-cloned into pET28b(+) (Novagen) using these restriction sites.

CYP102A1 (P450 BM3) in pET28a(+) was kindly provided by Prof. Vlada Urlacher (then at Institute for Technical Biochemistry, University of Stuttgart, Stuttgart, Germany). The N-terminal heme-domains (first 1182 bp of ORF) of 1-12G and 139-3C1 were synthesized by GenScript (supplied in pUC57) and were used to construct 1-12G and 139-3C1 by replacing these fragments in the complete open reading frames of CYP102A1 in pET28a using restriction sites *Bam*HI-*Pme*I. Similarly, the 139-3 and 47-51 mutants were created from CYP102A1 using the *Afl*II-*Pme*I and *Bam*HI-*Afl*II restriction sites, respectively. The 87-328 and 139-3C2 mutants were created using the "megaprimer" PCR method[38] with CYP102A1 and 139-3C1 as templates and primers: 102A1-F87V-F: 5′-CAGGAGACGGGTTAGTTACAAGCTGGAC-3′ and 102A1-A328F-R: 5′-GGAAAACGCAGGAAAAGTTGGCCATAAG-3′. NADH-dependent variants were similarly created by introducing the mutations R966D and W1046S into the respective parent plasmids by using primers: 102A1-R966D-F: 5′-CTTCATACCGCTTTTTCTGACATGCCAAATCAGC-3′ and 102 A1-W1046S-R: 5′-GAATTCTTACCCAGCCGACACGTCTTTTGCGTATC-3′. PCR reactions consisted of 1X KOD Hot Start Polymerase buffer, 1.5 mM $MgSO_4$, 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 0.02 $U \cdot \mu L^{-1}$ KOD Hot Start polymerase (Novagen), 0.4 $ng \cdot \mu L^{-1}$ template DNA and 0.1 μM of both forward and reverse primers. PCR conditions consisted of an initial denaturation step (95 °C, 2 min), followed by 7 cycles of denaturation at 95 °C (20 s), annealing at 62 °C (10 s) and elongation at 70 °C (15 s) and 22 cycles of denaturation at 95 °C (20 s) and annealing and extension of the megaprimer at 70 °C (4 min) with a final extension of 10 min at 70 °C. Template DNA was removed with *Dpn*I digestion (10 U) at 37 °C overnight, whereafter the PCR products were purified from a DNA agarose gel (Biospin gel extraction kit, BioFlux) and transformed into *E. coli* TOP10 (Invitrogen). Single colonies were selected and grown overnight in 5 mL LB medium containing 30 $\mu g \cdot mL^{-1}$ kanamycin. Plasmid DNA was isolated (Biospin plasmid DNA extraction kit, BioFlux) and verified by DNA sequencing.

The gene encoding GDH from *Bacillus megaterium* (BmGDH) was kindly provided by Dr. Dirk Holtmann (Dechema, Germany) in pETDuet (Novagen) multiple cloning site 2 (MC2): cloned using the *Nde*I and *Xho*I restriction sites.

Heterologous Expression of Enzymes

For expression of the CYPs and BmGDH, *E. coli* BL21-Gold(DE3) (Stratagene) was transformed with the relevant plasmids and transformants selected on LB-plates containing 30 $\mu g \cdot mL^{-1}$ kanamycin or 100 $\mu g \cdot mL^{-1}$ ampicillin. Expression was performed by using ZYP-5052 auto-induction medium[39] containing 1 mM 5-aminolevulinic acid and 0.05 mM $FeCl_3$. Cells were cultured for 48 h at 20 °C, after which they were harvested through centrifugation (6000 × *g*, 10 min) and 1 g (wet weight) resuspended in 10 mL or 2 mL Tris-HCl buffer (pH 8, 200 mM). CFEs were obtained by a single passage of the suspended cells through a One Shot Cell Disrupter (Constant Systems) at 207 MPa. The soluble fraction was separated from unbroken cells by centrifugation (20,000 × *g*, 20 min).

CYP450 concentrations were determined using CO-difference spectra[40] recorded in F8 Maxisorp Nunc-ImmunoTM Modules. Samples (2 × 200 μL) of a given preparation were added into wells in two different microtiter strips and reduced by adding a few grains of sodium dithionite. One well of each sample was saturated with carbon monoxide. Spectra were recorded between 400 and 500 nm using a Spectramax M2 Microtiter Plate Reader (Molecular Devices Corporation). The A450–A490 difference was corrected for a path length of 1 cm, and the CYP concentration was calculated using an extinction coefficient of 91 $mM^{-1} cm^{-1}$.

Biotransformations

All chemicals were from Sigma–Aldrich and were used without further purification. Biotransfromations were performed in 40-mL amber glass vials. The cell suspensions defined as the biotransformation reaction mixture (BRM) contained, unless otherwise stated, a final concentration of 0.05 g wet weight cells $\cdot mL^{-1}$ (or CFE from a corresponding cell suspension) and 100 mM of glucose and glycerol in 200 mM Tris-HCl buffer (pH 8). In the case of CFE reac-

tions the BRM also contained, unless otherwise stated, CFE from an equally concentrated cell suspension of a culture expressing BmGDH. In cases where the GDH was omitted GDH containing CFE was replaced with CFE from an empty vector control strain. NAD(P)H (1 mM) was, unless otherwise stated, added to CFE reactions. Four or 250 μL of alkane were added to each vial containing 1 mL of BRM before it was placed on an orbital shaker at 20 °C, 200 rpm, oscillation amplitude 26 mm. Vials were removed at specific time intervals for extraction. Reactions were stopped and extracted using an equal volume of ethyl acetate containing 2 mM 2-decanol or 1-undecanol as internal standard. GC-FID and GC-MS analyses were carried out on a Shimadzu GC and Thermo Trace GC ultra chromatograph with DSQ mass spectrometer, both equipped with a FactorFour VF-5 ms column (60 m × 0.32 mm × 0.25 μm, Varian). Alcohol concentrations were calculated using conversion factors derived from standard curves of authentic samples (see the Supporting Information, Section B).

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