Catalytic Hydroxylation in Biphasic Systems using CYP102A1 Mutants

Steffen C. Maurer, Katja Kühnel, Leonard A. Kaysser, Sabine Eiben, Rolf D. Schmid, Vlada B. Urlacher*

Institute of Technical Biochemistry, Stuttgart University, Allmandring 31, 70569 Stuttgart, Germany
Fax: (+49)-711-685-3196, e-mail: itbvur@itb.uni-stuttgart.de

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Abstract: Cytochrome P450 monooxygenases are biocatalysts that hydroxylate or epoxidise a wide range of hydrophobic organic substrates. Their technical application is, however, limited to a small number of whole-cell processes. The use of the isolated P450 enzymes is believed to be impractical due to their low stability, stoichiometric need of the expensive cofactor NAD(P)H and low solubility of most substrates in aqueous media. We investigated the behaviour of an isolated bacterial monooxygenase (mutants of CYP102A1) in a biphasic reaction system supported by cofactor recycling with the $NADP^+$-dependent formate dehydrogenase from *Pseudomonas sp* 101. Using this experimental set-up cyclohexane, octane and myristic acid were hydroxylated. To reduce the process costs a novel NADH-dependent mutant of CYP102A1 was designed. For recycling of NADH an NAD^+-dependent FDH was used. The stability of the monooxygenase mutants under the reaction conditions in the biphasic system was quite high as revealed by total turnover numbers of up to 12,850 in the NADPH-dependent cyclohexane hydroxylation and up to 30,000 in the NADH-dependent myristic acid oxidation.

Keywords: biotransformations; biphasic system; cofactor recycling; cofactor specificity; green chemistry; hydroxylation; P450 monooxygenase

Introduction

Cytochromes P450 belong to the heme-containing enzyme class of monooxygenases (EC 1.14.x.y). They are widely distributed in nature and play an important role in primary and secondary metabolism as well as in detoxification of xenobiotic compounds.[1] P450 enzymes catalyse the introduction of molecular oxygen into activated and even non-activated aliphatic or aromatic XH bonds (X = C, N, S). Moreover, a remarkable number of P450 enzymes are capable of epoxidising C=C double bonds.[2] Common to both reaction types is the incorporation of one atom of dioxygen into the substrate, while the other atom is reduced to water by electrons ultimately originating from the nicotinamide cofactor NAD(P)H. Electrons are transferred from NAD(P)H to the P450 heme iron *via* a flavin reductase or/and an iron-sulphur protein.[3]

Currently, the use of P450 enzymes in industrial processes is restricted to whole-cell biotransformations.[4,5] Advantages of such systems are stabilisation of the complex P450 systems with up to three individual proteins and simultaneous regeneration of cofactors in the cellular metabolism. Disadvantages of whole-cell processes might be a further degradation of products, toxicity of the educts or products for the cell, and complicated recovery of products from a complex fermentation broth. Considering these problems, the use of isolated oxygenases in enzyme reactors can be advantageous.[6–9]

From a technical point of view, microbial P450s are easier to handle than P450 enzymes from plants and animals. They are not membrane-associated and exhibit a relatively high stability and activity.[10]

Particularly appropriate for cell-free processes are so-called "self-sufficient" P450 monooxygenases. They consist of a heme domain fused to an FAD- and FMN-containing P450 reductase domain and, in contrast to the majority of P450s, do not have to be supplied with additional redox partners (reductases) apart from NADPH. The best characterised fusion protein CYP102A1 from *Bacillus megaterium* has been intensively studied during the last two decades.[11,12] This enzyme converts saturated and unsaturated fatty acids with chain lengths of C_{12} to C_{22} with high activity (up to 4000 min^{-1}) and partially also high stereoselectivity to their subterminally oxygenated derivatives.[13,14] Recently, cloning and characterisation of two further fusion enzymes CYP102A1 and CYP102A3 from *Bacillus subtilis*, exhibiting high homology to CYP102A1, was reported.[15–17] While a large number of publications exist describing novel evolved CYP102A1 mutants with altered substrate specificity,[18–26] there are only very few

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reports about the use of these biocatalysts in preparative organic synthesis.[7,27] The main drawback for *in vitro* applications of P450s is the dependence on the highly expensive cofactor NAD(P)H, which has to be added in stochiometric or even higher amounts (if uncoupling is taken into account). Several possibilities exist to overcome this problem, for example, the use of peroxides (so-called shunt-pathway)[28] or cathodic reduction of the heme iron.[29–31] Other approaches are electrochemical[32] or enzymatic reduction of the oxidised cofactor $NAD(P)^+$ during biotransformations. Dehydrogenases are currently utilised for cofactor recycling in various biocatalytic processes.[7,33,34]

In a previous paper we reported a cofactor recycling system for CYP102A1 by means of an $NADP^+$-dependent formate dehydrogenase (FDH, EC 1.2.1.2) from *Pseudomonas* sp. 101. It has been shown that both enzymes can be used in combination.[34]

Using this approach we have developed a process for oxidation of cyclohexane in a biphasic (cyclohexane/aqueous buffer) system. Industrial oxidation of cyclohexane is a process still open to improvements. Thus, on one hand, this reaction serves as a model for activation of an inert hydrocarbon, on the other hand there might be interest in an alternative approach for cyclohexane oxidation.

