# Immobilisation of P450 BM-3 and an NADP$^+$ Cofactor Recycling System: Towards a Technical Application of Heme-Containing Monooxygenases in Fine Chemical Synthesis

Steffen C. Maurer, Holger Schulze, Rolf D. Schmid, Vlada Urlacher*

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

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**Abstract:** Cytochrome P450 monooxygenases are potentially a very useful class of hydroxylation catalysts; they are able to introduce oxygen at activated and non-activated carbon-hydrogen bonds and thus lead to regio- and/or stereochemically pure compounds. However, this potential is lowered by their intrinsic low activity and inherent instability. P450-catalysed biotransformations require a constant supply of NAD(P)H, making the process an expensive one. To render these catalysts more suitable for industrial biocatalysis, the immobilisation of P450 BM-3 (CYP 102A1) from *Bacillus megaterium* in a sol-gel matrix was combined with a cofactor recycling system based on NADP$^+$-dependent formate dehydrogenase (EC 1.2.1.2) from *Pseudomonas* sp. 101 and tested for practical applicability. This approach was used for the conversion of β-ionone, octane and naphthalene to the respective hydroxy compounds with DMSO as cosolvent using sol-gel immobilised P450 BM-3 mutants.

**Keywords:** biotransformation; cofactor-recycling; cytochrome P450 BM-3; hydroxylation; immobilisation; sol-gel

**Abbreviations:** CYP: cytochrome P450; FDH: formate dehydrogenase; GC: gas chromatography; KPi: potassium phosphate; NAD(P)H: nicotinamide adenine dinucleotide (phosphate); 10-pNCA: *p*-nitrophenoxydecanoic acid; TEOS: tetraethoxy orthosilicate

## Introduction

Cytochromes P450 (CYPs) are heme-containing enzymes catalysing a broad range of monooxygenation reactions in catabolic and anabolic pathways.[1] Several groups have recently started to investigate cytochrome P450 monooxygenases for their potential use in biotransformations.[2,3] CYP P450 s catalyse the hydroxylation of a wide range of hydrophobic compounds at activated and non-activated carbons. Thereby, one oxygen atom of $O_2$ is transferred to the substrate. The other oxygen atom is reduced to $H_2O$ with the simultaneous oxidation of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H). These hydroxylations are in most cases regio- and stereospecific. As regio- and stereospecific oxidation of aliphatic carbons is a reaction which is difficult to perform by means of classical organic synthesis, the biocatalysis by monooxygenases is a promising option, especially with regard to fine chemistry.

P450 BM-3, which was originally cloned from *Bacillus megaterium*, is a 119 kDa natural fusion protein composed of a heme-containing monooxygenase domain and an FAD- and FMN-containing reductase domain.[4,5] This multi-domain architecture renders P450 BM-3 catalytically self-sufficient in terms of electron transfer from the cofactor NADPH to the heme iron. The turnover frequencies determined for P450 BM-3 ($>1000\ min^{-1}$) are, to date, among the highest reported for a P450 monooxygenase.[6] The natural substrates of P450 BM-3 are long-chain fatty acids ($C_{12}$ to $C_{20}$), which are exclusively hydroxylated at the subterminal positions ω-*1*, ω-*2* and ω-*3*, partially with high enantioselectivity.[6,7] P450 BM-3 has frequently been investigated using rational protein design and/or directed evolution aimed at developing a more suitable catalyst. Artificial mutants of P450 BM-3 also hydroxylate non-natural substrates like short-chain fatty acids ($C_8$ to $C_{10}$),[8,9] indole,[10] polycyclic aromatic hydrocarbons,[11] alkanes [2,12] and styrenes.[13]

The immobilisation of enzymes has proved very useful in *in vitro* biotransformations as this leads to enhanced operational and storage stability[14] and simple product separation. To date, this subject has received very little

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attention.[15–17] Therefore, the immobilisation of two P450 BM-3 mutants was examined on a broad variety of common immobilisation matrices. The best result was achieved by encapsulation of the enzyme in a sol-gel matrix derived from tetraethoxy orthosilicate (TEOS).[18] This approach resulted in a biocatalyst that revealed long-term activity when immobilised on the matrix.

A key problem preventing the use of P450 s in biotechnology is the supply of electrons to the heme iron. *In vivo*, the electrons are supplied by NAD(P)H, which is however far too expensive to be used in technical applications.[19] In addition, the use of whole cells in industrial transformation reactions is impeded by the complicated recovery of the products, even though considerable progress has been made in the field of *in situ* product removal techniques.[20] Therefore, an *in vitro* reaction process would be highly desirable if a cost efficient way of supplying electrons to the heme iron can be found. The use of electron mediators[25], direct electron supply from electrodes[26,27] as well as enzymatic[15,17] and organometallic[28] approaches have been suggested as sources of the required reduction equivalents.

In our report we suggest an enzymatic cofactor recycling system based on $NAD^+$-dependent FDH from *Pseudomonas* sp. 101. FDH catalyses the oxidation of formate anions to carbon dioxide with concomitant reduction of $NAD^+$ to NADH and thus is of considerable commercial interest as a catalyst for the regeneration of reduced coenzymes in fine chemical synthesis. The great advantage of FDH is the low cost of the substrate formate and the simple removal of the reaction product $CO_2$. An FDH mutant also exhibits high activity with $NADP^+$ and therefore can be used as an NADPH-regenerating enzyme in combination with P450 BM-3 (Figure 1).[29,30]

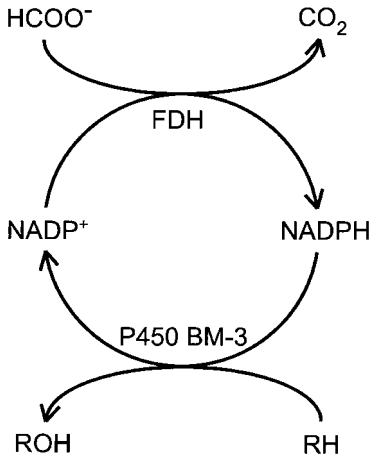


**Figure 1.** Cofactor recycling system for P450 BM-3 with $NADP^+$-dependent FDH.

## Results and Discussion

### Immobilisation of P450 BM-3

To our knowledge, to date no systematic investigation on immobilisation of bacterial P450 monooxygenases has been published.