In order to prove the general applicability of CYP102A1 mutants in biphasic reaction systems, the hydroxylation of octane and myristic acid was also investigated.

Further reduction of the process costs was achieved by switching the cofactor specificity of CYP102A1 from NADPH to NADH. The advantage of NADH is its lower price and higher stability.[35] Besides that, NAD^+-dependent dehydrogenases are more abundant in nature than their $NADP^+$-dependent analogues, opening up the possibility to combine CYP102A1-catalysed reactions with a large variety of dehydrogenases.

Results and Discussion

Identification of CYP102A1 Mutants Hydroxylating Cyclohexane

A set of CYP102A1 mutants constructed by site-directed mutagenesis was screened for activity towards cyclohexane by monitoring NADPH oxidation at 340 nm during the hydroxylation reaction. The results are summarised in Figure 1.

The highest activity (56 eq eq^{-1} min^{-1}) was detected for the CYP102A1 mutant R47L, Y51F. Both amino acid substitutions render the substrate access channel more hydrophobic causing easier access of cyclohexane to the active centre of the enzyme.[36–38] The mutant R47L, Y51F, F87V, L188Q with an enlarged binding pocket[39] also showed rather high activity of 38 eq eq^{-1} min^{-1}, however lower than the double mutant's activity.

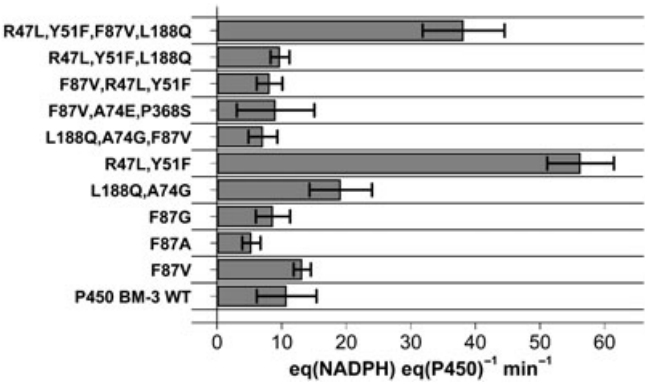

Figure 1. Results of screening a CYP102A1 mutant library for hydroxylation activity towards cyclohexane. NADPH consumption in the presence of 470 μM cyclohexane was tracked for 3 minutes.

Uncoupling of NADPH oxidation and substrate hydroxylation resulting in reduction of oxygen to hydrogen peroxide or water is an issue frequently encountered during monooxygenase catalysis.[38,40] Therefore product formation and its coupling to NADPH consumption was investigated by GC/MS analysis (data not shown). This revealed coupling efficiencies of 25% for the R47L, Y51F mutant, 19% for R47L, Y51F, F87V, L188Q, 16% for A74G, L188Q and only 6% for wild-type CYP102A1. Generally we found correlation between NADPH oxidation rates and corresponding coupling efficiencies. The faster the NADPH oxidation was the higher was the coupling efficiency. All mutants, for which coupling efficiency was measured, produced cyclohexanol as a single product (GC/MS data not shown). The double mutant R47L, Y51F was revealed as the most active (cyclohexane hydroxylation activity: 14 min^{-1} for R47L, Y51F compared to 0.75 min^{-1} for the wild-type enzyme) among the mutants investigated and therefore used in further experiments with this substrate. As activity of the wild-type enzyme in this reaction was almost negligible it was not investigated for preparative purposes.

Kinetic Characterisation

Kinetic data for the purified mutant R47L, Y51F were determined by monitoring of cyclohexane-dependent NADPH oxidation at a saturating concentration of the cofactor.

The plot of NADPH oxidation rate *versus* cyclohexane concentration shown in Figure 2 has non-hyperbolic characteristics. This indicates cooperativity in substrate binding according to the Hill kinetic model. Even at the maximum cyclohexane concentration ($\approx$500 μM) that

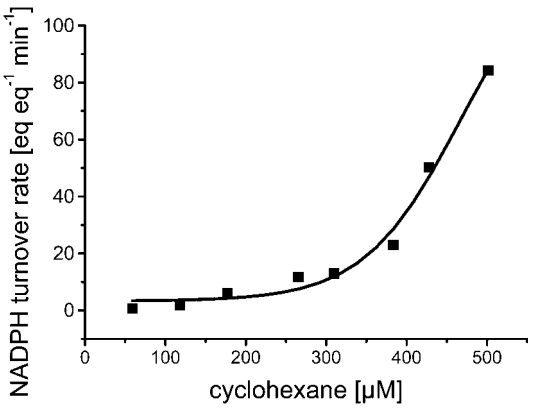

Figure 2. Steady-state NADPH turnover rates by purified CYP102A1 mutant R47L, Y51F at various cyclohexane concentrations. The plot demonstrates sigmoidal dependency of the reaction rate on substrate concentration. This indicates non-Michaelis–Menten kinetics.

is limited by its solubility in the aqueous phase, full saturation of the active centre of the enzyme was not reached.

Substrate binding to P450 usually causes a heme spin-state shift from low to high, which can be monitored as changes in P450 absorbance spectra (Figure 3). Cyclohexane binds to R47L, Y51F leading to an increase in the difference between the absorbance at 417 nm (trough) and that at 387 nm (peak). However, the maximum heme spin-state shift was not reached at a concentration of cyclohexane of 500 μM.

The plot of absorption differences at ~387 nm and 417 nm *versus* cyclohexane concentration indicates exponential growth of the spectral changes in the concentration range inferred through the solubility limit (small graph in Figure 3).

These measurements confirmed the steady-state kinetic data. This is, however, atypical for CYP102A1 that usually corresponds to Michaelis–Menten kinetics.

The results summarised in Figures 2 and 3 clearly demonstrate that application of the CYP102A1 mutant R47L, Y51F would benefit from an increase in cyclohexane concentration in the aqueous phase. Different detergents such as Tween 20, Tween 80, cholate, CHAPS, α-cyclodextrin, β-cyclodextrin and co-solvents such as methanol, ethanol, DMSO, acetone, butanol, THF were tested with CYP102A1 R47L, Y51F in order to increase substrate solubility. Only Tween 20 and 80 applied at 1% (v/v) displayed a positive effect leading to 1.5- and 2-fold higher cyclohexane hydroxylation activity. However, these beneficial effects could not be transferred to the two-phase reaction system. While Tweens enhanced the activity of the monooxygenase by increasing the cyclohexane concentration, the stability of monooxygenase and/or dehydrogenase was negatively affected.

Biphasic Reaction System Cyclohexane/Aqueous Buffer

We investigated the optimal conditions for hydroxylation of cyclohexane by CYP102A1 in a biphasic reaction system (Figure 4). In this system cyclohexane acts as a substrate pool and simultaneously as an organic solvent for extraction of the reaction product. Thus, the catalytic system is retained in the aqueous phase while the product may continuously be isolated from the organic phase. The cofactor recycling system is based on a mutant of FDH from *Pseudomonas* sp. which has been proven to be highly stable in organic solvents.[8,41]

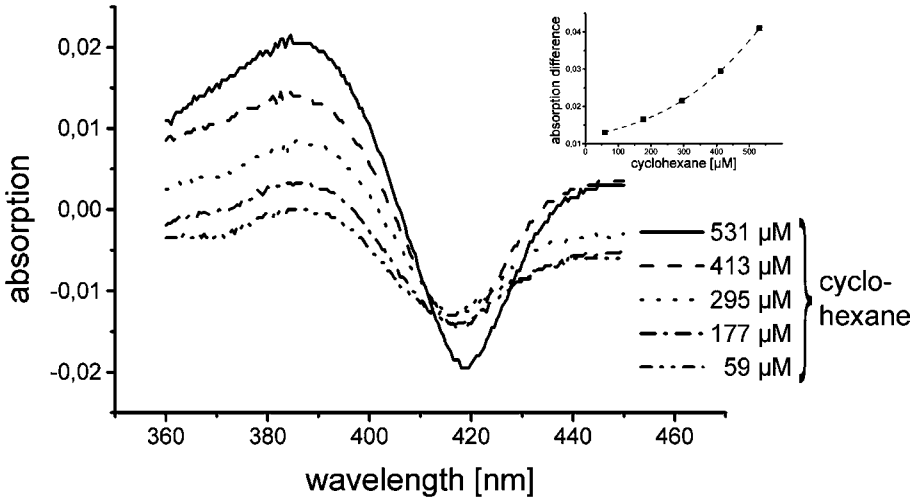

Figure 3. Cyclohexane binding difference spectra of CYP102A1 R47L, Y51F. Difference spectra were obtained by subtracting reference spectra of enzyme (8.6 μM) without substrate from spectra of samples containing the indicated cyclohexane concentrations and exactly the same enzyme concentration as the reference. The small chart at the right upper corner was generated by plotting A_{387} minus A_{417} data from the difference spectra *versus* the relevant cyclohexane concentrations.

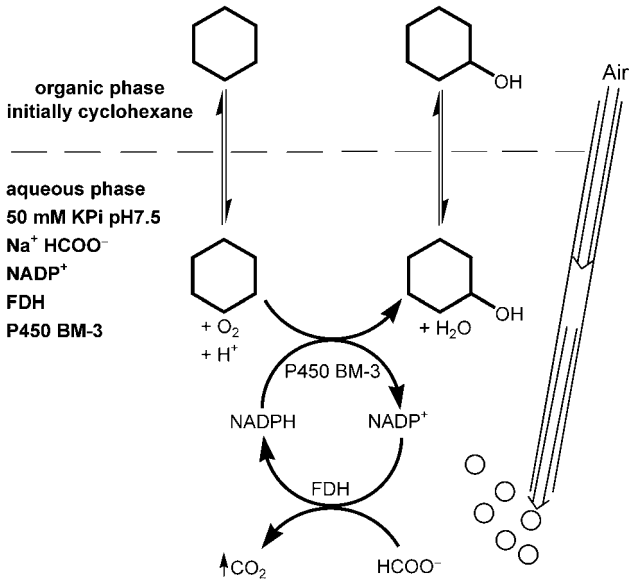

Figure 4. Scheme for CYP102A1-mediated hydroxylation of cyclohexane in a biphasic system. The organic phase serves as substrate pool and extracts the products from the aqueous phase.