The immobilisation procedure involved the highly purified P450 BM-3 mutant A74G, F87V, L188Q which hydroxylates a broad range of substrates.[12]

According to Taylor et al. a bacterial P450 can be immobilised on anion exchange materials.[17] The electrostatic interactions built up by anion exchangers like DEAE and SuperQ render them quite suitable for immobilisation of the negatively charged protein. In our investigations this could also be achieved with P450 BM-3. However, as expected, the products of 10-pNCA oxidation and other hydroxylation reactions (data not shown) were also adsorbed to the matrix. This renders DEAE and SuperQ unsuitable for our purposes. In addition, washes with higher ionic strength of course leached the enzyme from the carrier.

A variety of further matrices was tested using the respective standard immobilisation protocols (Table 1). Immobilisation procedures based on hydrophobic interactions proved unsuitable for efficient immobilisation of P450 BM-3, as judged from the throughout negative results obtained with EP100 and MP1000 (polypropylene derivatives), phenyl-, octyl- and butylsepharose. Also, the enzyme was virtually unable to adsorb on celite, a porous silicate matrix derived from diatomaceous soil. The covalent attachment to Eupergit

**Table 1.** Immobilisation matrices under investigation; references include standard immobilisation protocols. Identical procedures were used for immobilisation on phenyl-, octyl- and butylsepharose.

| Immobilisation matrix | EP100[22] | MP1000[23] | Phenylsepharose[24] | Octylsephaose | Butylsepharose | DEAE[17] | SuperQ[17] | Celite[21] | Eupergit[31] | Sol-Gel[18] |
|---|---|---|---|---|---|---|---|---|---|---|
| Immobilisation result | – | – | – | – | – | + | + | – | – | + |

+ : Immobilisation was observed; –: no immobilisation could be observed

  

git, an oxirane-functionalised polymer, worked for many proteins,[31] but not for active P450 BM-3.

Sol-gel entrapment was shown to be suitable for a broad range of labile biological macromolecules,[18] but to date no P450 monooxygenase was immobilised in this kind of matrix. Therefore, the encapsulation of the monooxygenase in a sol-gel matrix derived from TEOS was assayed. First tests were carried out using a slightly modified version of the protocol proposed by Shtelzer and co-workers.[32] Monooxygenase activity was observed indicating entrapment of P450 BM-3 (up to 7.2 mg corresponding to 6.4 U P450 BM-3 in 1 g sol-gel). During immobilisation about half of the hydroxylation activity was lost (see Figure 2). Repeated washing steps with 50 mM potassium phosphate (KPi) buffer lead to a negligible loss of P450 BM-3 (about 2% of total P450 BM-3). Further washing did not lead to leaching of any P450 BM-3.

A large amount of ethanol, which is expected to inactivate P450 BM-3,[33] is released during the sol-gel formation. Remarkably, we did not observe loss of the enzyme's activity.

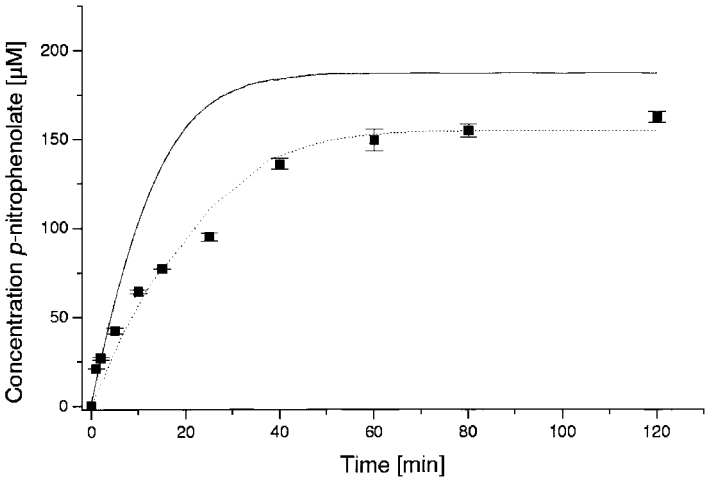


**Figure 2.** Turnover of 0.25 mM 10-pNCA by P450 BM-3 monitored by absorption measurement at 410 nm. Solid line: 0.061 nmol/mL (0.0125 U/mL, specific activity: 1.7 U/mg) of freshly purified P450 BM-3 in solution (continuous *in situ* measurement). Squares/dotted line: 0.061 nmol/mL (0.0065 U/ml) of sol-gel immobilised P450 BM-3 (measured at particular time intervals after removal of the matrix). Values displayed in this and the following figures originate from measurements made at least three times. Standard deviations were determined to be lower than 5% in all cases. These standard deviations are given as error bars for the individual data points. All curves shown in this work were generated by least-squares fitting of the experimental data points.