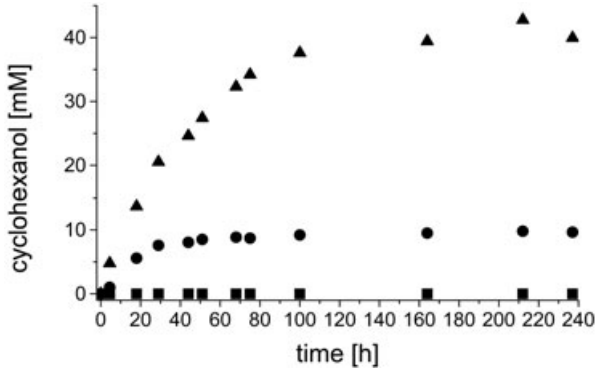

Figure 5. Production of cyclohexanol during reaction in the biphasic system. The reaction was performed in a stirred 20-mL flask containing 5 mL cyclohexane and 5 mL aqueous reaction medium. Squares: Negative control containing *E. coli* cell extract; circles: CYP102A1 mutant A74G, F87V, L188Q; triangles: CYP102A1 mutant R47L, Y51F.

The stability of this dehydrogenase in a 1:1 mixture of FDH reaction buffer and cyclohexane under vigorous stirring was tested. Studies on FDH activity revealed a half-life of at least one day for this cofactor regenerating enzyme in this system.

The effect of different protein stabilising agents – glycerol, bovine serum albumin (BSA), catalase, PEG derivatives – was studied. Addition of 10 mg mL^{-1} BSA and 0.2 mg mL^{-1} (600 U mL^{-1}) catalase from horse liver stabilised the CYP102A1 monooxygenases in the biphasic emulsion and led to higher hydroxylation activity for an extended period of time (data not shown).

Concentration of cyclohexanol in the organic phase measured during the biotransformation indicates that the mutant R47L, Y51F was active for at least four days (~100 h) when stabilised by addition of BSA and catalase (Figure 5).

Limitations in transfer of substrate from the organic to the aqueous phase can decrease reaction rates, at least if phase transfer is slower than the rate of hydroxylation.

The triple mutant displaying a lower reaction rate than the double mutant (Figure 1; 7 eq eq^{-1} min^{-1} compared to 56 eq eq^{-1} min^{-1}, respectively) also showed lower cyclohexanol production in the biphasic reaction system (Figure 5). For the best mutant R47L, Y51F the total turnover number (ttn) was 9620 (Table 1). An increase in ttn to 10280 was achieved by changing the reaction vessel from a stirred flask at room temperature to a flask shaken vigorously at 160 rpm and 18 °C.

In a further experiment, the reaction was scaled up to one litre volume (1.3 μmol of CYP102A1 R47L, Y51F) and was carried out in a fermenter under continuous aeration. On-line monitoring of oxygen partial pressure

Table 1. Comparison of the biotransformation reactions in biphasic reaction media.

Substrate	CYP102A1 mutant	Reaction time [h]	Reaction volume [mL]	Amount of CYP102A1 [nmol]	ttn for CYP102A1 mutant	ttn for cofactor NAD(P)$^{+}$[a]	Volumetric productivity (mg L^{-1} h^{-1})[b]
Cyclohexane	R47L,Y51F	100	10 (stirred flask)	66	9620	1270	63.5
Cyclohexane	A74G,F87V,L188Q	100	10 (stirred flask)	66	2370	313	15.6
Cyclohexane	R47L,Y51F	100	40 (shaking flask)	180	10280	925	46.3
Cyclohexane	R47L,Y51F	100	1000 (fermenter)	1300	12850	334	16.7
Octane	A74G,F87V,L188Q	100	10 (stirred flask)	65	2200	280	18.6
Myristic acid	A74G,F87V,L188Q	80	8 (stirred flask)	100	3300	825	101
Myristic acid	A74G,F87V,L188Q, R966D, W1046S (NADH-dependent)	24	10 (stirred flask)	5	30000	300	153

[a] NAD(P)H concentration was 0.1 mM in the aqueous phase.
[b] Calculated for the whole reaction time. Higher volumetric activities can be calculated for the beginning of the reactions.

and pH allowed us to control the reaction progress. The increase in oxygen partial pressure reflects a decrease in oxygen consumption during the hydroxylation reaction. As protons are stoichiometrically consumed during the reaction (Figure 4), no further increase in pH indicates loss in activity.

The total turnover number (ttn) in the fermenter reached 12,850, yielding 1.67 g of cyclohexanol (16.7 mmol). The course of product development indicates that the monooxygenase was active for at least 100 h. During the first 53 h productivity (12.6 mmol cyclohexanol) was higher than during the second half of the reaction (4.1 mmol). The space-time yield during the first 53 h of the reaction was 23.8 mg L^{-1} h^{-1} (Table 1). This result can be significantly improved by applying higher concentrations of CYP102A1 and FDH. In this case only substrate phase transfer will be the limiting factor.

Conversion of Octane and Myristic Acid

In order to demonstrate the general applicability of CYP102A1 in biphasic systems, the hydroxylation of octane and myristic acid were also investigated. The CYP102A1 mutant A74G, F87V, L188Q hydroxylates both substances with NADPH turnover rates of 1760 min^{-1} for octane[19] and 2100 min^{-1} for myristic acid.

Similar to the experiments with cyclohexane, octane was used as substrate and as organic phase. GC/MS analysis revealed a product mixture consisting of 2-, 3- and 4-octanol as well as the corresponding octanones. The formation of octanones by CYP102A1 was previously reported.[20] A possible mechanism for the formation of the ketones is a second hydroxylation of the alcohol to generate a *gem*-diol, which dehydrates to the corresponding ketone. Ketones might alternatively originate from *vic*-diols *via* pinacol rearrangement. In any case double hydroxylation is a prerequisite for ketone formation. For calculation of total turnover numbers the GC/MS signals of all reaction products were calibrated for quantification using authentic standards of known concentration. For octane hydroxylation a ttn of 2200 was measured.

For hydroxylation of myristic acid in the biphasic system, the substrate was dissolved in dodecane. In preliminary investigations dodecane was proven not to be a substrate of CYP102A1 A74G, F87V, L188Q. Fatty acid hydroxylation was performed under constant air bubbling. The total reaction volume of 8 mL consisted of 4 mL of the aqueous catalyst system and 4 mL 100 mM myristic acid (91 mg) in dodecane.

With myristic acid as a substrate for CYP102A1, usually a mixture of the subterminally hydroxylated products is obtained.[13,14] In the biphasic system, however, additionally to the monohydroxylated products dihydroxylated myristic acid derivatives were produced. A hydroxylation of 12-hydroxy- and 13-hydroxymyristic acid has previously been reported, even though with low reaction rate.[42] Totally, 92.9 mg of myristic acid and derivatives were extracted. This mixture consisted of 41.4 mg of myristic acid (predominantly found in the dodecane phase) and of 51.5 mg (55%) of hydroxylated products less soluble in dodecane as revealed by GC/MS. Hydroxylated products comprised 48% monohydroxy- (ω-1 to ω-7) and 52% dihydroxymyristic acid (mixture of 7 regioisomers). As stated in Table 1 this corresponds to ttns of 3300 for the P450 enzyme and 825 for the cofactor $NADP^+$.

These results indicate the applicability of the biphasic reaction system for conversion of different substrates by CYP102A1.

Conversion of Myristic Acid by an NADH-Dependent CYP102A1 Mutant

Multiple sequence alignment of NADPH-dependent cytochrome P450 reductases, which supply the P450 monooxygenases with electrons, revealed 5 highly conserved positions important for cofactor specificity.[43,44] These positions in CYP102A1 are S965, R966, K972 and Y974, which bind the 2′-phosphate group of NADPH and W1046, the so-called gate-keeping amino acid, involved in electron transfer. Döhr et al. changed the cofactor specificity of the human P450 reductase from NADPH to NADH *via* substitution of the gate-keeping tryptophan to alanine.[44]

The same effect was observed for the CYP102A1 mutant W1046A, that could accept NADH as cofactor [K_M (NADH) = 14 μM and k_{cat} (NADH) = 6300 min^{-1} compared to CYP102A1 wild-type reductase: K_M (NADPH) = 2.5 μM, k_{cat} (NADPH) = 7930 min^{-1}, K_M (NADH) = 1430 μM and k_{cat} (NADH) = 2810 min^{-1}] (Table 2) as revealed by reduction of cytochrome C. However, in our investigation serine at this position caused a stronger effect on the switch in cofactor specificity [K_M (NADH) = 4.4 μM und k_{cat} (NADH) = 4710 min^{-1}]. A further improvement in terms of catalytic activity of the CYP102A1 reductase with NADH was achieved by substitution of the phosphate binding arginine 966 by aspartate. Kinetic constants for the reductase double mutant R966D, W1046S were K_M (NADH) = 12 μM, k_{cat} (NADH) = 14601 min^{-1}. Thus, the catalytic efficiency (k_{cat}/K_M) of the CYP102A1 reductase in NADH-dependent cytochrome C reduction was improved 640-fold. Further details on reductase mutants will be described elsewhere.[45]

The two substitutions R966D and W1046S were introduced into the reductase domain of the CYP102A1 A74G, F87V, L188Q monooxygenase. The new NADH-dependent mutant was tested with myristic acid in the biphasic system. The NAD^+-dependent

FDH from *Pseudomonas* sp.[46] was used for cofactor recycling. Extraction of the reaction mixture and subsequent GC/MS analysis revealed 20% substrate conversion to hydroxylated products. The hydroxy- and dihydroxymyristic acid products identified were the same and at approximately the same ratio as in the NADPH-driven reaction. The lower yield of products (20% compared to 55% yield in the NADPH-dependent reaction) is most likely a result of the lower amount of P450 enzyme added to the reaction mixture (5 nmol compared to 100 nmol). The ttn calculated for this reaction reached 30000. To our knowledge this represents the highest value reported for P450 monooxygenases so far (Table 1). Further experiments on use of the NADH-dependent system in preparative organic synthesis are currently being performed.