## Activity and Stability of Sol-Gel Immobilised P450 BM-3 A74G, F87V, L188Q

The oxidation of *p*-nitrophenoxydecanoic acid (10-pNCA) was chosen as a model reaction, because it allows the simple photometrical measurement of *p*-nitrophenolate which is produced during the reaction.[34]

Upon its addition to the reaction mixtures, the immobilised enzyme forms a turbid suspension. Therefore, direct kinetic measurements are not feasible for determining the activity of encapsulated P450 BM-3. To circumvent this, the activity of sol-gel immobilised P450 BM-3 was assayed by incubating all the components of the standard pNCA assay with the respective immobilised enzyme preparations. Subsequent centrifugation separated the yellow reaction product, *p*-nitrophenolate, from the immobilised catalyst. The absorption of the supernatant was measured at 410 nm.

Figure 2 illustrates the hydroxylation reaction of 10-pNCA using 0.061 nmol of P450 BM-3 in solution and 0.061 nmol P450 BM-3 encapsulated in the sol-gel matrix. The specific activity of immobilised enzyme (0.89 U/mg P450 BM-3) was lower compared to that observed for the free enzyme (1.7 U/mg P450 BM-3). This may be due to diffusion limitation in the nanoporous catalyst particles or to enzyme denaturation, but is most likely due to the combination of both effects.

Investigations on long-term storage stability revealed advantages of the immobilised form of P450 BM-3. At 4 °C, no loss of activity was observed during a period of 36 days. In contrast, the free enzyme has a half-life of only 26 days when stored in 50 mM KPi. The dissolved enzyme could be stabilised by addition of 50% glycerol (Figure 3). According to Figure 3, the half-life of sol-gel immobilised P450 BM-3 is even longer than that of the glycerol-stabilised one. Estimates of half-lives were calculated assuming a first order exponential decay of enzymatic activity.

The immobilised enzyme was remarkably stable also at 25 °C. Half-lives of 29 days for the immobilised enzyme, 2 days for the enzyme dissolved in 50 mM KPi and 5 days for the enzyme dissolved in 50 mM KPi with 50% glycerol added were found (Figure 4). The stability of the immobilised enzyme at 25 °C is crucial for its application in a future P450 BM-3 bioreactor, because the enzyme is most active in this temperature range (see next section).

To determine the activity of the immobilised P450 BM-3 as a function of temperature, the reaction was studied at temperatures ranging from 4 °C to 50 °C (Figure 5). Both the free and the immobilised enzyme performed best at about 25 °C. Below 20 °C the immobilised enzyme was less active than P450 BM-3 in solution, which is probably due to restricted diffusion. On the other hand, sol-gel immobilised P450 BM-3 seems to retain its activity better at temperatures above 30 °C.

  

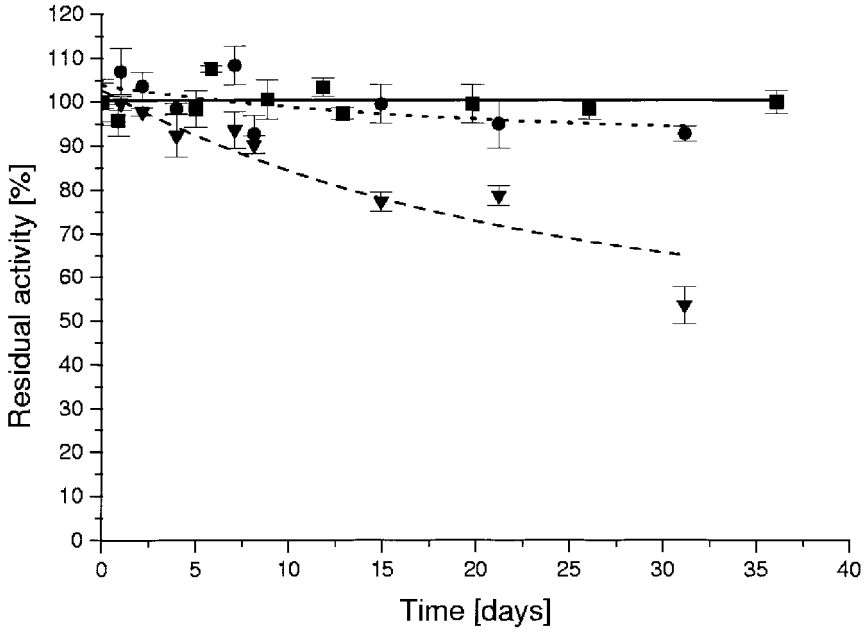


**Figure 3.** P450 BM-3 activity at 4 °C. *Squares/solid line:* Sol-gel immobilised enzyme (measured at particular time intervals after removal of the matrix). 100% activity correspond to 0.008 U/mL (0.075 nmol/mL of sol-gel immobilised P450 BM-3). *Circles/dotted line:* Enzyme dissolved in 50% glycerol (measured at particular time intervals to allow comparison with sol-gel immobilised enzyme). 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3). *Triangles/dashed line:* Enzyme dissolved in 50 mM KPi (measured at particular time intervals to allow comparison with sol-gel immobilised enzyme) 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3).

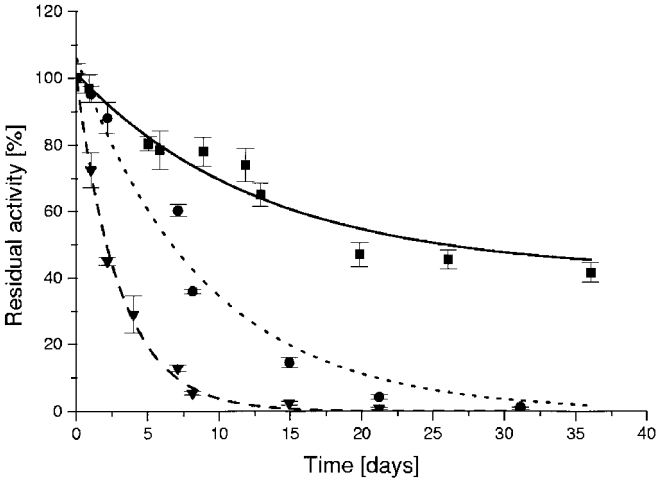


**Figure 4.** P450 BM-3 activity at 25 °C. *Squares/solid line:* Sol-gel immobilised enzyme (measured at particular time intervals after removal of the matrix). 100% activity correspond to 0.008 U/mL (0.075 nmol/mL of sol-gel immobilised P450 BM-3). *Circles/dotted line:* Enzyme dissolved in 50% glycerol (measured at particular time intervals to allow comparison with sol-gel immobilised enzyme). 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3). *Triangles/dashed line:* Enzyme dissolved in 50 mM KPi (measured at particular time intervals to allow comparison with sol-gel immobilised enzyme). 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3).

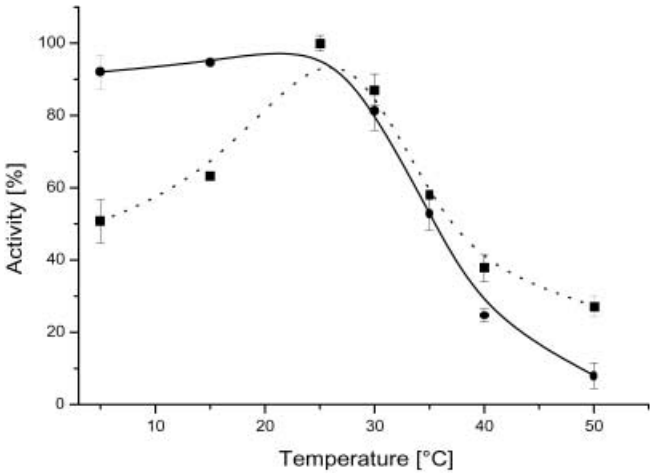


**Figure 5.** Temperature profile of P450 BM-3 activity. Prior to the experiments, the enzyme batches were incubated at the respective temperature for 10 minutes. *Squares/dotted line:* Sol-gel immobilised enzyme. 100% activity correspond to 0.008 U/mL (0.075 nmol/mL of sol-gel immobilised P450 BM-3). *Circles/solid line:* Enzyme dissolved in 50 mM KPi. 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3).

  

### Influence of Acetone and DMSO

Solubilisation of the mostly hydrophobic monooxygenase substrates requires at least small amounts of organic solvents. Previous investigations showed that acetone and DMSO are the most suitable solvents for this purpose.[9,11,12,35] Hence the activity of immobilised and free P450 BM-3 was compared using acetone and DMSO. DMSO is the more favourable co-solvent because it has a less deleterious effect on hydroxylation activity (Figures 6 and 7), this seems to be the case in particular for the immobilised enzyme (Figure 6).

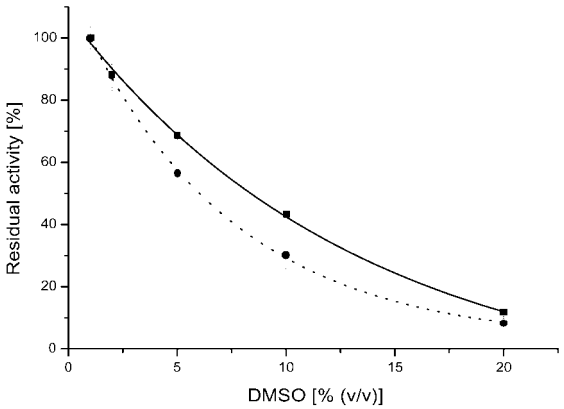


**Figure 6.** P450 BM-3 activity in presence of DMSO. *Squares/solid line:* Sol-gel immobilised enzyme. 100% activity correspond to 0.008 U/mL (0.075 nmol/mL of sol-gel immobilised P450 BM-3). *Circles/dotted line:* Enzyme dissolved in 50 mM KPi. 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3).

### Turnover of β-Ionone by Sol-gel Immobilised P450 BM-3 A74E, F87V, P386S; Hydroxylation of *n*-Octane and Naphthalene by P450 BM-3 A74G, F87V, L188Q

To test the general applicability of the immobilisation approach, the hydroxylation of three further substrates beside 10-pNCA was investigated. The conversion of β-ionone into 4-hydroxy-β-ionone[36] (Figure 8) by the P450 BM-3 A74E, F87V, P386S mutant was measured as well as the hydroxylation of *n*-octane and naphthalene by P450 BM-3 A74G, F87V, L188Q. For all these reactions, educts and products were quantified by GC.

The transformation of β-ionone is of great interest to the flavour industry.[37]

GC analysis of the reaction products of the heterogeneously catalysed reaction revealed that 83% of β-ionone was converted; reaction product was exclusively 4-hydroxy-β-ionone. Investigations on the enantiomeric excess of 4-hydroxy-β-ionone formed in this reaction are currently in progress.

In the case of naphthalene oxidation, GC analysis of the reaction extract revealed 77% conversion, mainly to 1-naphthol (85%). As minor component (15%) 2-naphthol was detected. These results show similarity to the observations reported by Appel et al. for this reaction with the same P450 BM-3 mutant in solution: In their investigation exclusively 1-naphthol was detected as reaction product.[12]

Finally we studied the hydroxylation of *n*-octane by immobilised P450 BM-3. 79% of the educt was converted. As reaction products the regioisomers 2-, 3- and 4-octanol were identified in molar ratio 1 : 2.1 : 1.6. As for naphthalene, these values correlate to those reported by Appel et al. for the free enzyme.[12]

As substrates belonging to diverse substance classes such as fatty acids (10-pNCA), terpenes (β-ionone), aromatics (naphthalene) and *n*-alkanes (*n*-octane) are hydroxylated by sol-gel immobilised P450 BM-3 mutants, we conclude that the method described here is applicable to a very broad range of substrates. We suppose that at least most substances which are substrates of free P450 BM-3 can also be transformed by the sol-gel immobilised enzyme.