Conclusions

The exploitation of isolated P450 monooxygenases in biocatalysis is a field of ongoing research. However, until now it is not clear if the properties of these enzymes allow their *in vitro* use. Although a few reports concerning the use of oxygenases (for a compilation see[47]) in synthetic applications do exist, bioengineering-related data are still scarce.

We investigated the enzymatic hydroxylation of different substrates of CYP102A1 in a biphasic system. The proposed biphasic reaction scheme (Figure 4) is especially fitted to the requirements of the catalytic system under debate: the aqueous phase dissolves – and retains – cofactor and proteins without need for expensive membrane technology. The organic phase acts as substrate pool dissolving substrates or representing the substrate itself. Also a substantial amount of reaction products accumulates in the organic phase. These can be continuously removed using extraction, distillation or adsorption to resins.

Our experiments (Table 2) can be compared to the first report of preparative *in vitro* use of a bacterial P450 monooxygenase.[7] The authors reported a synthesis of 14,15-epoxyeicosatrienoic acid from arachidonic acid (500 mg, 1.64 mmol) using CYP102A1 F87V (600 U, 500 nmol) and glucose 6-phosphate dehydrogenase for NADPH recycling. Their attempt was to dissolve the hydrophobic substrate using a large volume of aqueous buffer. 0.74 mmol of product were obtained from 4-L reaction mixture, resulting in a ttn of approximately 1500. Our results revealed not only technical advantages of the biphasic system, but also its high stability expressed in a ttn of 12800 with cyclohexane and 30000 with myristic acid as substrate. The total turnover numbers of the cofactor ranging from ~300 up to ~1300 are among the highest reported for a preparative application of isolated oxygenases.

The total turnover numbers, calculated for CYP102A1 are higher than the value of up to 2867 reported for the asymmetric epoxidation of styrene by styrene monooxygenase (StyAB) in a two-phase dodecane/aqueous buffer system.[8] In this system a maximum turnover number of 87 was determined for the cofactor NAD^+.

Lutz et al. investigated the preparative application of 2-hydroxybiphenyl 3-monooxygenase coupled to enzymatic cofactor regeneration. The authors reported a ttn for NAD^+ of maximally 503.[47]

Zambianchi et al. used isolated cyclohexanone monooxygenase for Baeyer–Villiger oxidation of 10 g L^{-1} bicyclo[3.2.0]hept-2-en-6-one. The process was running under enzymatic NADPH regeneration and continuous substrate feeding.[48]

While volumetric productivities reported in our study are quite low (maximum 0.15 g L^{-1} h^{-1}), higher values found for other oxygenases and reported in literature (up to 1 g L^{-1} h^{-1})[8] are usually calculated for 10 h or less reaction time. The values were determined for the whole reaction time (up to 100 h).

Switching the cofactor specificity of CYP102A1 from NADPH to NADH allows us to reduce the cofactor costs to about 20%. The new NADH-dependent mutant demonstrated a comparable stability to that of its NADPH-dependent homologue.

The summarised results and the comparison with other oxygenases pointed out the potential of P450 enzymes for cell-free applications. Further investigations are, however, needed and will address problems of low substrate solubility, low enzyme activity and low regio- and/or stereoselectivity. The CYP102A1 monooxygenase is a highly "evolvable" biocatalyst. Protein engineering by directed evolution and site-directed mutagenesis has generated many mutants with versatile properties,

Table 2. Comparison of K_M and k_{cat} values for reductase mutants. Values were calculated for NADPH- or NADH-dependent cytochrome C reduction. The monooxygenase domain in all cases carried mutations A74G, F87V and L188Q.

	WT-reductase	W1046A	W1046S	R966D, W1046S
K_M (NADPH)	(2.5 ± 0.2) μM	(0.82 ± 0.08) μM	(0.78 ± 0.05) μM	(1.6 ± 0.2) μM
k_{cat} (NADPH)	(7930 ± 430) min^{-1}	(524 ± 5) min^{-1}	(366 ± 4) min^{-1}	(5410 ± 430) min^{-1}
K_M (NADH)	(1430 ± 70) μM	(14.0 ± 0.75) μM	(4.4 ± 0.1) μM	(11.6 ± 0.7) μM
k_{cat} (NADH)	(2810 ± 100) min^{-1}	(6300 ± 100) min^{-1}	(4710 ± 40) min^{-1}	(14600 ± 1200) min^{-1}

including the activity towards a tremendous number of substrates. Application of these CYP102A1 variants in biphasic systems represents an efficient and feasible approach for synthesis of many useful compounds.