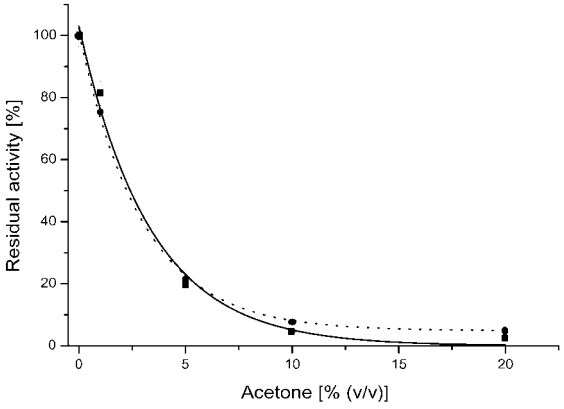


**Figure 7.** P450 BM-3 activity in presence of acetone. *Squares/solid line:* Sol-gel immobilised enzyme. 100% activity correspond to 0.008 U/mL (0.075 nmol/mL of sol-gel immobilised P450 BM-3). *Circles/dotted line:* Enzyme dissolved in 50 mM KPi. 100% activity correspond to 0.009 U/mL (0.044 nmol/mL of free P450 BM-3).

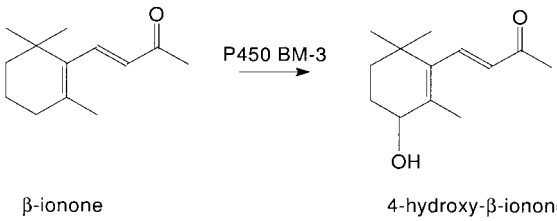


**Figure 8.** Conversion of β-ionone to 4-hydroxy-β-ionone catalysed by the P450 BM-3 A74E, F87V, P386S mutant.

### Cofactor Recycling with FDH

First we investigated whether dissolved P450 BM-3 and FDH are active in the same buffer. To make sure that only NADPH that is recycled from $NADP^+$ is used for

  

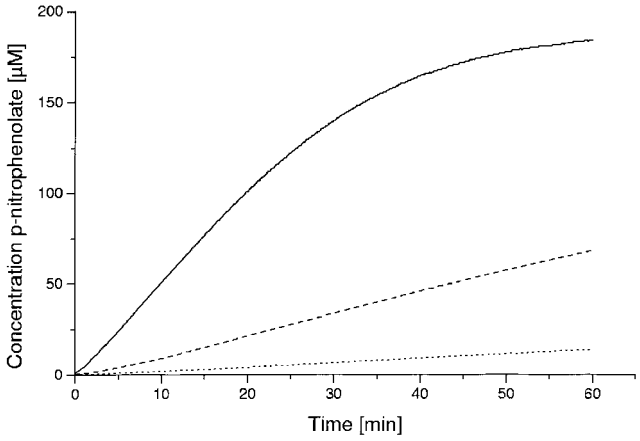


**Figure 9.** Turnover of 0.25 mM 10-pNCA by 0.02 U/ml P450 BM-3 monitored by absorption measurement at 410 nm (continuous *in situ* measurement). No NADPH was supplied externally and the reduction equivalents were generated by 0.025 mM (*solid line*), 0.005 mM (*dashed line*), 0.0025 mM (*dotted line*) $NADP^+$ and 0.02 U/ml FDH.

the oxidation of 10-pNCA no external NADPH was added to the reaction mixture. A 10-fold excess of 10-pNCA over $NADP^+$ leads to a quantitative oxidation reaction within one hour reaction time (Figure 9). Decreasing the $NADP^+$-concentration results in lower reaction speed. In case of a 50- and 100-fold excess of 10-pNCA over $NADP^+$, the reaction rate decreases to 26% and 4.4% of the value obtained with a 10-fold excess of 10-pNCA, respectively.

Encouraged by this result, sol-gel immobilisation of FDH was examined in two approaches. In the first set of experiments, co-immobilisation of both enzymes simultaneously (P450 BM-3 and FDH) was investigated. In the second set, the two proteins were immobilised separately and then mixed in a ratio of 1:1 (m/m). Co-immobilised enzymes from the first set and the mixture from the second set were used in pNCA assays, which again contained a 10-fold excess of 10-pNCA over oxidised $NADP^+$. After 3 hours, the co-immobilised enzymes showed a conversion of up to 28% of the 10-pNCA used, whereas the mixture of the separately immobilised enzymes led to 75% conversion of the educt.

This result could be reproduced thrice in repeated batch experiments, indicating the theoretical possibility to design a continuously operated bioreactor based on sol-gel encapsulated P450 BM-3.

## Conclusions

The exploitation of P450 s in technical applications has attracted great attention. However, not much is known about using P450 monooxygenases in organic synthesis.[3,15,17]

Thus, the combination of sol-gel immobilised P450 BM-3 and FDH in repeated batch reactions represents as far as we know the first solid phase example of combining a P450 monooxygenase with a cheap cofactor recycling system. Sol-gel immobilisation of P450 BM-3 facilitates the separation of the biocatalyst from the product and greatly improves the retention of enzymatic stability and activity. Therefore, the approach offers the possibility of designing a P450 bioreactor that can be operated over a long period of time. The use of immobilised FDH in such a system allows the exchange of stoichiometric quantities of expensive NADPH with cheaper $NADP^+$.[38] Combined with the broad substrate spectrum of P450 BM-3, which can certainly be extended further, a step towards the technical application of heme-containing monooxygenases in fine chemical synthesis has been made. The transformation of β-ionone presented here involves the hydroxylation at an activated carbon atom. The possibility to hydroxylate even non-activated carbons is demonstrated in the reactions with *n*-octane and naphthalene described above. The full potential of the approach will be exploited in stereo- and regioselective hydroxylation reactions at non-activated carbons. Therefore, deeper insights into the suggested system are required and potential improvements are certainly possible, which will also involve the further reduction of $NADP^+$. Experiments on long-term oxidation reactions using the methods described in this paper are currently under way.

This work presents a simple approach to practically useful and stable hydroxylation catalysts based on P450 BM-3.

## Experimental Section

### Chemicals

All chemical reagents were purchased from Fluka, Aldrich, Sigma or Riedel-de-Haën. NADPH tetrasodium salt was procured from Jülich Fine Chemicals (Germany). All chemicals used were of analytical grade or higher. 10-pNCA was synthesised as described elsewhere.[34] β-Ionone was procured from Merck, the 4-hydroxy derivative was synthesised according to Broom et al.[39] The substrates β-ionone and 10-pNCA were added to the reaction mixtures as 10–25 mM stock solutions in DMSO, which were prepared fresh every day. 10 mM NADPH and $NADP^+$ were dissolved fresh each day in 50 mM KPi pH 8.1 and pH 6.5, respectively.

### Enzymes

$NADP^+$-dependent formate dehydrogenase (FDH) was obtained from Prof. V. I. Tishkov (Department of Chemical Enzymology, Lomonosov Moscow State University, 119899 Moscow, Russia). FDH was stored in 0.1 M KPi, 1.2 M

  

$NH_4SO_4$, 20% (v/v) glycerol, pH 7 at 4 °C. The obtained enzyme batch displayed an activity of 47 U/mL.

DNA-modifying enzymes were purchased from MBI Fermentas.

## Construction of a P450 BM-3 Expression System

The genes encoding P450 BM-3 mutants A74G, F87V, L188Q and A74D, F87V, P386S were amplified from pT-USC1BM-3[29] and pET22-BM-3mt, respectively, by PCR using primers designed to facilitate cloning into pET28a(+) vector. The oligonucleotide primer for the *5′*-end of the gene: *5′*-GCGGATCCATGACAATTAAAGAAATGCCTCAGC was designed to introduce a *BamHI* restriction site upstream of the start codon. The primer for the *3′*-end of the gene: *5′*-GCGAATTCTTACCCAGCCCACACGTCTTTTGCG introduced an *EcoRI* restriction site downstream after the stop codon. The gene was amplified in 25 cycles of 2 min 95 °C, followed by 2 min annealing at 62 °C and extension 4 min at 72 °C in an Eppendorf Thermal Cycler. The amplified gene was subsequently cloned into the expression vector by *BamHI*/*EcoRI* restriction sites. Initial cloning has been done in strain DH5α (Clontech, Heidelberg, Germany), which gives high transformation efficiency and good plasmid yield followed by heterologous expression in BL21(DE3) *Escherichia coli* cells (Novagen, Madison, USA) with N-terminal $His_6$ tag to facilitate purification of the protein on a nickel affinity column. Attachment of the $His_6$ polypeptide had no effect on the activity of the gene product.

## Expression and Purification of P450 BM-3

The first culture (2 mL) was inoculated from a single colony into 2 mL Luria-Bertani (LB)-medium supplied with kanamycin (30 μg/mL) and grown at 37 °C with shaking at 150 rpm until $OD_{578}$ reaches 0.6 – 1.0. This culture was used to inoculate 200 mL LB-medium, supplied with kanamycin (30 μg/mL). The cells were grown at 37 °C with shaking at 160 rpm to an $OD_{578}$ ~0.8 and expression of the gene was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.35 mM. After a further 6 h of growth at 30 °C the cells were harvested by centrifugation (20 min, 6000 rpm, 4 °C). The cell pellet was then resuspended in loading buffer (50 mM KPi, 800 mM NaCl, 0.1 mM PMSF, pH 7.5) and lysed by sonication on ice (5 × 2 min, output control = 4, duty cycle 40%; BransonSonifier W250, Dietzenbach, Germany).

Purification took advantage of the $His_6$ tag, utilising Quiagen Ni-NTA superflow resin. After centrifugation cell lysates were 0.22 μm-filtered and loaded onto the pre-equilibrated affinity column. The column was subsequently washed with loading buffer, washing buffer A (50 mM KPi, 800 mM NaCl, pH 6.2) and washing buffer B (50 mM KPi, 800 mM NaCl, 250 mM glycine, pH 7.5). As in our experiments the standard Ni-NTA eluting agent imidazole led to partial aggregation and inactivation of BM-3, enzyme elution was performed using L-histidine (50 mM KPi, 80 mM L-histidine, pH 7.5). The purified protein was supplemented with 50% glycerol and stored at – 20 °C. Prior to use the buffer was exchanged to 50 mM KPi by ultrafiltration utilising a 50 kDa Amicon membrane. P450 concentrations were measured by CO difference spectroscopy as previously described,[40] using an extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ for the 450 minus 490 nm peak.