Experimental Section

Chemicals, Enzymes and Strains

All chemical reagents were of analytical grade or higher and purchased from Fluka, Aldrich, Sigma or Riedel-de-Haën. NADPH tetrasodium salt and FDH from *Pseudomonas* sp. 101 were procured from Jülich Fine Chemicals (Jülich, Germany). *E. coli* strain BL21 (DE3) and vector pET28a+were obtained from Novagen (Madison, Wisconsin, USA).

Cyclohexane was distilled prior to use in order to remove traces of cyclohexanol and cyclohexanone.

Saturated solutions of substrates in aqueous buffer were prepared by adding 5 mL of organic substrate to 50 mL of 50 mM potassium phosphate buffer (KPi), pH 7.5. The biphasic mixture was vigorously stirred overnight, the substrate-saturated aqueous phase was separated using a separatory funnel and used immediately.

Design of NADH-Dependent Reductase Mutants

For creation of an NADH-dependent mutant of CYP102A1 a sequence alignment with the rat cytochrome P450 reductase was performed and two positions important for cofactor specificity of CYP102A1 were identified. The corresponding mutants were constructed by site-directed mutagenesis using the Stratagene QuickChange™ Kit (Stratagene, La Jolla, Ca, USA) according to the manufacturer's protocol. Mutations were introduced to plasmid pT-USC1BM3[49] using the following oligonucleotide primers:

Primer W1046S forward: 5′-gatacgcaaaagacgtgTCGgctgggtaagaattc-3′

Primer W1046S reverse: 5′-gaattcttacccagcCGAcacgtcttttgcgtatc-3′

Primer W1046A forward: 5′-gatacgcaaaagacgtgGCGgctgggtaagaattc-3′

Primer W1046A reverse: 5′-gaattcttacccagcCGCcacgtcttttgcgtatc-3′

Primer R966D forward: 5′-gcttcataccgctttttctGACatgccaaatcagccg-3′

Primer R966D reverse: 5′-cggctgatttggcatGTCagaaaaagcggtatgaagc-3′

Reductase activity was measured by standard cytochrome C reduction assay as described previously.[50]

Expression and Purification of CYP102A1 Mutants

The expression system pET28a+CYP102A1 was described previously.[34] Mutations were introduced by PCR using the QuikChange™ site-directed mutagenesis kit from Stratagene according to the manufacturer's protocol.[51] High level protein expression was achieved using a slightly modified version of the protocol already published.[34] By incubating the starter culture for only 4 h, using terrific broth (TB)-medium for expression and prolonging cultivation time after induction to 16 h at 25 °C, 800 to 1200 nmol (96 to 144 mg) of soluble CYP102A1 per litre of cell culture were obtained. Aliquots of the crude cell lysates containing 25 to 50 μM CYP102A1 were stored at −20 °C or directly used for preparative reactions.

For kinetic measurements purified enzyme was used. Purification was performed according to a two-step strategy using a Ni-sepharose HP (Amersham Biosciences, Sweden) column with elution by 100 mM imidazole first. For further purification and to remove imidazole, the eluate was loaded onto a fractogel EMD DEAE 650S (Merck KgaA, Darmstadt, Germany) column and eluted by applying a linear sodium chloride gradient.

Activity Assays and Characterisation of Mutants

Substrate binding difference spectra, NADPH oxidation and CO difference spectra were recorded on an Ultrospec 3000 UV/vis spectrometer (Amersham Biosciences, Sweden). Concentration of correctly folded P450 enzymes was determined from the CO-binding difference spectra of the reduced heme iron using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ as reported elsewhere.[52]

For selection of mutants with activity towards cyclohexane 160 μL cyclohexane-saturated 50 mM KPi, pH 7.5 (0.59 mM cyclohexane) per well (96-well microtitre plate) was mixed with 20 μL of cell lysate and the reaction was started by addition of 20 μL 1 mM NADPH stock solution in 50 mM KPi. Reaction progress was monitored by measuring the absorption of NADPH at 340 nm for 5 minutes. For each mutant a blank containing no substrate was recorded and subtracted from the slope recorded in the presence of substrate. Each of these measurements was repeated eight times and the average slope during the first 3 min of the reaction was calculated. For calculations of turnover numbers an extinction coefficient for NADPH of 6.22 $mM^{-1}\,cm^{-1}$ at 340 nm was used.

Kinetic measurements were performed in sealed cuvettes at least in triplicates to reduce standard errors.

Coupling efficiency was measured by incubation of purified CYP102A1 (3 μM), 50 U mL^{-1} catalase with 0.4 mM cyclohexane and 0.2 mM NADPH in a sealed cuvette. After total consumption of NADPH the reaction mixture was extracted three times with 300 μL 1-butanol or diethyl ether. The combined organic layers were dried over $MgSO_4$ and analysed by quantitative GC/MS. From the amount of substrate converted, the coupling of NADPH oxidation to product formation can be deduced.

Substrate binding difference spectra were recorded using standard procedures.[13,53]

Myristic acid was oxidised in aqueous buffer by adding 20 μL of 5 mM myristic acid dissolved in DMSO to 930 μL of 1 μM CYP102A1 in 50 mM KPi, pH 7.5. The reaction was started by addition of 50 μL 10 mM NADPH in 50 mM KPi, pH 7.5. After 15 min the reaction mixture was acidified using dilute HCl and extracted thrice with diethyl ether and analysed as described in the section on product identification.