## Sol-Gel Immobilisation

The fluoride-catalysed formation of the silica sol was started by mixing 5 volumes of TEOS with 1 part of 20% (w/v) polyethylene glycol 6000 in 50 mM KPi and 0.25 parts of 0.2 M NaF on ice. After 3 minutes, 2.5 volumes of a 2 – 12 μM solution of P450 BM-3 in 50 mM KPi buffer was added. The mixture was stirred on ice for another 15 minutes or until a homogeneous emulsion had formed. This hydrogel was subsequently stored at 4 °C for at least 3 days to allow formation of the xerogel. The resulting hard and brittle solid was washed 5 times in a centrifugation tube with 50 mM KPi to remove all traces of non-immobilised P450 BM-3. The samples were subsequently lyophilised. The obtained immobilisate was 60% of the theoretically possible yield calculated from the amount of TEOS used. The P450 BM-3 content [nmol $g^{-1}$] of the sol-gels was determined by dividing the total amount of P450 BM-3 used minus the P450 amount in the washing solutions by the total mass of the sol-gel obtained. Depending on the P450 BM-3 concentration of the particular enzyme batch used for immobilisation, loadings of 13 – 60 nmol catalyst per g sol-gel were obtained. The residual P450 BM-3 activity after immobilisation was 50% of the original activity. Immobilised enzyme preparations were stored at – 20 °C until required.

FDH was immobilised in the same way as P450 BM-3.

Prior to use, sol-gel immobilised enzymes were ground to a fine powder in order to reduce mass transfer limitation effects in the nanoporous catalyst particles.

## Activity Assays with 10-pNCA

All activity measurements were executed at least three times to minimise standard errors.

P450 BM-3 activity assays with the enzyme in solution were performed as previously described by following the kinetics of *p*-nitrophenolate production at 410 nm.[29]

For immobilised enzymes this procedure was modified: 5 mg of sol-gel immobilised P450 BM-3 were incubated with 965 μL 50 mM KPi, pH 8.1, 10 μL 25 mM 10-pNCA in DMSO and 25 μL 10 mM NADPH stock solution in a shaker at 1200 rpm. After 1 hour the solid was removed by centrifugation. *p*-Nitrophenolate production was measured by determining the absorption difference at 410 nm of the supernatant against a blank, which was treated exactly as the actual sample except that no P450 BM-3 was encapsulated in the used sol-gel.

When activity of immobilised P450 BM-3 was compared to activity of the enzyme in solution, activity of the free enzyme was assayed as described for the immobilised enzyme, using the same amount of P450 BM-3 in both cases.

The pNCA assay with FDH and P450 BM-3 in solution was executed by adding 2.5 μL 10 mM $NADP^+$ stock solution to 1 mL of FDH/BM-3 assay solution (containing 0.25 mM 10-pNCA, 0.05 μM P450 BM-3, 0.02 U/mL FDH, 100 mM KPi, 300 mM formate, pH 8.1) resulting in a 10-fold excess of 10-pNCA over $NADP^+$. Immediately after $NADP^+$ addition, absorption measurement at 410 nm was started.

  

For activity assaying of immobilised FDH in combination with immobilised P450 BM-3 5 mg of each immobilised enzyme preparation (or 10 mg of the co-immobilised enzymes) was added to 1 mL of FDH/BM-3 assay solution. The reaction was started by addition of 2.5 μL 10 mM $NADP^+$, again leading to a 10-fold excess of 10-pNCA over $NADP^+$. Further steps were carried out as described for immobilised BM-3 without FDH, except that the reaction time was prolonged to 3 hours. To quantify pNCA turnover, an extinction coefficient of *p*-nitrophenolate at 410 nm of 13.2 $mM^{-1}\,cm^{-1}$ was used. For determining 10-pNCA turnover the respective reaction and reference solutions were diluted in the ratio 1 to 10 with 50 mM KPi, pH 8.1 to give absorption values in the linear range of the photometer detector.

### Turnover of β-Ionone, Naphthalene and *n*-Octane with Immobilised P450 BM-3 Mutants

100 mg of the respective immobilised P450 BM-3 mutant (35 nmol/g) was suspended in 9.3 mL 50 mM KPi pH 7.5, supplemented with 100 μL 20 mM substrate in DMSO and 600 μL 10 mM NADPH. The reaction mixture was subsequently shaken for 2 h. After centrifugation the supernatant was extracted twice with 4 mL diethyl ether. The combined organic layers were dried with sodium sulphate and concentrated to a volume of 100 μL.

Reaction products and unreacted educts were identified by GC analysis using a Fisons GC 8000 gas chromatograph equipped with a flame ionisation detector (FID) and a 60 m Zebron ZB 1 (polydimethylsiloxane) column. The temperature gradients were as follows: β-Ionone : 1) 90 °C for 5 min, 2) 90 to 300 °C at 10 °C/min, 3) 300 °C for 10 min. Naphthalene: 1) 100 °C for 10 min, 2) 100 to 140 °C at 2 °C/min, 3) 140 to 250 °C at 10 °C/min, 4) 250 °C for 5 min. Octane: 1) 45 °C for 8 min, 2) 45 to 100 °C at 2 °C/min, 3) 100 to 240 °C at 10 °C/min. Pure samples of substrates and potential reaction products were available. Equal amounts of these substances dissolved in diethyl ether were applied to the column. From the resulting GC trace the ratio of the peak areas corresponding to the substrates and products were calculated. These ratios were used to determine the molar ratios of substrates and products emerging from the biotransformations. Therefore equal diethyl ether-water partition coefficients for educts and products were assumed.

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## References and Notes

[1] J. T. Groves, Y.-H. Han, in *Cytochrome P450: Structure, Mechanism and Biochemistry*, (Ed.: P. R. E. Ortiz de Montellano), Plenum Press, New York, **1995**, pp 3 – 48.
[2] E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold, *Adv. Synth. Catal.* **2001**, *343*, 601 – 606.
[3] V. Urlacher, R. D. Schmid, *Curr. Opin. Biotechnol.* **2002**, *13*, 557 – 564.
[4] L. P. Wen, A. J. Fulco, *J. Biol. Chem.* **1987**, *262*, 6676 – 6682.
[5] A. J. Fulco, R. T. Ruettinger, *Life Sci.* **1987**, *40*, 1769 – 1775.
[6] S. S. Boddupalli, R. W. Estabrook, J. A. Peterson, *J. Biol. Chem.* **1990**, *265*, 4233 – 4239.
[7] G. Truan, M. R. Komandla, J. R. Falck, J. A. Peterson, *Arch. Biochem. Biophys.* **1999**, *366*, 192 – 198.
[8] T. W. Ost, C. S. Miles, J. Murdoch, Y. Cheung, G. A. Reid, S. K. Chapman, A. W. Munro, *FEBS Lett.* **2000**, *486*, 173 – 177.
[9] Q. S. Li, U. Schwaneberg, M. Fischer, J. Schmitt, J. Pleiss, S. Lutz-Wahl, R. D. Schmid, *Biochim. Biophys. Acta* **2001**, *1545*, 114 – 121.
[10] Q. S. Li, U. Schwaneberg, P. Fischer, R. D. Schmid, *Chemistry* **2000**, *6*, 1531 – 1536.
[11] Q. S. Li, J. Ogawa, R. D. Schmid, S. Shimizu, *Appl. Environ. Microbiol.* **2001**, *67*, 5735 – 5739.
[12] D. Appel, S. Lutz-Wahl, P. Fischer, U. Schwaneberg, R. D. Schmid, *J. Biotechnol.* **2001**, *88*, 167 – 171.
[13] Q. S. Li, J. Ogawa, R. D. Schmid, S. Shimizu, *FEBS Lett.* **2001**, *508*, 249 – 252.
[14] W. Tischer, F. Wedekind, *Top. Curr. Chem.* **1999**, *200*, 95 – 126.
[15] P. Fernandez-Salguero, C. Gutierrez-Merino, A. W. Bunch, *Enzyme Microb. Technol.* **1993**, *15*, 100 – 104.
[16] S. B. Lamb, D. C. Lamb, S. L. Kelly, D. C. Stuckey, *FEBS Lett.* **1998**, *431*, 343 – 346.
[17] M. Taylor, D. C. Lamb, R. J. Cannell, M. J. Dawson, S. L. Kelly, *Biochem Biophys. Res. Commun.* **2000**, *279*, 708 – 711.
[18] I. Gill, *Chem. Mater.* **2001**, *13*, 3404 – 3421.
[19] Catalogue price of NADPH and NADH about 800 €/g and 90 €/g, respectively.
[20] G. J. Lye, J. M. Woodley, *TIBTECH* **1999**, 17, 395 – 402.
[21] C. H. Lee, K. L. Parkin, *Biotechnol. Bioeng.* **2001**, 75, 219 – 227.
[22] A. Sanchez, J. L. del Rio, F. Valero, J. Lafuente, I. Faus, C. Sola, *J. Biotechnol.* **2001**, 84, 1 – 12.
[23] J. C. Chen, S. W. Tsai, *Biotechnol. Prog.* **2000**, 16, 986 – 992.
[24] N. S. Dosanjh, J. Kaur, *Biotechnol. Appl. Biochem.* **2002**, 36, 7 – 12.
[25] U. Schwaneberg, D. Appel, J. Schmitt, R. D. Schmid, *J. Biotechnol.* **2000**, *84*, 249 – 257.
[26] G. Gilardi, Y. T. Meharenna, G. E. Tsotsou, S. J. Sadeghi, M. Fairhead, S. Giannini, *Biosens. Bioelectron.* **2002**, *17*, 133 – 145.
[27] J. Kazlauskaite, A. C. G. Westlake, L.-L. Wong, H. A. O. Hill, *Chem. Commun.* **1996**, *18*, 2189 – 2190.
[28] F. Hollmann, B. Witholt, A. Schmid, *J. Mol. Cat. B* **2002**, *791*, 1 – 10.
[29] V. I. Tishkov, A. G. Galkin, V. V. Fedorchuk, P. A. Savitsky, A. M. Rojkova, H. Gieren, M. R. Kula, *Biotechnol. Bioeng.* **1999**, *64*, 187 – 193.

  

[30] K. Seelbach, B. Riebel, W. Hummel, M.-R. Kula, V. I. Tishkov, A. M. Egorov, C. Wandrey, U. Kragl, *Tetrahedron Lett.* **1996**, *37*, 1377 – 1380.
[31] E. Katchalski-Katzir, D. M. Kraemer, *J. Mol. Cat. B* **2000**, *10*, 157 – 176.
[32] S. Shtelzer, S. Rappoport, D. Avnir, M. Ottolenghi, S. Braun, *Biotechnol. Appl. Biochem.* **1992**, *15*, 227 – 235.
[33] U. Schwaneberg, *PhD thesis*, Universität Stuttgart, Germany, **1999**.
[34] U. Schwaneberg, C. Schmidt-Dannert, J. Schmitt, R. D. Schmid, *Anal. Biochem.* **1999**, *269*, 359 – 366.
[35] O. Lentz, Q. S. Li, U. Schwaneberg, S. Lutz-Wahl, P. Fischer, R. D. Schmid, *J. Mol. Cat. B* **2001**, *15*, 123 – 133.
[36] V. Urlacher, manuscript in preparation.
[37] V. Krasnobajew, D. Helmlinger, *Helv. Chim. Acta.* **1982**, *65*, 1590 – 1601.
[38] Catalogue price of NADPH and $NADP^+$ about 800 €/g and 260 €/g, respectively.
[39] S. J. Broom, R. M. Ede, A. L. Wilkins, *Tetrahedron Lett.* **1992**, *33*, 3197 – 3200.
[40] T. Omura, R. J. Sato, *J. Biol. Chem.* **1964**, *239*, 2370 – 2378.

  