FDH activity assays were performed to estimate the stability of FDH when stirred with cyclohexane. The same assay was used during reactions to measure residual FDH activity. The assay was performed by adding 50 μL FDH-containing solu-

tion to a cuvette containing 950 μL FDH reaction buffer (50 mM KPi, 300 mM Na^+ $HCOO^-$, 0.1 mM $NADP^+$, pH 7.0) and measuring NADPH development at 340 nm.

Preparative Incubation in Biphasic Systems

The aqueous phase consisted of 250 mM sodium formate, 50 mM KPi, 10 mg mL^{-1} BSA for stabilisation, 600 U mL^{-1} catalase to destroy traces of hydrogen peroxide, 0.1 mM $NADP^+$. A 1.5-fold excess of FDH over CYP102A1 was used in the reaction to avoid limitation due to insufficient cofactor recycling. Then the organic substrate was added. In the cases of cyclohexane and octane as substrates the distilled solvents were used as organic phase. In the case of myristic acid conversion, the organic phase consisted of a 100 mM solution of myristic acid in dodecane. Additionally 1% DMSO was added to the aqueous phase to enhance solubility of the organic substrate. To start the reactions the respective CYP102A1 mutant was added. For cyclohexane and octane conversions the amount of CYP102A1 added is given in the results section. Hydroxylation of myristic acid was performed by 100 nmol CYP102A1 A74G, F87V, L188Q (NADPH dependent) and alternatively by 5 nmol CYP102A1 A74G, F87V, L188Q, R966D, W1046S (NADH dependent).

For hydroxylation of cyclohexane in a Labfors 3 l fermenter (Infors AG, Bottmingen, Switzerland) the following parameters were used: 1.3 μmol CYP102A1 R47L, Y51F, stirrer speed 350 rpm, aeration 0.5 L min^{-1} and readjustment of pH by 1 M KPi, 400 mM Na^+HCOO^- pH 6. The biphasic reaction emulsion consisted of 500 mL of aqueous phase and 500 mL of cyclohexane. For online monitoring of the reaction parameters IRIS software was applied. To collect evaporated educt and product the fermenter was equipped with a cryo trap charged with dry ice. To prevent foam formation, 100 μL of antifoam (silicone oil) were added.

During the reaction course FDH activity assays were performed. When no activity could be detected any more, FDH was added (maximally once per reaction batch; half the amount of FDH as in the beginning of the reaction).

Monitoring of the reaction progress was achieved by taking samples from the organic phase, drying them over $MgSO_4$ and analysis by quantitative GC/MS. In the case of myristic acid, samples were taken from the organic and the aqueous phase (1:1), acidified by addition of dilute HCl, extracted with diethyl ether and evaporated to dryness. Trimethylsilylation was achieved by dissolving the solid in *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane and incubation at 60 °C for 30 min in a sealed vial.

For quantitative determination of total products, the reaction mixture was extracted three times with diethyl ether, dried and analysed by GC/MS. For cyclohexanol the phase partitioning between cyclohexane and the aqueous reaction medium was determined. Below 20 g L^{-1} a linear relation of cyclohexanol in aqueous and organic phase was found: [cyclohexanol in cyclohexane] = (0.936 +/- 0.011) [cyclohexanol in aqueous phase].

For myristic acid the acidified (pH 2) reaction mixture was extracted thrice with diethyl ether. The combined organic layers were evaporated under vacuum until only high-boiling dodecane was left. At this stage a white solid had formed. The solid was separated by centrifugation and washed twice with dodecane. Analysis of this solid revealed it consisted of 90% mono- and dihydroxymyristic acid. In contrast the dodecane phase contained 95% myristic acid and only traces of hydroxylated compounds.

Product Identification

Products were identified on a Shimadzu GC/MS-QP2010 equipped with a 30 m FS-Supreme column (internal diameter 0.25 mm, film thickness 0.25 μM) using helium as carrier gas at linear velocity of 30 cm s^{-1}.

Cyclohexane: 1) 50 °C for 5 min; 2) 50 to 200 °C at 20 °C min^{-1}; 3) 2 min at 200 °C. The GC/MS signals of cyclohexane and cyclohexanol were calibrated for quantification using 10 standard concentrations ranging from 0.05 to 15 mM and fitted to a quadratic function.

Octane: 1) 40 °C for 1 min; 2) 40 to 80 °C at 2 °C min^{-1}; 3) 1 min at 80 °C; 4) 80 to 200 °C at 30 °C min^{-1}. Linear calibration curves using in each case 10 standard concentrations of 2-, 3- and 4-octanol and 2-, 3- and 4-octanone ranging from 0.01 to 1 mM were created.

Myristic acid: 1) 175 °C for 1 min; 2) 175 to 275 °C at 5 °C min^{-1}; 3) 1 min at 275 °C. Trimethylsilylated hydroxy derivatives of myristic acid were identified by their characteristic MS fragmentation patterns.

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